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Optical proxy for phytoplankton biomass in the absence of photophysiology: Rethinking the absorption line height

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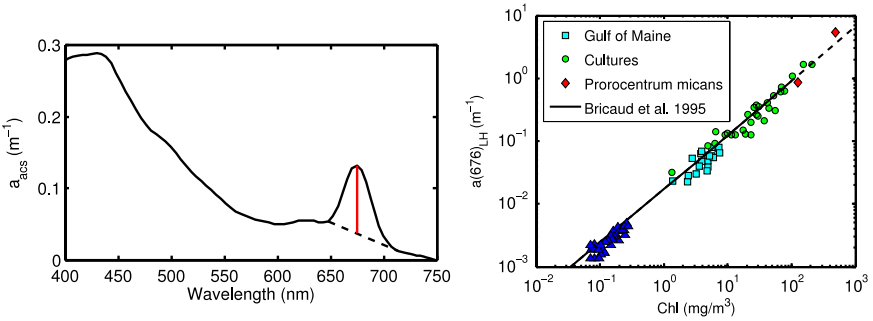
GRAPHICAL ABSTRACT



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ABSTRACT

The pigment absorption peak in the red waveband observed in phytoplankton and particulate absorption spectra is primarily associated with chlorophyll-*a* and exhibits much lower pigment packaging compared to the blue peak. The minor contributions to the signature by accessory pigments can be largely removed

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Fluorescence
Pigment-based taxonomy
Light saturation irradiance
Bio-optics

by computing the line height absorption at 676 nm above a linear background between approximately 650 nm and 715 nm. The line height determination is also effective in removing the contributions to total or particulate absorption by colored dissolved organic matter and non-algal particles, and is relatively independent of the effects of biofouling. The line height absorption is shown to be significantly related to the extracted chlorophyll concentration over a large range of natural optical regimes and diverse phytoplankton cultures. Unlike the *in situ* fluorometric method for estimating chlorophyll, the absorption line height is not sensitive to incident irradiance, in particular non-photochemical quenching. The combination of the two methods provides a combination of robust phytoplankton biomass estimates, pigment based taxonomic information and a means to estimate the photosynthetic parameter, E_K, the irradiance at which photosynthesis transitions from light limitation to light saturation.

1. Introduction

1.1. Historical perspective on optical sensing of chlorophyll-a absorption

Nearly two decades ago WET Labs released a small CHLorophyll Absorption Meter (CHLAM) which consisted of three wavelengths for absorption determination. The theory behind the instrument was that the red peak of phytoplankton absorption at 676 nm was primarily determined by Q-band chlorophyll absorption. By calculating the height of the peak absorption above the baseline absorption between 650 nm and 715 nm, most of the contribution by interfering particles and dissolved matter could be removed. An early version of the sensor, a three-wavelength absorption and attenuation (ac) meter, was deployed in the Bering Sea for six months (Davis et al., 1997). Davis and colleagues introduced the baseline correction as a means to remove biofouling from the signal. This approach compared favorably to that derived from sensors that employed chemical anti-biofouling features. However, the CHLAM sensor was not widely adopted by the optics research community, perhaps because *in situ* absorption technology was not as familiar as fluorescence and the slightly more complicated technical details required for use.

Over the past decade the user community has gained more experience with absorption meters such as the WET Labs ac9 and acs. As greater opportunities for deploying optical sensors on remote platforms arises (Dickey et al., 2008), uncertainties in the estimation of chlorophyll concentration from fluorescence suggest that the time may have come for a revival of the chlorophyll absorption meter. In this paper we present an analysis of the absorption line height as an *in situ* proxy for chlorophyll absorption and hence chlorophyll concentration. We compare and contrast this approach to standard *in situ* fluorometry, quantifying the strengths and weakness of each approach. Our hypothesis in setting out this analysis is that absorption and fluorescence target different characteristics of the chlorophyll molecule specifically, but more generally of phytoplankton ecology and physiology. Thus, while each is a robust proxy for concentration under certain conditions, each provides additional information such that the combination of approaches is more than the sum of the parts.

1.2. Variability in Fluorescence yield and absorption yield per chlorophyll a

The use of simple *in situ* fluorometers to obtain a proxy for chlorophyll concentration is one of the most common aquatic biological measurements. These devices rely on excitation of the chlorophyll-*a* molecule using a blue light source, and measuring the re-emission of photons of red light in the

process known as fluorescence. This process can be represented by the following equation:

$$F = E[CHLa]a^*\Phi_f \tag{1a}$$

where E is the excitation irradiance (μmol photons/m^2/s), *[CHLa]* is the concentration of chlorophyll-*a* (mg/m^3), a^* is the chlorophyll-specific absorption coefficient (m^2/mg, often defined as a^*_ϕ, the chlorophyll-specific *phytoplankton* absorption), and Φ_f is the quantum yield of fluorescence (dimensionless but quantifying the ratio of photons fluoresced to photons absorbed). The equation is rearranged to express *[CHLa]* as a function of the measured fluorescence:

$$[CHLa] = F/Ea^*\Phi_f. \tag{1b}$$

In principle, *in situ* chlorophyll fluorometers provide a known and constant light excitation energy ($\sim E$, but see Neale et al. (1989)), and measure the resultant fluorescence response (F) in a water sample of known volume. Typically, calibrations of these fluorometers are performed using a monospecific (single species) culture of phytoplankton, grown under known and controlled environmental conditions (e.g. light, nutrient, temperature) and growth phase (e.g. exponential). A dilution series of the culture is used to measure the instrument response as a function of extracted chlorophyll-*a* and a slope factor is determined. In this method, it is assumed that the denominator in Eq. (1b) ($Ea^*\Phi_f$) is constant for all species of phytoplankton, for all phases of growth and under all environmental conditions. As we will discuss below, this is certainly not true in the natural environment. The method of a monoculture of phytoplankton does, however, provide a consistent method by which to calibrate many fluorometers to a high degree of precision and reasonable accuracy for that specific species under those growth conditions.

There are a number of factors that impact the ratio of *in situ* chlorophyll fluorescence to the extracted chlorophyll concentration, called the fluorescence yield per chlorophyll (herein called the fluorescence yield), among them taxonomic difference, pigment packaging and non-photochemical quenching. When large data sets of paired observations are compiled, these multiple sources of variation lead to extreme variations in the relationship (Fig. 1). Looking at the spread of data points leaves one with a sense of hopelessness for using *in situ* fluorescence as a proxy for chlorophyll concentration. Yet if one breaks up such large data sets into points on an individual profile or within a single region, or by depth or time of day, strong linear relationships between *in situ* fluorescence and extracted chlorophyll concentration are found. However the slope of those relationships (i.e. the fluorescence yield) varies tremendously. This is quite problematic as fluorescence is a relative measure and requires calibration in order to be quantitative and comparable between samples, over time and between investigators (Briggs et al., 2011). Below we identify and quantify some of the sources of variability in the fluorescence yield and compare them to variations in the absorption line height yield (the ratio of the red absorption peak – the Q-band of chlorophyll absorption – to the chlorophyll *a* concentration). The goal of this analysis is to identify robust proxies for phytoplankton chlorophyll concentration and to elucidate optical approaches that separate biomass from photophysiological characteristics of phytoplankton *in situ*.

1.2.1. Species-specific variations

Phytoplankton orders differ significantly in their pigment composition. With few exceptions, pigments can be used as a taxonomic tool; the basis for the software program CHEMTAX (Mackey et al., 1996) that discriminates community composition from pigment composition analysis (Kozlowski et al., 2011; Lewitus et al., 2005; Wright et al., 1996). The pigment composition, in turn, determines the spectral dependence of the absorption coefficients (Hoepffner and Sathyendranath, 1992) and hence the relative absorbed energy that excites chlorophyll fluorescence (Yentsch and Phinney, 1985). Second order effects on both spectral absorption and fluorescence excitation are variations in the absolute cellular pigment concentration and relative cellular pigment composition induced by physiological responses to environmental factors such as light history and nutrient status.

Species composition is perhaps the largest source of variability in the fluorescence yield parameter. Proctor and Roesler (2010), in investigating the fluorescence yield from three different excitation wavelengths (440, 470, and 532 nm) found that the fluorescence yield with a 440 nm excitation varied

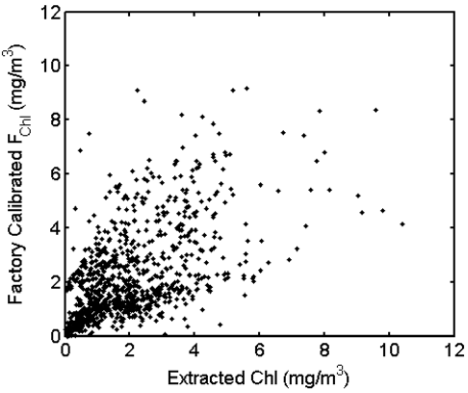

Fig. 1. Chlorophyll concentration calculated from in situ fluorometers with up-to-date factory calibrations versus extracted chlorophyll concentration from water bottles; Massachusetts Bay, 1068 paired measurements.
Source: Modified from Libby et al. (2009).

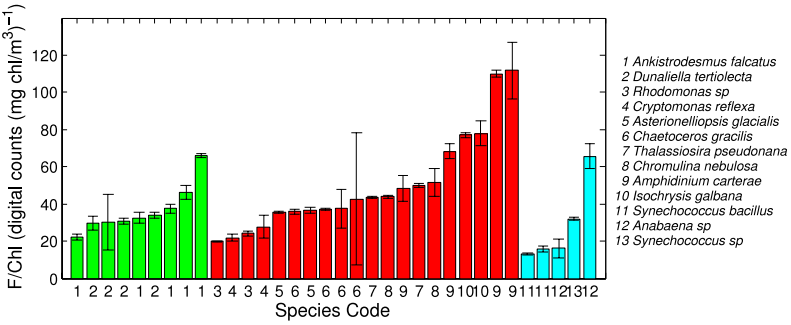

Fig. 2. Natural variability in the chlorophyll-specific *in vivo* fluorescence yield. In vivo fluorescence (470 nm excitation) per extracted chlorophyll ratio for 13 monospecific cultures grown under saturating and limiting irradiances and measured during different growth phases. Cultures are grouped by pigment lineage (green, red and cyanobacterial lineages, following Falkowski et al. (2004)) and sorted by increasing yield value within group. Error bars represent yield standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Source: Modified from Proctor and Roesler (2010).

nearly six fold between acclimated monospecific cultures (Fig. 2), even within each pigment lineage. This range was comparable to the ranges observed for the other two excitation wavelengths as well, and the spectral variations in fluorescence yields were quite variable between species. This led the authors to conclude that the ratios of fluorescence yields from any two excitation wavelengths were statistically distinct and detectable and therefore provided a robust approach to estimating taxonomic variations *in situ*.

The taxonomic variability in pigment composition leads to large spectral variations in phytoplankton absorption coefficients (Fig. 3). The relative magnitudes and locations of specific pigments vary between species and are the sources of the observed variations in fluorescence yield. The red peak associated with chlorophyll *a* Q-band absorption at approximately 676 nm exhibits less variability in spectral shape as there are fewer spectral overlaps with accessory pigments compared to the Soret chlorophyll *a* absorption peak at approximately 440 nm. This has been noted by a number of investigators as a reduced variability in the phytoplankton absorption coefficient versus extracted pigment relationships at 676 nm compared to 440 nm (Babin et al., 2003; Bricaud et al., 1995). These relative ranges are more extreme when total particulate absorption or total particulate plus dissolved absorption at the two wavelengths are independently compared to chlorophyll concentration due to the added absorption at 440 nm by the addition of non-algal particles and colored dissolved organic

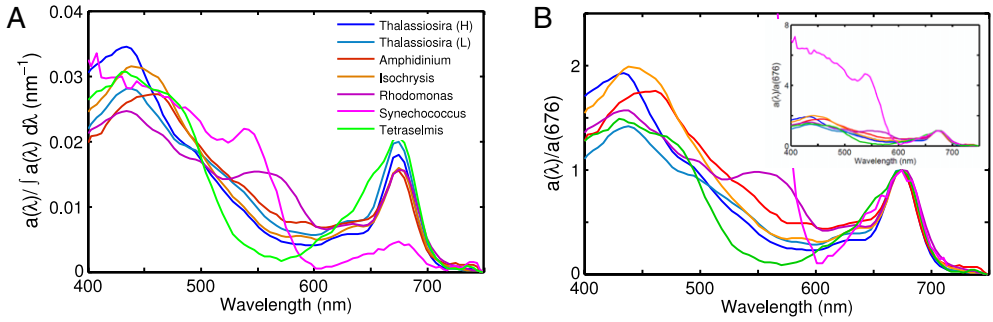

Fig. 3. Phytoplankton absorption spectra for diverse phytoplankton species scaled to the A. value of the integrated absorption over 400–750 nm; B. red chlorophyll absorption peak, inset shows scale expanded for *Synechococcus sp*. H and L indicate adaptation to high and low irradiances (600 and 50 $\mu E/m^2/s$, respectively). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

matter, respectively (Barnard et al., 1998; Bricaud et al., 1998). The relatively small contribution of non-algal material to absorption at 676 nm was the premise for the model to separate phytoplankton components from particulate or total absorption spectra proposed by Roesler et al. (1989) and implemented by others (Chang and Dickey, 2001; Roesler and Zaneveld, 1994).

1.2.2. Photoacclimation and pigment packaging

Phytoplankton cells respond to variations in instantaneous as well as average irradiance by changing their absorption cross sections. Behavioral responses to high and low irradiance include contracting or expanding chloroplasts (Kirk, 1983) and vertical migration (Cullen, 1982; Cullen and MacIntyre, 1998; Geider et al., 1998). Longer term acclimation generally includes changing the size and number of light harvesting complexes (LHC) (Perry et al., 1981; Prezelin and Matlick, 1980; Sukenik et al., 1990). Changes in the both the size and number of LHC serves to increase or decrease the absolute and relative pigment concentration, which impacts the so-called pigment packaging. While increases in cellular pigment concentration increase the overall absorption efficiency of the cell, the pigment-specific absorption is decreased. This will be manifested as changes in the absorption and fluorescence yield per chlorophyll. Theoretical and observational analyses have been used to quantify the variability in the absorption yields (Bricaud et al., 1983; Geider and Osborne, 1992; Morel and Bricaud, 1981; Sosik et al., 1989; Sosik and Mitchell, 1991). As the absorption yield per chlorophyll *a* changes, so too will the fluorescence yield, both proportionally (due to the product of $E[CHLa]a^*$) and as a function of the physiology (i.e. Φ_f). Proctor and Roesler (2010) found a minimum of 3% and a maximum of 120% variability in fluorescence yield as a function of growth irradiance for exponentially growing cultured cells of different species, with an average of 25% difference between low and high light acclimated cells.

1.2.3. Diel non-photochemical quenching (NPQ)

In vivo fluorescence varies with instantaneous irradiance due to non-photochemical quenching (NPQ). This is manifested as reduced fluorescence yield in high light surface regions compared to deeper lower light locations, leading to the erroneous identification of a deep chlorophyll maximum (Cullen, 1982). For moored observations, the onset of NPQ can be late morning to early afternoon, with reduced fluorescence yields for 5–6 h until recovery is obtained. NPQ occurs when phytoplankton cells are exposed to excessive ambient light, triggering energy transfer of absorbed photons to pigment complexes that dissipate the excess energy as heat. This process competes with the photosynthetic and fluorescence energy transfer pathways, and is dependent on the light exposure history of phytoplankton cells (Chekalyuk and Hafex, 2011). NPQ stops the transfer of photon energy between light harvesting complexes and thus to the chlorophyll *a* molecules in the PSII reaction center, ceasing the release of light energy via fluorescence (Krause and Weis, 1991). It does not,

Table 1
Comparison of the 676 nm chlorophyll-specific Q-band phytoplankton absorption, a^*_ϕ, and chlorophyll-specific absorption line height, a^*_{LH}, for laboratory and field samples. The slope of a linear regression is reported along with the 95% confidence intervals. Mean and standard deviation values were computed from the relationship between absorption and chlorophyll developed by Bricaud et al. (1995) that would be modeled based upon the chlorophyll concentrations observed in this data set.

ID	a^*_ϕ (676) (m^2/mg)		a^*_{LH} (676) (m^2/mg)	
	Slope	±95% CI	Slope	±95% CI
Cultures	0.017	0.009	0.009	<0.001
Prorocentrum micans bloom	0.010	na	0.009	na
Gulf of Maine	0.014	0.004	0.008	0.003
Equatorial Pacific	0.025	0.006	0.009	<0.001
All obs this study	0.020	0.008	0.010	<0.001
Bricaud et al. 1995[a]	0.019	0.007	0.016	0.006

[a] Computed statistics for chlorophyll values measured in these studies. Values are mean and standard deviation of the a_{LH} to chlorophyll ratios rather than slope statistics.

however, impact the absorption of light energy by pigments. NPQ can decrease fluorescence yield up to 90% (Huot and Babin, 2011). It is against these sources of variability in the fluorescence yield that we re-examine the sources of variability in the yield of the absorption line height with the goal of evaluating these proxies for chlorophyll concentration.

2. Methods

2.1. Laboratory analysis

Six monospecific phytoplankton cultures, representing a range of cell sizes and pigment compositions (Table 1), were grown in sterile filtered L1 medium (a vitamin, trace metal and nutrient enriched seawater; Guillard and Hargraves (1993) as modified by Guillard and Morton (2003)) under controlled light conditions. Each culture was acclimated to its respective growth irradiance in a small (∼250 ml) flask, and then stepped up to a 2-l flask for approximately 3 days and then inoculated into 80-l tanks. Daily water samples were collected for extracted chlorophyll analysis (modified from Holm-Hansen et al. (1965)) and particulate and dissolved spectrophotometric absorption analysis (following Roesler (1998)) to assess the culture concentration and establish the growth phase. Once exponential growth phase was established subsamples were collected and placed in 5-l aspirator bottles for gravity feeding through the absorption and attenuation tubes of a WET Labs ac9 and an acs (the 9-wavelength and hyperspectral versions, respectively, of the dual path absorption, a, and beam attenuation, c, meters). At least 60 s of observations were collected once the system was purged of air bubbles and the observed variations were less than 0.1% of the signal. The ac sensors were calibrated daily before each sampling session with degassed Nanopure®water. Values for pure water were within 0.005 m^{-1} absorption and within 0.01 m^{-1} attenuation of the calibration values, on the order of the factory designation instrumental noise.

2.2. Field analysis

Time series observations of paired *in situ* chlorophyll fluorescence and multispectral absorption were measured hourly with a WET Labs Environmental Characterization Optics series chlorophyll fluorometer and turbidity combination sensor (ECO FLNTU) and ac9, respectively. Sensors were deployed at 2.5 m depth on moorings as part of the ongoing Gulf of Maine Integrated Coastal Ocean Observing System (GoMICOOS, formerly GoMOOS; Pettigrew and Roesler (2005)). Chlorophyll fluorometers and ac sensors were calibrated prior to each deployment; dark readings and pure water calibrations were taken prior to deployment, upon recovery and after cleaning in order to independently assess biofouling and drift during deployment (Roesler and Boss, 2008). Hourly PAR values were derived from seven channel irradiance observations made with a Satlantic OCR 507a deployed on top of the buoy tower using the approach of Gregg and Carder (1990).

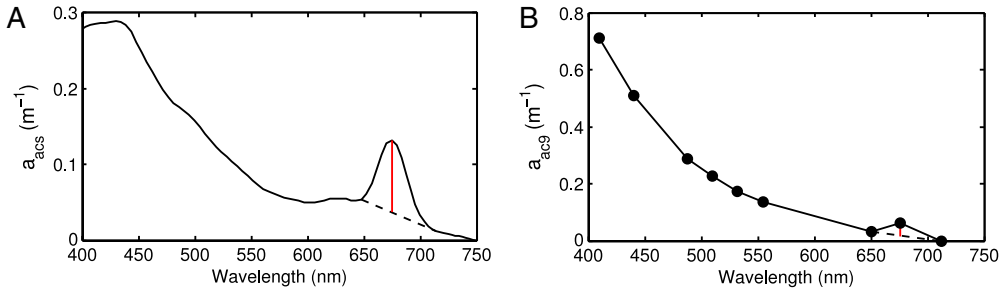

Fig. 4. Schematic of the absorption line height for A. a monospecific culture of the diatom *Thallasiosira pseudonana* grown at high light (~600 μE/m^2/s) and measured in exponential growth phase with a WET Labs acs. This is comparable to that which would be obtained using the quantitative filter technique on a spectrophotometer; B. an *in situ* absorption spectrum, which includes non-algal particles and colored dissolved organic matter, measured with multispectral absorption meter such as the WET Labs ac9. Dashed lines are the baseline absorption between 650 nm and 715 nm, the red line is the absorption line height of the peak above baseline absorption. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.3. Computation of absorption line height

The absorption value of the baseline between 650 and 715 nm at a reference wavelength is given by the linear equation:

$$a_{BL}(\lambda_{ref}) = \frac{a(715) - a(650)}{715 - 650} * (\lambda_{ref} - 650) + a(650) \quad (1)$$

of the form $y = m * x + b$. The absorption line height at the red Q-band absorption peak is computed from the difference of the baseline absorption from the observed absorption (Fig. 4):

$$a_{LH}(676)(m^{-1}) = a(676) - a_{BL}(676) \quad (2)$$

where the baseline value of absorption at 676 nm is computed from (1) with $\lambda_{ref} = 676$ nm. This approach serves not only to remove the measured null absorption value at wavelengths >715 nm that is associated with scattering offset in the absorption meter, but also to remove the tail of absorption by pigments that peak near that of chlorophyll *a*, such as chlorophyll *b* and some phycobilipigments. This equation is also applicable to particulate absorption spectra measured either spectrophotometrically on glass fiber filters (i.e. the Quantitative Filter Technique (Mitchell, 1990)) or with ac meters after removing the dissolved fraction (Roesler, 1998; Slade et al., 2010) as well as the total absorption measured with ac meters (Fig. 4B). Application of the model to ac meter observations required that the measured absorption values used in Eq. (2) be corrected for the temperature and salinity-dependence of pure water absorption (Pegau et al., 1997; Sullivan et al., 2006) prior to computing the absorption line height.

3. Results

3.1. Laboratory results

The range in spectral absorption coefficients for the 6 laboratory cultures measured with a hyperspectral acs is shown in Fig. 3. Each spectrum is normalized to the spectrally integrated absorption coefficient to show how the center of mass of each spectrum varies, i.e. at what wavelength is the average absorption found. *Synechococcus* absorption is heavily weighted towards the blue end while *Tetraselmis* is weighted towards the red end, with all other species in between. The same absorption spectra scaled to the absorption at 676 nm show not only the large variations in spectral dependence of absorption in the spectral range of the accessory pigments, but also the relative invariance in shape around the Q-band chlorophyll *a* peak. There is, however increased variations in the relative magnitudes of absorption at 650 nm, the value used in the computation of the absorption

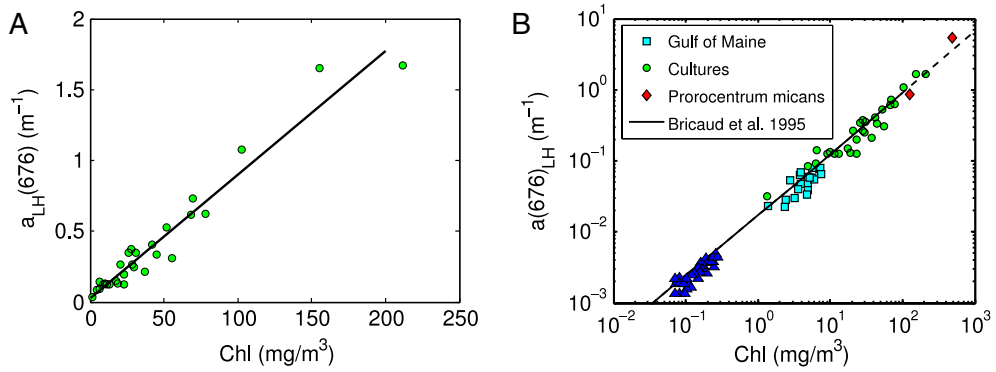

Fig. 5. Absorption line height at 676 nm versus extracted chlorophyll concentration for A. the monospecific cultures shown in Fig. 3 measured in different growth phases and grown at different irradiances; B. cultures in A (green circles) and *in situ* samples from oceanic Equatorial Pacific (blue triangles), coastal Gulf of Maine (cyan squares) and two different red tide blooms of *Prorocentrum micans* in Boothbay Harbor, Maine (red diamonds; Etheridge (2002)). Statistics in Table 1. The analytic function of Bricaud et al. (1995) is recomputed here from their spectral data using the line height absorption correction at 676 nm versus chlorophyll concentration (solid black line, both figures) for range presented in their paper, dotted line is extrapolation to 1000 mg/m^3 chlorophyll. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

line height. The largest absorption at 650 nm is found in the green lineage *Tetraselmis sp.* which is associated with chlorophyll *b* absorption.

The relationship between the 676 nm a_{LH} and the extracted chlorophyll concentration for each of these cultures as a function of growth phase and growth intensity shows a strong linear dependence (Fig. 5A). The slope of the line is 0.0087 m^2/mg chl, with 95% confidence limits ±0.0007; the intercept is 0.03 m^{-1} with 95% confidence limits ±0.005 ($r^2 = 0.95, p < 0.01$). The variability in the absorption line height yield reveals the underlying variability that is caused by differences in pigment composition that arise not only within a species due to differential pigment synthesis but also between species with large diversity in pigment composition. Thus this level of variation is expected in field data collected in a single location through a seasonal succession of species or collected over a range of ecological environments. Additional variability is expected due to the absorption in the line height region by material other than algal pigments, specifically non-algal particles and colored dissolved organic matter.

3.2. Field results

Field observations from three diverse field locations and seasons were collected for analysis. Observations from the equatorial Pacific region were collected on 5 cruises as part of the Equatorial Box Project (Wang et al., 2009) in 2006. These stations were located from 145W to 170E and from 10N to 10S. Chlorophyll concentrations, measured by HPLC (NASA contract to Horn Point Laboratory) ranged from 0.07 to 0.3 mg/m^3, absorption line height ranged from 0.0016 to 0.0073 m^{-1}. Time series observations were collected from Harpswell Sound and the Gulf of Maine through a single spring to summer transition bracketing the spring bloom when chlorophyll concentration and species succession exhibit the largest range. Chlorophyll concentrations ranged from 1 to 10 mg/m^3 and absorption ranged from 0.02 to 0.08m^{-1}. Two blooms of *Prorocentrum micans* observed in West Boothbay Harbor in September 1999 and October 2001 (Etheridge, 2002) were also included in this analysis because of the extreme concentrations and nearly monospecific composition in a natural environment. The chlorophyll concentration for these two extreme blooms was approximately 126 and 480 mg/m^3; the absorption line height, measured using the quantitative filter technique was 0.86 and 5.4 m^{-1}, respectively.

The absorption line height as a function of extracted chlorophyll concentration for the field observations is shown along with the culture results (Fig. 5B). The slope and intercept for the

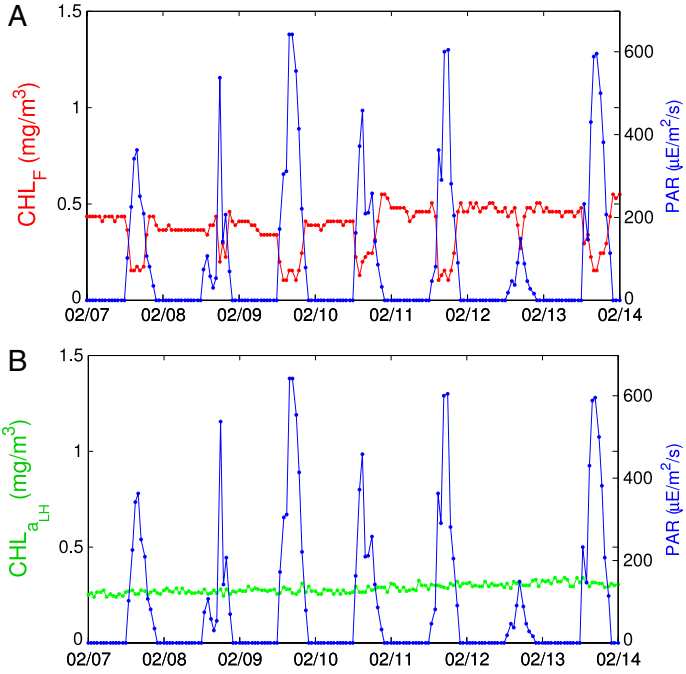

Fig. 6. Time series hourly observations from the Gulf of Maine Integrated Coastal Ocean Observing System Buoy I in February 2009. PAR (blue symbols; $\mu E/m^2/s$, right axes) computed from above water observations with a Satlantic OCR-507 (seven-channel irradiance sensor; Gregg and Carder (1990)) and chlorophyll concentration (left axes) estimated from A. calibrated chlorophyll fluorescence (red; mg/m^3); and B. 676 nm absorption line height (green, mg/m^3) from the *in situ* chlorophyll fluorometer and ac9, respectively, deployed at 2.5 m. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

complete set of observations are 0.0104 m^2/mg (95% confidence limits of ± 0.0004 m^2/mg) and -0.012 m^{-1} (± 0.022 m^{-1}), respectively ($r^2 = 0.98, p < 0.01$). The relationship between the chlorophyll-specific absorption at each wavelength and chlorophyll parameterized by Bricaud et al. (1995) was used to derive the absorption line height at 676 nm as a function of chlorophyll (solid line in Fig. 5B) for the range of chlorophyll values in their paper. The relationship was extended for the range of chlorophyll values observed here (dotted line).

The chlorophyll-specific absorption line height, a^*_{LH}, did vary slightly between the data sets (Table 1), with the highest values observed in the Equatorial Pacific and the lowest values in the Gulf of Maine. Overall a^*_{LH} values were much lower than the chlorophyll-specific absorption at 676 nm uncorrected for the baseline absorption a^*_ϕ by up to a factor of two. More significantly the variability in the slope of the relationship is significantly lower. Results were consistent with those predicted by the Bricaud et al. (1995) model, although the observed confidence limits are much lower than the modeled ones (indicating that the uncertainty is greatly reduced by subtracting the baseline which accounts for presence or absence of non-chlorophyll *a* absorption).

3.3. Diel non-photochemical quenching (NPQ)

Hourly *in situ* observations of PAR and calibrated chlorophyll fluorescence (CHL_F) clearly show the impact of high surface irradiance on non-photochemical quenching of fluorescence (Fig. 6A). Quenching initiates early each day the first hour after sunrise and begins recovery at about 4 h after local noon on average. Cloudy days with low PAR (below 200 $\mu E/m^2/s$) exhibit much weaker quenching and only during the hour around local noon but any PAR values above 80 $\mu E/m^2/s$ were associated with quenched fluorescence at 2.5 m. Quenched fluorescence values are nearly half of

unquenched evening values for data shown but have exhibited greater than 50% reduction. In contrast, the hourly observations of a_{LH} for the same period shows no diel variability. For comparison we computed the absorption based chlorophyll estimates as:

$$CHL_{a_{LH}} = a_{LH}/a^*_{LH} \tag{3}$$

where a^*_{LH} is the chlorophyll-specific absorption line height ($\approx$0.0104 m^2 mg^{-1}, average from Table 1). Note that both the hourly $CHL_{a_{LH}}$ and the nighttime CHL_F signals show a slight trend of increasing phytoplankton biomass over the 7-day interval.

3.4. Impacts of bio-fouling

The fluorescence signal detected by *in situ* fluorometers is very resistant to contamination by surface film biofouling as biofilms are not significantly fluorescent and do not significantly absorb the blue excitation light emanating through the optical head nor the red fluorescence from the environment entering through the optical head into the detector. Copper heads and wipers are effective agents for limiting large scale biofouling such as algae or invertebrates on the sensors themselves (Manov et al., 2004), and have greatly extended the deployment duration with high quality data. Conversely, absorption meters are highly prone to biofouling, particularly biofilms which not only attenuate the collimated beam but also impact the scattering properties of the optical surfaces and tubes. In productive coastal waters biofouling can have significant impacts (i.e. 10% of the signal) within one to two weeks. Although the absorption measurements are less impacted than the beam attenuation, the quantity of robust high quality data is significantly reduced.

While biofouling is a clear disadvantage for the absorption measurement, that absorption is measured in geophysical units (m^{-1}) relative to a known standard (i.e. pure water) makes it an optically robust measurement compared to fluorescence, although the sensitivity of absorption to chlorophyll concentration is much lower than that of fluorescence for current technologies. This is in part due to the optical sensing technology used in each method, but also due to the propagation of errors in the line height equation. However, a calibration-independent method for determining particulate absorption and, by extension, absorption line height using inline filters for ac sensors was developed (Slade et al., 2010) and successfully employed extensively on an inline optical system (Boss et al., 2013). By subtracting the absorption measured on filtered samples from those measured on the whole water samples, all calibration uncertainties, including biofouling, are removed. These authors have routinely demonstrated the ability to resolve line height absorption to better than 0.001 m^{-1}, which translates < 0.1 mg/m^3 chlorophyll *a*.

Moored applications do not yet have the capability for routine paired filtered and non-filtered observations, although that technology is just on the horizon. A four month time series of fluorescence and absorption line height is shown in Fig. 7. Twice during this deployment, a diver serviced all optical instrumentation, clearing away large macroalgal growth and invertebrates from the cage, cleaning all optical heads, and replacing tubes and tubing from the ac9 (indicated by arrows). A spectral view of the absorption indicates that the absorption signature of the biofouling material resembles the absorption spectrum of non-algal particles and in fact is similar in composition. Thus the absorption line height calculation efficiently removes the biofouling signature in the same way that non-algal particle and CDOM absorption contribution is removed from the total absorption spectrum. A comparison of time series observations of $a(676)$ and $a_{LH}(676)$ shows that the $a(676)$ time series observations are clearly impacted by biofouling and cleaning, while the $a_{LH}(676)$ time series show no impact of biofouling or cleaning.

For comparison, the calibrated chlorophyll fluorescence time series is also shown. Not only are the fluorescence and absorption line height times series extremely coherent with respect to their temporal patterns, but when both are converted to chlorophyll concentration they also exhibit nearly identical values, demonstrating the robust value of the average chlorophyll-specific line height absorption in Table 1. The significant relationship is made possible only because the midnight observations of fluorescence, which are not impacted by NPQ, were used. In the following section we address both the differences between two time series on the daily scale, shown in Fig. 7, and on an hourly scale, shown in Fig. 6, in which NPQ effects are observed.

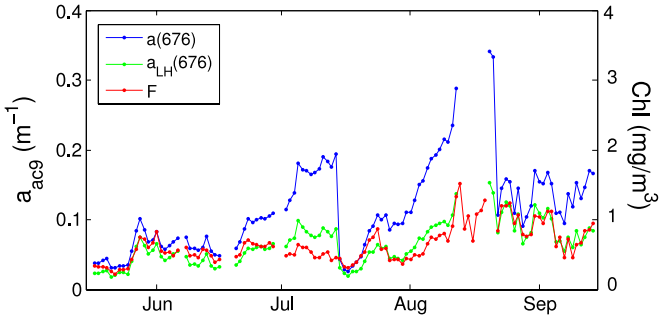

Fig. 7. Four-month time series measurements of WET Labs ac-9 and ECO chlorophyll fluorometers moored in the productive coastal waters of Harpswell Sound, Maine, in 2008 (GoMICOOS buoy DO2). The absorption coefficient at 676 nm is corrected for scattering by subtraction of 715 nm absorption (blue; Zaneveld et al. (1994)); absorption line height is computed from Eq. (1) (green, left axis) with the corresponding scale for chlorophyll concentration shown on the right axis. The two sharp decreases in absorption in mid-July and late August indicate times of diver servicing for bio-fouling removal. For comparison, the time series of midnight observations of calibrated chlorophyll fluorescence is also shown (red), right axis scale. Midnight fluorescence values are not sensitive to NPQ variations inherent in the chlorophyll fluorescence time series. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Beyond phytoplankton biomass

Our analysis in this paper is by no means meant to suggest that absorption-based chlorophyll proxies for phytoplankton biomass replace fluorometric ones. In fact, it is our supposition that the combination provides even more useful information than either in isolation. That the absorption line height is most resilient to variations in species composition and has no sensitivity to incident solar radiation make it the measure of choice for estimating chlorophyll concentration on a variety of platforms for which such natural variations impact chlorophyll fluorescence observations. The absorption line height method is therefore inherently more related to phytoplankton chlorophyll concentration than is chlorophyll fluorescence, and thus offers an improved method to understand and compare chlorophyll concentrations across many environments and over time. However, that *in situ* chlorophyll fluorescence *is* sensitive to incident irradiance and that the calibration *is* dependent upon the pigment composition and hence taxonomy, provides a new tool for moving beyond mere chlorophyll estimation. In fact, it can provide an improved understanding of why chlorophyll concentrations do vary over time. The combination of the two measures opens up new avenues for ecophysiological observations, potentially providing insights into remote detection of primary productivity (e.g. Huot et al., 2007).

In situ fluorometry is measured in relative and non-geophysical units and therefore requires calibration with a known standard. Unlike bench-top fluorometers used to determine extracted chlorophyll concentration, *in situ* fluorometry requires calibration with viable cells. This is in part because the standard excitation is 470 nm, a wavelength not absorbed by the chlorophyll molecule. Thus *in situ* fluorometers make use of the nearly perfect energy transfer from accessory pigments that absorb at 470 nm to chlorophyll which fluoresces. Secondly, acetone and other solvents used to extract chlorophyll or dissolve standards are damaging to optical sensors. Calibration with living cells is challenging because a choice of which species or combination of species should be used must be made, or one must rely upon vicarious calibration methods. Regardless, which ever calibrating standard is used, it will not represent the natural population in a different location or at a different time. Thus, selecting a single but invariant calibration standard may be preferable. *T. pseudonana* is a ubiquitous diatom found throughout the world's oceans and brackish environments. It is easy to culture and grows quickly. Its optical properties are robust when replicate cultures are grown year after year under the same growth conditions. Interestingly, it also has a fluorescence yield that represents the median value of a diverse range of phytoplankton species (Proctor and Roesler, 2010). Thus calibrating with this culture will not provide the accurate *in situ* chlorophyll value for every condition (that would require continuous *in situ* validation), but it will provide an accurate measure *on*

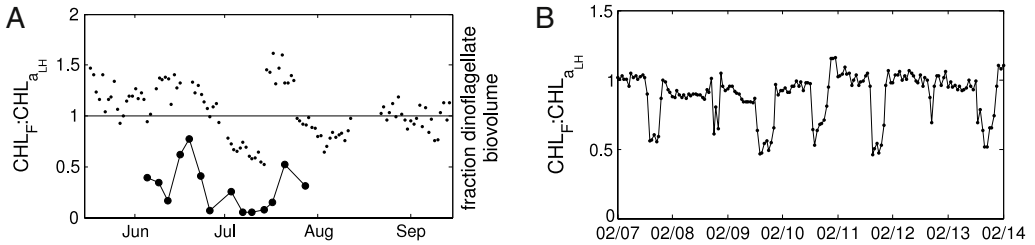

Fig. 8. Application of paired chlorophyll fluorometers and ac meters to compute the proxy for fluorescence quantum yield (CHL_F : $CHL_{a_{LH}}$) for determination of A. species composition changes from daily observations over a four month times series (data from Fig. 7) and B. the hour of the onset or release of NPQ determined on hourly observation over a one week period (data from Fig. 6). Dinoflagellate fraction of total biovolume (filled circles) shown in part A from G. Teegarden (unpublished results) varies from zero to 1 on left scale.

average. More importantly, it will provide a consistent and repeatable value for comparison between stations, over time and between investigators.

The ratio of the calibrated chlorophyll fluorescence and the chlorophyll absorption line height (Eq. (2)) provides a quantitative proxy for the fluorescence quantum yield:

$$\Phi_F = CHL_F / CHL_{a_{LH}}. \tag{4}$$

An investigation of this ratio on a range of time and space scales provides additional information beyond the phytoplankton biomass. Below we describe two such applications, the first as a proxy for pigment-based phytoplankton taxonomy, and the second as a proxy for determining E_K, the half saturation irradiance for photosynthesis.

3.5.1. Phytoplankton community structure

The fluorescence yield proxy, when evaluated during hours when NPQ is not a contributing factor, provides a mechanism for qualitatively assessing patterns in species succession (Fig. 8A). Proctor and Roesler (2010) established that the calibrated fluorescence yields (fluorescence per chlorophyll concentration) varied predictably between species. In their paper the fluorescence yields were presented as calibration slopes for a single characterized fluorometers determined for a range of monospecific cultures (Fig. 2). Specifically, the fluorescence yield of dinoflagellates was found to be statistically larger than that for diatoms (and in particular to the calibrating diatom *T. pseudonana*), and the fluorescence yield for cyanobacteria was significantly smaller than that for diatoms. Having established that the chlorophyll absorption is less sensitive than fluorescence to accessory pigment variability, then the ratio of chlorophyll estimated via calibrated fluorescence to that of absorption line height provides a proxy for fluorescence yield and a qualitative proxy for pigment-based algal taxonomy.

As a test of this concept, we selected the four month time series of fluorescence and absorption line height data collected from sensors deployed at 2.5 m depth in the productive Harpswell Sound in eastern Casco Bay, Maine (GoMICOOS buoy D; time series shown in Fig. 7). The period of observations spans mid-May through mid-September in 2008 (Fig. 8A). Midnight observations of the fluorescence were used in order to remove the impacts of NPQ; values were calibrated to chlorophyll using the standard species *T. pseudonana* (Proctor and Roesler, 2010). Daily median absorption line height values were converted to chlorophyll concentration via Eq. (3). When the proxy for fluorescence quantum yield computed via Eq. (4), is equal to one, that suggests that the population has both fluorometric and absorption properties similar to the calibration species *T. pseudonana*. When values exceed 1, the population has properties more similar to dinoflagellates; values less than 1, the population has properties more similar to other diatoms, and, as values decrease further, cryptophytes and cyanophytes. Microscopic evaluation of the phytoplankton community (G. Teegarden, unpublished data) supports the optical proxy. The phytoplankton populations were dominated by diatoms and dinoflagellates during this period and the percent of total biovolume attributed to dinoflagellates has peaks when the optical proxy ratio is greater than one (Fig. 8A).

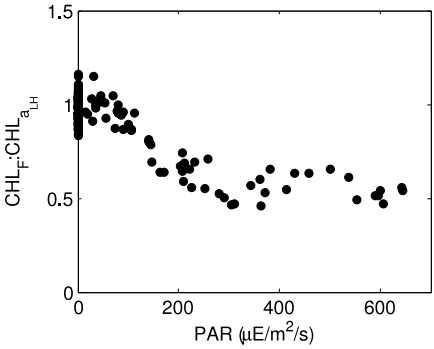

Fig. 9. Hourly observations of the proxy ratio for fluorescence yield versus instantaneous PAR measured *in situ* on a mooring (derived from data shown in Fig. 6).

Thus the fluorescence yield ratio, investigated on daily time scales (without NPQ effects) provides a useful proxy for identifying changing phytoplankton community composition. The potential for real time *in situ* opens up opportunities for targeted sampling, for identifying target species, and for investigating the successional patterns through a bloom. Furthermore, by utilizing additional fluorescence measurements resulting from a different excitation waveband (i.e. 532 nm excitation), Proctor and Roesler (2010) showed improved ability to identify phytoplankton community compositional changes based on fluorescence ratios. While a comparison of the two optical approaches warrants a systematic analysis, the above analyses provide a demonstration of potential in utilizing combined optical methods. By incorporating all of this information (the combination of species- and growth-rate-specific fluorescence and absorption information) species community shifts, physiological status, *and* chlorophyll concentration can be obtained from two optical signatures.

3.5.2. Phytoplankton photophysiology

The fluorescence NPQ index is generally computed from variable fluorescence, the relative difference between maximum fluorescence measured under light exposure, F'_m, to that measured in the dark-adapted state, F_m

$$NPQ = \frac{F_m - F'_m}{F'_m}. \tag{5}$$

It is well established that the dependence of NPQ on incident irradiance varies depending upon light acclimation and the light-response curve has been modeled for different organisms, including phytoplankton (Serôdio and Lavaud, 2011). Assuming that variations in phytoplankton biomass due to advection are negligible on these time and space scales, the NPQ index can be estimated from the moored observations where F_m and F'_m are observations taken in the evening to early morning and mid-morning to mid-afternoon, respectively.

Alternatively, the assumption of invariant time and space scales of advection may be a poor one for this approach and thus hourly ratios of fluorescence quantum yield proxy ($CHL_F : CHL_{a_{LH}}$) can be used to indicate the timing of when NPQ impacts the fluorescence (and hence photosynthesis; Fig. 8B). Each day when the CHL_F signal diverges from the $CHL_{a_{LH}}$ signal indicates the time at which photosynthesis is saturated. Simultaneous observations of PAR would therefore provide an instantaneous estimation of E_K, the saturation irradiance for photosynthesis, enabling more accurate bio-optical modeling of photosynthesis and growth (e.g. Cullen et al., 2012). We investigate the behavior of the fluorescence yield ratio for the seven days of hourly observations on GoMICOOS buoy I in February 2009 (data set shown in Fig. 6). The onset of NPQ occurs each day at approximately 9 am. Over this seven day interval, the saturating PAR values exceed 200 $\mu E/m^2/s$ and the half saturation irradiance, E_K, is approximately 100 $\mu E/m^2/s$ (Fig. 9). This figure also demonstrates that once quenching occurs, it remains nearly constant regardless of PAR, and for this 7-day interval, remains at a value of approximately 0.6. These results warrant further investigation over longer timescales and different seasons.

This same approach could be invoked for profile optical measurements shipboard or via remote vehicle. E_K would be the PAR value measured at the depth where profiles of CHL_F diverge from profiles $CHL_{a_{LH}}$. This *in situ* determination of the E_K parameter from simple optical sensors could open up new capabilities for remote detection of primary production and physiological assessments (Huot and Babin, 2011; Jolliff et al., 2012; Laney et al., 2005).

4. Conclusions

In this paper, we have presented an examination of fluorescence and absorption line height based methods for determination of chlorophyll concentration. While each method has its own set of advantages/disadvantages, we contend that by making both measurements, a greater understanding of phytoplankton ecology and physiology can be achieved. In terms of estimating chlorophyll concentration, we show evidence that in general, the absorption line height method is a more accurate, though slightly less sensitive method of determining *in situ* bulk chlorophyll concentration compared to standard *in situ* fluorometers. We find that pigment packaging effects and species-specific variations are the main contributors to the absorption to chlorophyll relationship, although the variability is significantly reduced compared to the specific absorption coefficient when not corrected for line height. We also find that at least an order of magnitude of variability in the chlorophyll specific fluorescence can be attributed to pigment packaging and species specific variations. The effects of NPQ can induce an additional order of magnitude of variability in the chlorophyll specific fluorescence, whereas the absorption by chlorophyll is unaffected by NPQ. For these reasons, we postulate that the absorption line height determination of chlorophyll can provide an improved method to estimate chlorophyll concentration as compared to typical single fluorescence excitation/emission pair based determinations. Furthermore, the combination of these two measurement techniques allows for more in depth assessment of the phytoplankton community structure and photophysiological status of the phytoplankton population.

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