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Recommendations for obtaining unbiased chlorophyll estimates from in situ chlorophyll fluorometers: A global analysis of WET Labs ECO sensors

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Abstract

Chlorophyll fluorometers provide the largest in situ global data set for estimating phytoplankton biomass because of their ease of use, size, power consumption, and relatively low price. While in situ chlorophyll *a* (Chl) fluorescence is proxy for Chl *a* concentration, and hence phytoplankton biomass, there exist large natural variations in the relationship between in situ fluorescence and extracted Chl *a* concentration. Despite this large natural variability, we present here a global validation data set for the WET Labs Environmental Characterization Optics (ECO) series chlorophyll fluorometers that suggests a factor of 2 overestimation in the factory calibrated Chl *a* estimates for this specific manufacturer and series of sensors. We base these results on paired High Pressure Liquid Chromatography (HPLC) and in situ fluorescence match ups for which non-photochemically quenched fluorescence observations were removed. Additionally, we examined match-ups between the factory-calibrated in situ fluorescence and estimates of chlorophyll concentration determined from in situ radiometry, absorption line height, NASA's standard ocean color algorithm as well as laboratory calibrations with phytoplankton monocultures spanning diverse species that support the factor of 2 bias. We therefore recommend the factor of 2 global bias correction be applied for the WET Labs ECO sensors, at the user level, to improve the global accuracy of chlorophyll concentration estimates and products derived from them. We recommend that other fluorometer makes and models should likewise undergo global analyses to identify potential bias in factory calibration.

Quantifying the distribution and variability in global phytoplankton biomass is a challenge that has been attempted for nearly a century using a range of approaches. The earliest studies incorporated cell enumeration (Booth 1993) and determination of water color (Wernand and Van Der Woerd 2010). Later studies relied on the uniqueness of the pigment chlorophyll *a* (Chl) to identify the presence and concentration of phytoplankton, although the relationship between Chl and cell carbon varies tremendously between species and within species as a function of environmental conditions (Banse 1977; Geider 1987; Cloern et al. 1995) and growth phases (Riemann et al. 1989; Geider et al. 1997). Despite the uncertainty in Chl as a proxy for phytoplankton biomass, the fact that all phytoplankton have Chl and only phytoplankton have Chl has led to its ubiquity and resilience as a biomass proxy with general acceptance. But sometimes forgotten are aspects of the uncertainty due to natural variations in phytoplankton carbon to Chl ratio. The optical characteristics of the Chl molecule enable the quantification of the in situ concentration via its distinct absorption (Lorenzen 1967) and fluorescence (Holm-Hansen et al. 1965)

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features in extracts of natural samples. Further, the in vivo absorption and fluorescence properties are easily monitored with in situ absorption meters (Davis et al. 1997; Boss et al. 2013; Roesler and Barnard 2013) and Chl fluorometers (e.g., Lorenzen 1966; Falkowski and Kiefer 1985), respectively. Chl fluorescence measurements are easy and economical to collect, which has given rise to increased numbers of fluorometers deployed around the world on a variety of platforms. Often overlooked is that the ratio of fluorescence to Chl concentration, the quantity required for sensor calibration, varies as a function of species, photoacclimation, nutrient limitation and acclimation, growth phase, and non-photochemical quenching (NPQ). The challenges lie in the details of the measurements and the assumptions of the chain of proxies between what is measured and what is actually phytoplankton biomass (Cullen 1982).

Recent technological advances have reduced sensor size, power requirements, and cost, enabling deployment from autonomous platforms, including underway inline systems, moorings, profiling floats, gliders, and Autonomous Underwater Vehicles (AUVs). With strong input from the community, the quality control on these sensors has improved substantially so that side-by-side deployments provide replicated values within uncertainty levels reported by factory specifications (e.g., D'Ortenzio et al. 2010; Briggs et al. 2011; IOCCG 2011; Xing et al. 2012; Schmechtig et al. 2015). One aspect of sensor deployment protocols is the collection and HPLC analysis of discrete water samples adjacent to and synchronous with sensor observations. HPLC analysis of total Chl concentration [Chl], is the community-accepted validation product (e.g., Claustre et al. 2004). Furthermore, the comparison between HPLC-derived [Chl] and in situ calibrated Chl fluorescence is the most robust and rigorous way for community-wide validation to be implemented (e.g., direct calibration as in Lavigne et al. 2012; merging of a large HPLC-fluorescence database for a posteriori calibration of fluorescence profiles using a neural network approach as in Sauzède et al. 2015). However, direct fluorescence to HPLC comparisons are not straight forward. In vivo phytoplankton fluorescence is quenched (reduced) following exposure to high light by a phenomenon reported as NPQ (Huot and Babin 2011). Hence fluorescence values used for calibration need to be taken either at low light or corrected for the quenching effect. In addition, it has been reported that chlorophyll fluorometers also excite Colored Dissolved Organic Matter (CDOM) fluorescence (e.g., Proctor and Roesler 2010), which can be a significant bias at high CDOM concentration (e.g., Black Sea, river plumes) or at depth where chlorophyll is zero but CDOM concentration can be high (e.g., Xing et al. 2017).

As the number of deployments increases and the number of point-by-point validations is presented at scientific meetings and workshops, we have become aware of an apparent bias in the calibrated Chl fluorescence values observed globally using WET Labs ECO series Chl fluorometers. The bias appears regardless of the source of data used for validation, including HPLC and other validation data sources such as ocean color (e.g., Boss et al. 2008), radiometry (e.g., Xing et al. 2011), red peak absorption line height (e.g., Roesler and Barnard 2013). Most critically, we observe the bias *after* correction for or removal of NPQ and CDOM fluorescence contamination of in situ Chl fluorescence observations. In this article, we describe the sources of uncertainty, provide evidence for the bias, quantify the bias, and make recommendations to the community of users so as to both improve the accuracy and reduce the uncertainty in global estimates of Chl concentration and hence phytoplankton biomass. We also propose that while this bias was identified for a specific make and model series of in situ fluorometers (e.g., WET Labs ECO series), similar biases may exist in other commercially available in situ Chl fluorometers. Each manufacturer independently determines a factory-specific calibration standard, which may or may not correspond to the global mean. We recommend that each sensor make/model undergo a comparable global analysis as was performed here.

Data and analysis

Principles of in vivo fluorometric Chl analysis

Regardless of manufacturer, in vivo chlorophyll fluorometers are all designed around common principles (Huot and Babin 2011) which are presented here in simplified terms. Briefly, a volume of water is illuminated with an excitation light source (typically in the blue-green region of the spectrum which stimulates absorption by either Chl or photosynthetic accessory pigments), the phytoplankton within that target volume absorb the excitation energy and fluoresce red light energy within a narrow spectral band. A fraction of that emitted energy from the target volume is detected by the sensor. The detected energy is reported in raw analog or digital form in relative units (volts or digital counts), and this signal is calibrated with a standard to convert the output into concentration units of (mg Chl m^{-3}) or equivalent. The differences between manufacturers lie with the configuration of the target volume, the lamp source, the detector, and the calibration standard. Commercial fluorometers typically target fluorescence by the Chl *a* molecules in photosystem II, for which the peak fluorescence emission is approximately 685 nm, although not all Chl *a* is in photosystem II. The underlying theory behind the measurement is that the intensity of the measured Chl fluorescence (F_{Chl} [μmol photons fluoresced $m^{-3}s^{-1}$]) is given by

$$F_{\mathrm{Chl}} = \Phi_F \int_{400}^{750} E(\lambda) * a^*_{\mathrm{Chl}}(\lambda) * \mathrm{Chl}\ d\lambda \qquad (1)$$

where $E(\lambda)$(μmol photons $m^{-2}s^{-1}nm^{-1}$) is the available spectral scalar irradiance at wavelength λ (nm), the sensor excitation irradiance, $a^*_{\mathrm{Chl}}(\lambda)$ is the spectral Chl-specific absorption coefficient (m^2 $mgChl^{-1}$), Φ_F(μmol photons fluoresced)

[μmol photons absorbed]$^{-1}$ is the fluorescence quantum yield, and the product is integrated over the photosynthetically available radiation (PAR) range from 400 nm to 750 nm. For a Chl fluorometer, the equation is rearranged to solve for the Chl concentration:

$$\mathrm{Chl} = {}^{F_{\mathrm{Chl}}}\Big/_{\Phi_F \int_{400}^{750} E(\lambda) * a^*_{\mathrm{Chl}}(\lambda)\ d\lambda.} \quad (2)$$

Assumptions for in vivo fluorometric Chl analysis

In reality, the terms in the ratio of Eq. 2 are not quantitatively measured. Only a fraction of the total fluorescence is detected. The Chl-specific absorption coefficient, while constant and known for extracted Chl (Jeffrey and Humphrey 1975), is neither constant nor known a priori for living cells due to variations in cellular pigment concentration, packaging, and pigment composition (Morel and Bricaud 1981; Bricaud et al. 1983). The quantum yield of fluorescence is likewise not constant and varies with phytoplankton composition, light history, and other environmental and physiological factors (Falkowski and Kiefer 1985; Mitchell and Kiefer 1988; Sosik and Mitchell 1991; Greene et al. 1994; Falkowski and Kolber 1995; Vassiliev et al. 1995; Babin et al. 1996; Behrenfeld and Kolber 1999; Parkhill et al. 2001; Kruskopf and Flynn 2006; Richardson et al. 2010). The underlying assumptions for in vivo fluorescence to be linearly related to [Chl] is that the excitation energy, E, is saturating and constant among measurements (Neale et al. 1989), and that both the Chl-specific absorption coefficient and the quantum yield of fluorescence are constant, their product is constant or they linearly covary with fluorescence. With these assumptions, the intensity of the measured fluorescence is proportional to the [Chl]. The proportionality constant, which is essentially the spectrally integrated product of Chl-specific absorption and fluorescence quantum yield, is determined by calibration. Calibrations are performed by measuring the in vivo fluorescence and extracted [Chl] for a dilution series of phytoplankton (a standard curve). Thus the two sources of uncertainty in this approach are (1) the variability in the Chl-specific absorption and (2) the variability in the fluorescence quantum yield. There is no consensus on what calibration standard should be used to enable global comparisons amongst fleets of deployed Chl fluorometers and between manufacturers (Table 1).

Observations of calibration bias in WET Labs, ECO Chl fluorometers

Paired HPLC and in situ Chl fluorescence

We assembled a data base of paired discrete HPLC total [Chl] analyses and in situ Chl fluorescence observations, collected with WET Labs ECO-series digital Chl fluorometers (F_{Chl}) deployed on shipboard profiling packages, underway flow through systems, moorings, and profiling floats (Table 1). Because a significant source of variability in the fluorescence quantum yield is due to NPQ, and since there is no community consensus on correction, we selected our paired observations to exclude fluorescence observations that were located at depths and/or times for which PAR exceeded 200 μEin m^{-2} s^{-1}, a value at which quenching is observed to exceed 10% reduction in in vivo fluorescence (Marra and Langdon 1993; Morrison 2003; Morrison and Goodwin 2010; Serôdio and Lavaud 2011; Xing et al. 2012; Roesler and Barnard 2013). That said, we recognize that a majority of in vivo fluorescence observations are impacted by NPQ because they are collected (1) during daylight hours, (2) in the upper part of the euphotic zone above the level of light saturation and/or (3) in regions of potential iron limitation (Falkowski and Kolber 1995; Claustre et al. 1999; Behrenfeld and Boss 2006; Behrenfeld et al. 2006; Sackmann et al. 2008; Lavigne et al. 2012; Guinet et al. 2013; Roesler and Barnard 2013; Cetinić et al. 2015; Sauzède et al. 2015; Swart et al. 2015). However, uncorrected quenched in vivo fluorescence is not a robust proxy for [Chl], yielding significantly underestimated [Chl] values; quenched fluorescence observations should be utilized only after robust correction for NPQ is applied. The uncertainty associated with NPQ is not an issue of calibration but of a light-dependent physiological response that is not the goal of this analysis.

The point-by-point comparisons for paired HPLC Chl and in situ factory-calibrated Chl fluorescence observations yield large scatter in the data distribution (Fig. 1A). While this particular data set represents observations made with one model series from one manufacturer, the data spread is comparable to those observed with any make/model of in situ fluorometer. The global data set might leave the impression that there is sufficient uncertainty in the relationship to negate the utility of in situ fluorometry for quantification of phytoplankton biomass. However, when data sets are restricted spatially and/or temporally, the linearity of the relationship between the factory-calibrated in vivo fluorescence-derived Chl and the HPLC-derived total Chl strengthens significantly and the variability reduces, however, the regional slopes remain quite variable (Fig. 1B). Here, we define the ratio between the factory-calibrated in vivo fluorescence-derived Chl and the HPLC-derived Chl as the dimensionless "slope factor." This is not to be confused with the calibration slope obtained from laboratory standard curve analyses of raw fluorescence and extracted Chl that has units (digital counts [mg Chl m^{-3}]$^{-1}$ (Figure 1)).

A regional analysis of the slope factor demonstrates that it varies from approximately 1 in the Arabian Sea coastal upwelling region to greater than 6 in the Southern Ocean province south of New Zealand (Fig. 2). The observed regional differences in the slope factor cluster around biogeochemical provinces. Following the approach of Longhurst (1998), a number of authors have utilized synchronous satellite-based observations of the state and climatological physical and biological properties to define biogeographic provinces (Banse and English 2000; Oliver and Irwin 2008; D'Ortenzio and Ribera D'Alcalà 2009). A comparison of the

Table 1. Location and dates of observational programs that contributed to the global analysis. The slope factor (factory calibrated Chl/HPLC Chl) is derived by regression with ± 95% confidence limits on the regression. Uncertainty propagation for global statistics calculated following JCGM (2008). Associated Longhurst biogeographical province numbers in parentheses taken from http://www.marineplan.es/ES/fichas_kml/biogeog_prov.html. Entries ordered by latitude ranges N to S.

Project/ cruise	Oceanic province (Longhurst province)	Lat/Lon ranges		Dates	Slope factor (HPLC)	Citation(s)
MALINA	Arctic Ocean (27)	71N	127W	Jul 2007	1.27±2.01	Coupel et al. (2015)
NAB08	Iceland Basin (25)	60–62N	25–28W	May 2008	1.70±0.51	Cetinić et al. (2015)
					2.60±0.78*	
GEOVIDE	Subarctic Atlantic	56–60N	27–39W	Jun 2014	4.15±0.46	unpublished results†
NAT-LAS	Ocean (24)	58N	51W	May 2013		
Bio-ArgoMED	Western	41–44N	7–12E	May 2015	1.62±0.28	unpublished results†
BOUSSOLE	Mediterranean (43)	43N	8E	Jul 2013		Antoine et al. (2008)
DEWEX		42N	5E	Feb–Apr 2013		Lavigne et al. (2015)
MOOSE		41–42N	5–6E	Jul 2014		D'Ortenzio et al. (2014) Lavigne et al. (2015)
Bio-ArgoMED	Eastern	34–38N	19–29E	May 2015	1.72±0.23	unpublished results†
BOUM	Mediterranean (43)	34N	33E	Jun 2008		Crombet et al. (2011)
SS286	Arabian Sea	18–21N	67–70E	Mar 2011	2.15±1.59	Do Rosário Gomes et al. (2014); Thibodeau et al. (2014)
	offshore gyre (32)	21–22N	66–70E		1.04±0.15	
	Monsoonal upwelling (22)					
OUTPACE	South Pacific Ocean (51)	19S	165–171W	Mar 2015	2.80±0.81	unpublished results†
SOCLIM	South Indian Ocean (52)	43–53S	52–72E	Jan 2015	3.46±0.35	unpublished results†
SOCCOM	Southern Ocean (23, 53)	39–68S	13E–144E	Mar 2014–Mar 2016	6.44±1.31	Schuller et al. (2015); Boss and Haëntjens (2016)
	Global				2.63±0.29	
	Data set mean				2.15	
	Data set median				4.00±0.48	
	Areally-averaged mean					

*Three models of fluorometers were used on the NAB08 cruise; FLNTU and ECOBBFL2 both exhibited a slope factor of 1.7, while the ECOTriplet exhibited a slope factor of 2.6.
†HPLC method as in Ras et al. (2008).

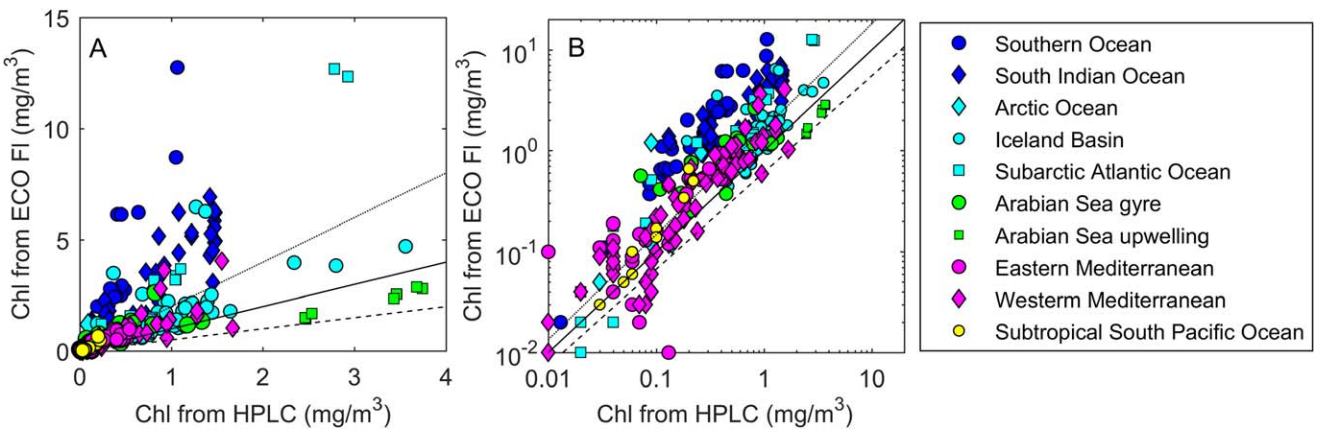

Fig. 1. Regression between factory-calibrated in vivo chlorophyll fluorescence observations from WET Labs ECO sensors and paired HPLC total Chl samples for the regions described in Table 1 on (**A**) linear and (**B**) log scales. Lines indicate slope factors of 0.5 (dash), 1 (solid), and 2 (dotted).

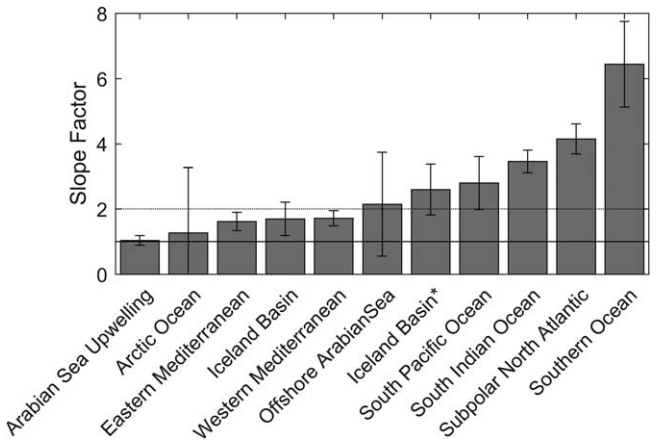

Fig. 2. Mean slope factors derived from observations of paired HPLC and in situ Chl fluorescence from major oceanographic regions (Table 1). Error bars indicate 95% confidence limits on slope from linear regression of all observations within each region. Lines indicate slope factors of 1 (solid) and 2 (dotted).

province-specific slope factor values and the biogeographic provinces proposed by Oliver and Irwin (2008) indicates that there is a strong correlation between the slope factor values and these independently-determined provinces. The Arctic Ocean, Iceland basin, Mediterranean Sea, and Arabian Sea upwelling zone all have slope factors within the range of 1.2–1.7 and are all characterized by similar biogeochemical provinces. The oligotrophic waters of the South Pacific and the monsoonal gyre province in the Arabian Sea (as defined by Longhurst 2006), are similar on province scales and have slightly higher slope factors, 2.15–2.80. Finally, the subpolar Indian, Atlantic, and Southern Oceans have increasingly higher slope factors, from 3.46 to 6.44. All are characterized by mixtures of three polar biogeochemical provinces, although the Southern Ocean sampled by SOCCOM profiling float deployment cruises is dominated by only one of the three polar provinces.

Only two of the sampled oceanic provinces have slope factors between 1 and 1.5, four have slope factors between 1.5 and 2.5, and the remaining four have slope factors in excess of 2.5. The median and mean slope factor values for this data set are 2.15 and 2.63, respectively. However, these statistics equally weight the slope factors without accounting for the global distribution and the spatial extent of the observations. A better statistic is to use the province definitions and province areas described by Longhurst (2006) to compute an areally-averaged slope factor. For this data set, that yields a value of 4.00 ± 0.48, with the sampled provinces representing approximately 40% of the global province area. An examination of the sampled vs. non-sampled provinces suggests this value to be heavily weighted by large slope factors the high latitudes. More paired observations in the tropical and subtropical gyre regions, which exhibit lower slope values and have larger areal coverages lowers the global areally averaged value to 2. This suggests that the in situ fluorometric estimate of Chl obtained by WET Labs ECO fluorometers using the factory-provided calibration overestimates Chl throughout most of the ocean by a factor of 2, however, the polar regions diverge from the global median by at least a factor of 4 in the subarctic Atlantic and 6 in the Southern Ocean. Globally, the median slope factor suggests there is a factor of 2 overestimation of Chl on which regional variations of the fluorescence-to-Chl ratio are superimposed.

Additional supporting observations of bias: Paired radiometry and in situ fluorescence from Biogeochemical-Argo floats

At the time of deployment, most of the Biogeochemical-Argo floats are associated with a Conductivity Temperature Depth (CTD)-rosette cast aiming at acquiring reference data for validation and/or calibration of the various sensors. For the Chl fluorometer, HPLC determination of [Chl], represents the reference calibration measurement. However, while such calibrations typically hold for the time and location of the deployment, they might become less valid with time as the float explores new waters and different seasons. Changes in environmental factors (nutrient availability, light regime, temperature), possibly associated with modifications in the structure of phytoplankton communities, likely affect the relationship between fluorescence and [Chl] cached in the initial reference calibration. Acquisitions of long Biogeochemical-Argo time series (up to 4–5 yr) are clearly a program's objective to make it cost-effective. It is therefore necessary to develop novel approaches to assess changes in the sensor calibration over time or to quantify the natural variations in the fluorescence to Chl ratio that occur seasonally and regionally over the lifetime of individual floats (Boss et al. 2008).

For Biogeochemical-Argo floats equipped with both a Chl fluorometer and radiometer acquiring downwelling irradiance at 490 nm, Xing et al. (2011) proposed a self-consistent "radiometric" calibration method of the fluorescence profile in [Chl]. The approach is to establish a direct relationship between radiometry and fluorometry (i.e., downwelling irradiance at 490 nm, E_d490, and Chl fluorescence), based upon the optical definition of the diffuse attenuation coefficient at 490 nm (K_d490) and its bio-optical relationship to [Chl] (Morel et al. 2007), which is then compared to the in situ observations of Chl fluorescence. In this analysis, the slope factor is radiometrically determined as the ratio of the paired Chl observations retrieved from the factory calibrated fluorometer to those derived from radiometry.

We applied the Xing et al. (2011) method to 7945 vertical profiles acquired by 98 Biogeochemical-Argo floats (type: "Provor-CTS4"; (Organelli et al. 2016) deployed between October 2012 and January 2016 in various open ocean areas representative of a wide range of oceanic conditions prevailing in the so-called Case 1 waters (Morel and Prieur 1977) (Table 2 and Fig. 3). This array of floats acquired 0–1000 m

Table 2. Number of floats and profiles for each of the 16 oceanic regions included in the Biogeochemical-Argo database. Mean slope factor (factory calibrated Chl/radiometric Chl) for each region computed from lifetime median slope factor for each float in the region.

Region	Basin	Abbreviation	N° float	N° profiles	Slope factor (radiometric)
Black Sea	Black Sea	BLACK_SEA	3	214	0.65 ± 0.03
Mediterranean Sea	Northwestern	MED_NW	9	630	1.47 ± 0.12
	Southwestern	MED_SW	6	555	1.56 ± 0.10
	Tyrrhenian Sea	MED_TYR	6	357	1.47 ± 0.09
	Ionian Sea	MED_ION	8	719	1.64 ± 0.14
	Levantine Sea	MED_LEV	7	502	1.80 ± 0.11
Red Sea	Red Sea	RED_SEA	2	78	1.74 ± 0.11
North Atlantic Subpolar Gyre	Labrador Sea	NASPG_LAS	12	878	2.15 ± 0.37
	Irminger Sea	NASPG_IRM	10	455	2.48 ± 0.15
	Iceland Basin	NASPG_ICB	7	833	2.49 ± 0.31
Subtropical gyres	North Atlantic	NASTG	4	341	2.22 ± 0.20
	South Atlantic	SASTG	3	349	3.34 ± 0.16
	South Pacific	SPSTG	3	263	2.91 ± 0.64
Southern Ocean	Atlantic sector	SO_ATL	2	246	4.30 ± 1.36
	Atlantic to Indian sector	SO_ATOI	10	878	3.89 ± 0.69
	Indian sector	SO_IND	6	647	4.13 ± 0.65

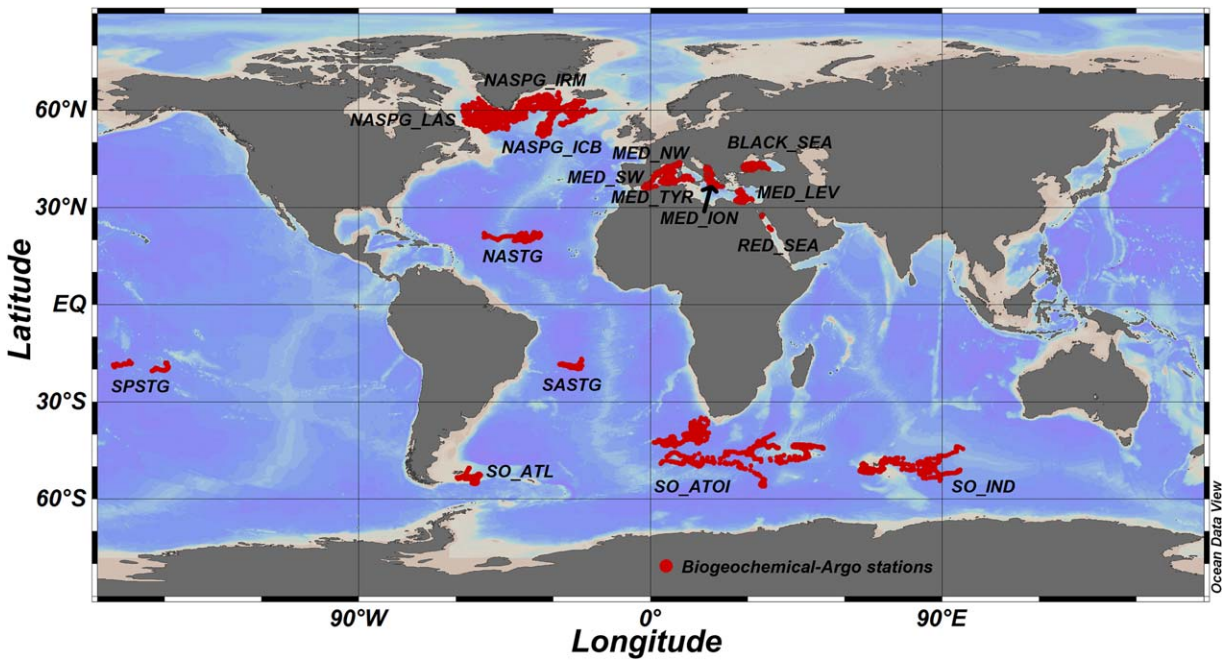

Fig. 3. Stations sampled by 98 Biogeochemical-Argo floats between October 2012 and January 2016 (Table 2).

upward casts every 1–10 d, around local noon. A WET Labs ECOPUCK fluorometer (excitation 470 nm, emission 695 nm) provided vertical profiles of Chl fluorescence. A Satlantic OCR-504 multispectral radiometer supplied 0–250 m profiles of E_d490. Raw data of both variables were first converted to geophysical units (mg Chl m^{-3} and $\mu W\ cm^{-2}\ nm^{-1}$) using manufacturer-provided calibration coefficients. Chl profiles were quality controlled following Schmechtig et al. (2015), corrected for NPQ (Xing et al. 2012) and the influence of dissolved and detrital organic material fluorescence (Xing et al. 2017).

The median value of the (radiometric) slope factors for each float was calculated over the lifetime of the float to address both the accuracy of the factory calibration with

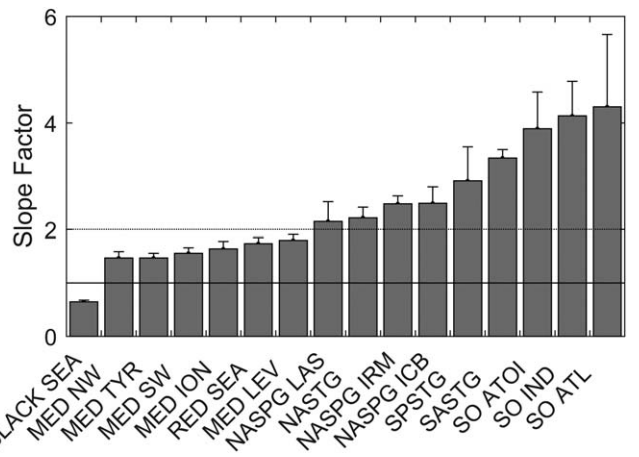

Fig. 4. Mean slope factors derived from ratio of factory calibrated Chl fluorescence to radiometrically-derived Chl (*see* text for details) obtained from profiling biogeochemical Argo floats described in Table 2. Error bars indicate 95% confidence limits on slope derived from regression of all observations within each region. Lines indicate slope factors of 1 (solid) and 2 (dotted).

respect to the temporal evolution of the environment and associated phytoplankton community that a float can encounter during its life-time. The Biogeochemical-Argo database was then split in 16 different regions (Table 2) and the mean slope factor value among the floats within the region was computed.

Except for the Black Sea, there is an overall overestimation of [Chl] at the global scale using the factory calibration as compared to the "radiometric" calibration (Fig. 4). Admittedly, the radiometric method is heavily dependent on the choice of the bio-optical relationship linking K_d490 to [Chl] (Morel et al. 2007), which varies regionally and includes CDOM absorption, and hence influences the magnitude of overestimation. Nevertheless, this relationship was established from a global database representative of the diversity of open ocean waters, actually from the ultra-oligotrophic waters of the South Pacific Gyre to eutrophic waters associated to upwelling conditions along the South African coast. Thus, relying on this bio-optical relationship is sensible for investigating regional anomalies in the Chl fluorescence calibration slope factor.

The range of [Chl] overestimation by the factory calibrated fluorometer as assessed with the radiometric slope factor is consistent with that determined using the HPLC reference calibration (Fig. 2). In particular, it is clear that the factory calibration is overestimating [Chl] in Southern Ocean waters by at least a factor of 4. Similarly, radiometrically-determined [Chl] is overestimated in the three basins of the North Atlantic Subpolar gyre by a factor of 2.2–2.5. As found for the HPLC-derived slope factor, the factory calibrated fluorometers overestimated the radiometrically-determined [Chl] in the Mediterranean Sea by a factor of 1.5–1.8 with the previously observed trend from the western mesotrophic waters (lowest values) to the eastern oligotrophic waters (highest values). The radiometric slope factor in the subtropical gyres is higher in the southern hemisphere (3.4 in the Atlantic and 2.9 in the Pacific, similar to the HPLC-derived slope factor) than in the northern hemisphere (2.2 in the Atlantic). Finally, the particular case of the Black Sea (slope factor of 0.7) reinforces the utility of the radiometric approach to refine and better constrain the calibrations for float time series. Indeed, the underestimation clearly illustrates that the global K_d490 vs. [Chl] relationship expectedly does not apply to waters with known high CDOM content (Suetin et al. 2002; Kopelevich et al. 2010). Additionally, the contribution of fluorescence by CDOM, detected by chlorophyll fluorometers (Proctor and Roesler 2010; Xing et al. 2017), will further reduce the apparent slope-factor.

Additional supporting observations of bias: Paired absorption line height and in situ fluorescence

We have compiled two data sets for which paired absorption line height and in situ Chl fluorescence observations were collected. The first is from an underway flow through bio-optical package (WET Labs acs and WETStar Chl fluorometer) during the TARA-Oceans cruise (Fig. 5A, Boss et al. 2013). The second is derived from moored bio-optical sensors (WET Labs ac9s and ECO Chl fluorometers) deployed in the Gulf of Maine (Roesler and Barnard 2013). In both data sets, the absorption line height was calibrated with HPLC-based [Chl]. The two line height conversion factors presented in the two papers do not result in statistically different estimates of Chl. The inclusion of both data sets is intended to provide a sense of how the slope factor varies spatially along a cruise transect and through time at one location, respectively.

While much of the Tara Oceans absorption-based slope factors are close to unity with a median value of 1.36 ± 1.10 (Fig. 5B), there are strong regional patterns. Observations from the South Pacific subequatorial waters provide slope factors consistently close to 3 (with some values in excess of 6), as was found with the HPLC-derived slope factors. The patterns are also reminiscent of spatial patterns of iron limitation identified by Behrenfeld et al. (2006). The absorption-based slope factor observations from the North Pacific subtropical waters are between 0.5 and 1, clearly lower than those derived either from HPLC or radiometry. At this point, it is unclear if this is an anomaly of the method or a true anomaly as we do not have HPLC-based or radiometer-based slope factors for these waters.

The observational time series from the Gulf of Maine mooring from May 2008 to September 2008, exhibited a median absorption-based slope factor of 2.04 ± 0.05, with values consistently above 1 (range: 1.03–3.19; Fig. 6). The temporal patterns in the slope factor vary on time scales consistent with succession of phytoplankton community

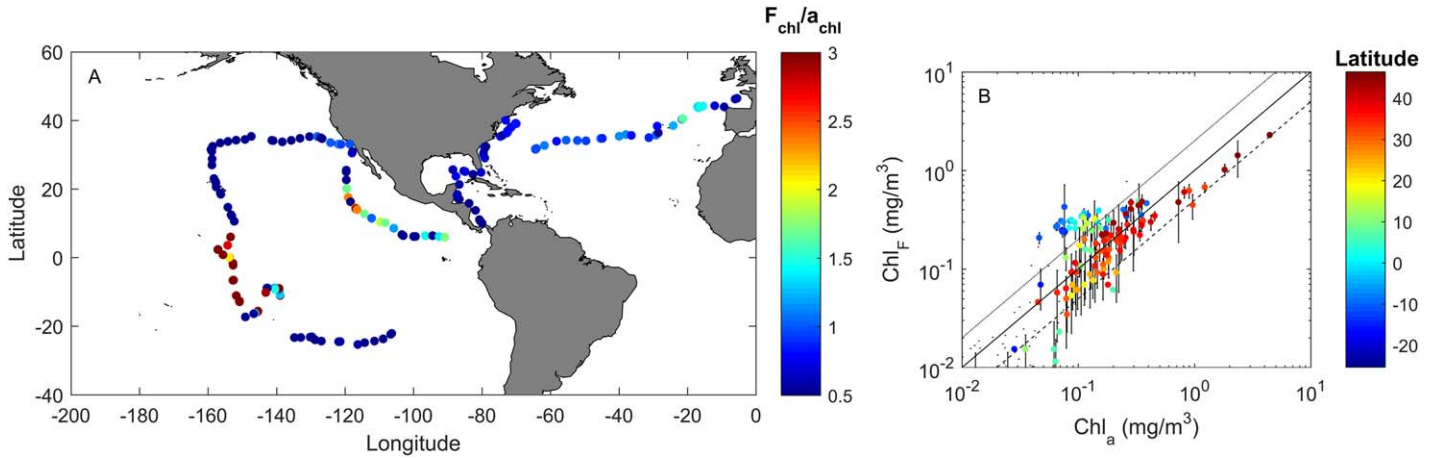

Fig. 5. (**A**) Variations in the underway slope factor derived from the ratio of factory calibrated Chl fluorescence and the Chl derived from absorption line height (Boss et al. 2013; Roesler and Barnard 2013) along the cruise track of the R/V Tara. (**B**) Daily observations of paired factory calibrated Chl fluorescence and the Chl derived from absorption line height. Observations impacted by NPQ were removed from the daily median, error bars indicate the standard deviation of observations in the daily median. Symbols color coded by latitude for comparison with cruise track locations in (**A**). Lines indicate slope factors of 0.5 (dash), 1 (solid), and 2 (dotted).

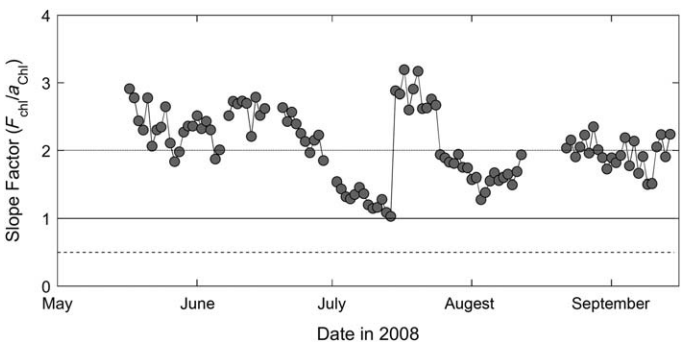

Fig. 6. Time series observations of the absorption-based slope factor derived from paired estimates of [Chl] from factory calibrated Chl fluorescence and ac9 absorption line height (Roesler and Barnard 2013) for 4 months in 2008. Data were collected from mooring D02 as part of the Gulf of Maine Ocean Observing System (GoMOOS) in Harpswell Sound, Casco Bay. Lines indicate slope factors of 0.5 (dash), 1 (solid), and 2 (dotted).

composition, as was confirmed with microscopic evaluations (Roesler and Barnard 2013). Slope factors values between 1 and 2 were associated with diatom-dominated communities, while values between 2 and 3 were associated with dinoflagellate-dominated communities.

Sources of variability in the calibration slope

The choice of calibration standard determines the factory calibration. Unlike benchtop fluorometers which can be calibrated with a solution of purified Chl in acetone, WET Labs ECO series fluorometers provide excitation with a 470 nm LED, which does not excite Chl in extract, but does excite photosynthetic accessory pigments in vivo. Current standard factory protocols for WET Labs ECO Chl fluorometers include gain adjustments for each sensor in a uranine solution to yield a constant calibration factor. The factory has quantified a transfer function between the uranine solution and a single calibration with a monospecific culture of the diatom *Thalassiosira weisflogii* with unspecified growth conditions. In contrast, the experiments conducted by Proctor and Roesler (2010), and expanded by recent experiments at Laboratoire d'Océanographie de Villefranche-sur-Mer (LOV) (using additional cultures of RCC42 *Synechococcus* sp., RCC233 *Tetraselmis* sp., and RCC834 *Micromonas pusilla* obtained from Roscoff RCC), were made on various cultures under controlled conditions and revealed that calibration slopes vary by over an order of magnitude depending upon phytoplankton species, consistent with Strickland (1968), and that the slope factor varied from approximately 0.5 to nearly 6, consistent with the global range (Fig. 7). This source of variability is captured by the Chl-specific absorption coefficient in Eq. 2 which is controlled by the cell size distribution and composition of photosynthetic accessory pigments, and by the fluorescence quantum yield which is controlled by light history and nutrient status.

There are three important conclusions to be drawn from these results. (1) There is a very large variability in calibration slope between species (even within taxonomic orders) that greatly exceeds the variability within a species due to photoacclimation or growth phase (error bars in Fig. 7). (2) The WET Labs calibration relies on only one species at a single (unspecified) growth condition. Thus the selection of the calibration standard can easily lead to significant over- or underestimation of in situ [Chl]. (3) The median slope factor for this set of 18 cultures grown under a range of conditions and in a variety of growth stages is 1.97, consistent with the global median bias. This median value is statistically identical to that

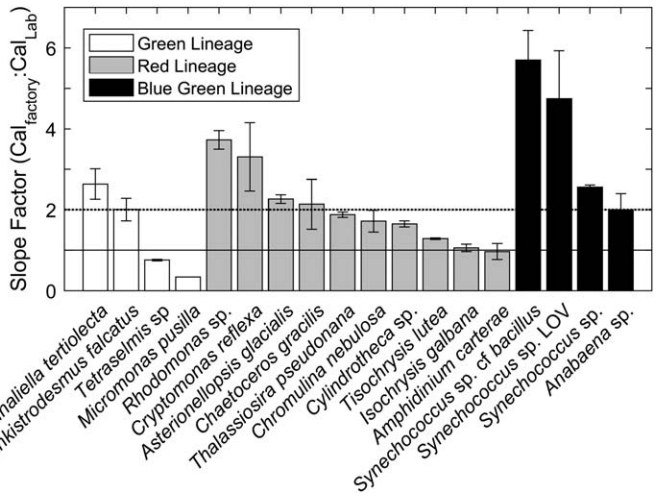

Fig. 7. Slope Factors derived from Chl calibration experiments using the WET Labs ECO series 470 nm excitation, 695 nm emission fluorometers. Each laboratory calibration slope was calculated using a type II regression of a standard curve for monospecific cultures in the laboratory (Cal_{Lab}). The raw fluorescence observations were converted to Chl using the factory calibration ($Cal_{factory}$), the ratio yielding the slope factor. For each species, the error bar represents the uncertainty propagated from the standard deviation of the calibration slopes obtained from cultures grown in limiting and saturating irradiances, cultures harvested in exponential growth phase and those obtained from a time course growth experiment hence providing quantitation of the natural variations within a species. Lines indicate slope factors of 1 (solid) and 2 (dotted). Figure modified from Proctor and Roesler (2010) with additional species.

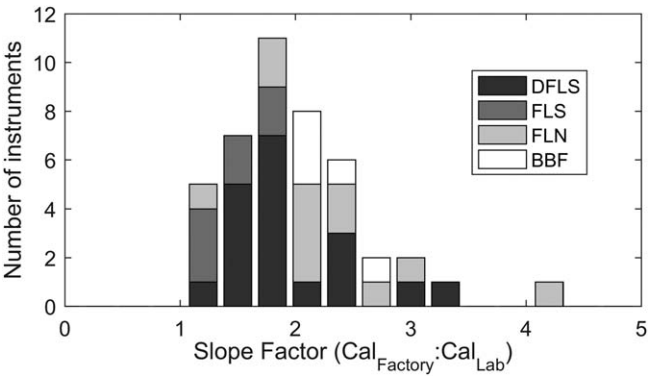

Fig. 8. Histogram of median slope factors for 55 WET Labs, digital fluorometer sensors in the ECO series from 2001 through 2016. The slope factor for each sensor was computed over the lifetime of the sensor and comprises from 3 to 17 separate calibrations (factory and laboratory) depending upon the lifetime of the sensor.

obtained from the culture of *Thalassiosira pseudonana*, a centric diatom. The standard deviation on the median is very low but represents multiple calibration experiments obtained from four purchased cultures (2004, 2010, 2011, and 2016), from two sources (Bigelow CCMP, now NCMA, https://ncma.bigelow.org/, and Roscoff RCC, http://roscoff-culture-collection.org/), grown and harvested under multiple conditions of light (50 μmol photons m^{-2} s^{-1} and 250 μmol photons m^{-2} s^{-1}) and growth phase (early and late exponential and stationary phase in batch cultures, exponential phase in semi-continuous culture) and performed in four different research laboratories (Bigelow Laboratory, University of Maine, Bowdoin College, LOV). The only consistent factor in these calibration experiments is the principal investigator in charge of them (C. Roesler).

WET Labs ECO series digital Chl fluorometers have been deployed on the Gulf of Maine moored array since July 2001 (Pettigrew and Roesler 2005). Over time a number of different instrument models have been used, ranging from DFLS, FLS, BBFL2, and FLN, which represent the time course of evolution of the modern ECO version. Factory calibrations for each sensor were compared with those derived from laboratory calibrations using cultures of *T. pseudonana* for 55 sensors over the time course 2001–2016. Each laboratory calibration is based upon approximately nine dilutions of the culture, where the [Chl] for each dilution is determined from triplicate samples of extracted fluorometry. [Chl] is validated for each calibration experiment with HPLC. The median extracted fluorometry slope factor (i.e., ratio of [Chl] derived from factory calibrated in situ fluorometer to [Chl] derived from extracted samples) for each sensor is shown in Fig. 8. The factory calibration values exceed the laboratory calibration in each case; the slope factors have a range of 1.25–4.25. The slope factor is lower for older generation sensors such as the DFLS (mean 1.7) and FLS (mean 1.5) compared to the newer generation sensors BBF (mean 2.18) and FLN (mean 2.16). The median slope factor for the most recent ECO sensors (BBF and FLN) calibrated in the last 5 yr was 2.08 ± 0.05.

Recommendations

Accuracy and precision are the two important considerations in obtaining quality observations. It can be argued that precision is less important as it can be improved by making more measurements, while this is not true for accuracy (bias). Precision becomes more important in the open ocean where signals are low and approaching sensor limits. Both precision and accuracy are critical when selecting sensors to provide insights into global processes that will be assessed by a large number of researchers on diverse platforms and under extremely different oceanographic regimes. The WET Labs ECO series digital Chl fluorometers have been a workhorse in terms of sheer numbers of deployed sensors, and the quantity and quality of retrieved data. As the demands for precision requirements increased, industry improvements have been provided. However, the in situ validation of the factory calibrated observations against paired HPLC Chl values has pointed to a bias in the global data base. The bias is consistently observed in other bio-optical proxies of Chl calibrated to HPLC such as in situ radiometry and absorption line height. A critical aspect to these analyses

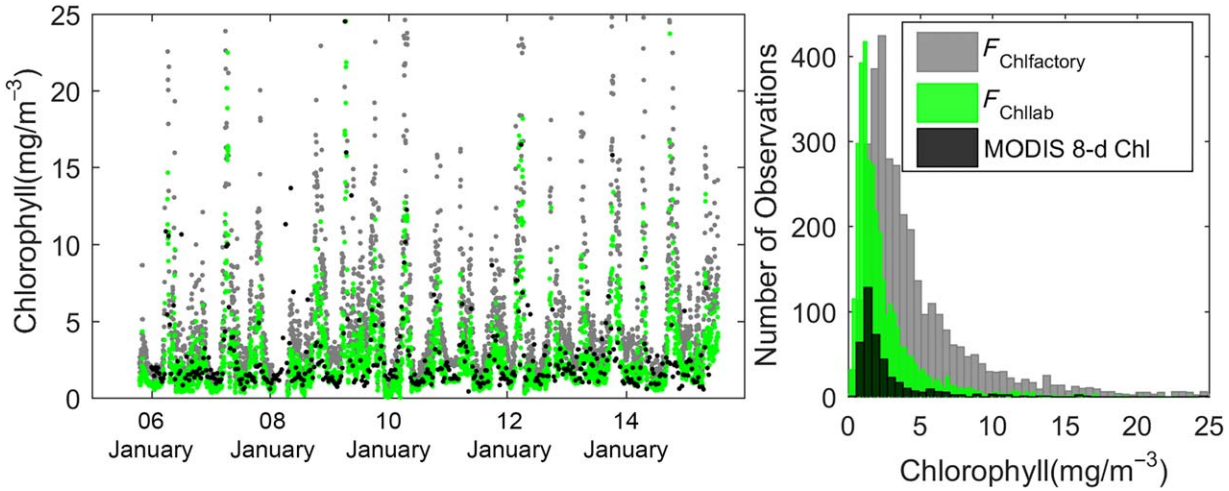

Fig. 9. Time series of daily observations of in situ Chl fluorescence calibrated by the factory (grey) or in the lab with cultures of *T. pseudonana* (green). MODIS Aqua 8-d Chl observations for the 0.1° × 0.1° box around NERACOOS mooring A off Cape Ann, Massachusetts (data modified from Roesler 2014).

is the removal or correction of NPQ from the in situ factory calibrated fluorescence observations. NPQ is maximal at the surface and decreases exponentially with depth and with the downwelling irradiance. NPQ can be observed in the near surface waters as early as 1 to 2 h after sunrise and may persist until sunset. The bias quantified here does not include observations impacted by NPQ (except potentially the radiometric approach, for which a correction was applied). Including NPQ-impacted fluorescence reduces the apparent slope factor *but in a manner not well constrained* (i.e., it may provide a closer estimate of [Chl] but for the wrong reasons). In lieu of HPLC validation samples, extractive fluorometric chlorophyll is often measured. While this method is a standard oceanographic protocol (Holm-Hansen et al. 1965), it carries known uncertainties, including variations in chl-specific absorption coefficient and fluorescence quantum yield, and interference of Chl *b* and/or pheophytin (Lorenzen and Newton Downs 1986; Welschmeyer 1994). Fluorometry was also found to underestimate Chl concentration compared to HPLC methods (Trees et al. 1985; Dos Santos et al. 2003).

In many situations, HPLC validation samples may not be available, such as for moored or floating platforms after deployment. We recommend that using satellite ocean color estimates of Chl can provide some measure of real time validation (and sensor drift), as this product is also calibrated to HPLC Chl (Boss et al. 2008; Lavigne et al. 2012). We caution that this approach be used only for regions for which in situ validation of the ocean color algorithm is in good agreement with HPLC (Szeto et al. 2011). As an example of this strategy, Fig. 9 exhibits a time series of daily Chl obtained from WET Labs factory-calibrated in situ fluorometers deployed off Cape Ann, Massachusetts (grey symbols). These same sensors were calibrated in the laboratory with cultures of *T. pseudonana* as described above (green symbols). The time series of MODIS 8-d Chl estimates (black symbols) computed from a 0.1° × 0.1° box around the mooring exhibits general agreement to the in situ observations. Histograms of the three data sets indicates that the factory calibrated in situ fluorescence has the factor of 2 bias relative to the laboratory calibrated values (median 3.10 mg Chl m^{-3} and 1.65 mg Chl m^{-3}, respectively). The MODerate resolution Imaging Spectroradiometer (MODIS) estimated Chl exhibits similar median (1.73 mg Chl m^{-3}) and spread of observations consistent with the laboratory calibrations.

The sources of natural variability in the calibration slope are well known and have been quantified, further supporting the observed in situ bias. Given these field and laboratory results, we suggest that the oceanographic community take into account the bias *at the data processing step* to correct their data. We *recommend against an industry correction of the bias* because such corrections lead to uncertainty on the user end of which calibration applies to which data set. By placing the responsibility on to the user, each data set can be evaluated for the statement in the metadata and in publications "*The community-established calibration bias of 2 for the WET Labs ECO-series fluorometer was applied to these in situ fluorometric chlorophyll values*."

Conclusions

Quantifying phytoplankton biomass in situ using in vivo Chl fluorescence is at once the most routine observation and one of the most complicated. There are many sources of uncertainty arising from measurement and data analysis, most of which have been well documented. There is a large source of natural variability which may be viewed as an impediment to quantifying Chl concentration and hence phytoplankton biomass. However, the importance of the measurement, combined with the large and meaningful dynamic range of values,

outweigh the measurement and natural sources of uncertainty. As the community expands the global coverage of fluorometric Chl observations, clear patterns in natural variations in the ratio of fluorescence to Chl concentration emerge and, in turn, provide insight into community composition and structure. It is still critical, in light of the natural variations in fluorescence to Chl ratio, to provide improvements in the quantitative retrieval of Chl concentration. Here, we present evidence for a bias in factory calibrations observed from field data and explained by controlled laboratory calibrations. We recommend a correction of this bias at the data processing step with the goal of improving the accuracy of in situ estimates of chlorophyll concentration from WET Labs ECO series Chl fluorometers. The bias of 2 is significant; a factor of two improvement in accuracy is critical for correcting global data bases and providing accurate inputs to global biogeochemical models. Our results imply a recommendation for the community to evaluate both (1) the instrument specific biases for other manufacturers using global data sets of matched in situ Chl fluorescence and HPLC and (2) local biases induced by natural variations in the fluorescence to Chl ratio.

The ongoing implementation of the Biogeochemical-Argo program (Biogeochemical-Argo Planning Group 2016; Johnson and Claustre 2016), where both radiometric and Chl fluorescence have been identified as core measurements, is progressively contributing to a drastic increase in the amount of acquired Chl fluorescence profiles. The radiometric calibration method might appear as a way to complement the initial HPLC reference calibration and its nuances over the float lifetime (albeit with the uncertainty induced by a necessary NPQ correction for near-surface daytime measurements). Indeed the radiometric calibration allows profiles acquired by different floats in different areas to become potentially intercomparable because of the link to a single currency: the bio-optical relationships. Additionally, the method might also work with other Chl fluorescence sensors because it is the shape of the fluorescence profile, not the magnitude that is used to calculate the slope factor. Finally, these methods might help to identify variations in the fluorescence to Chl relationships (linked to phytoplankton communities, light, and nutrient status) or in the K_d to Chl relationships (as due to varying proportions of phytoplankton, CDOM and non-algal particle optical properties and concentrations). In any case, such variations are not instrumental but reveal natural variability that deserves to be better addressed. Densifying the data base through float acquisition might be seen as a way to identify key oceanic areas or time scales where this natural variability could be more mechanistically understood through dedicated cruises.

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Conflict of Interest

None declared.

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