Volume 12 (2) June 2003

Pages 29-35

LIMNOLOGY AND OCEANOGRAPHY
BULLETIN

Published by the American Society of Limnology and Oceanography

THE BLANK CAN MAKE A BIG DIFFERENCE IN OCEANOGRAPHIC MEASUREMENTS

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It is hard to imagine a topic that seems more boring and trivial than the measurement of nothing. In a sense, determination of an analytical blank is exactly that — measurement of the signal associated with the absence of the property being detected. Although the magnitude and variability of an analytical blank may be very small compared to the oceanographic measurement, it must be determined as part of a calibration routine. For example, the blank for samples of particulate matter collected on filters consists of a filter that has been treated identically to field samples, except that no water with particles is drawn through it. Analysis of dissolved constituents requires measurements on samples that are analytically equivalent to field samples, but with no analyte present. It is the analyst's responsibility to determine what constitutes the appropriate blank (e.g., purified water, artificial sea water, filtered sea water treated to remove the analyte); generally, this requires a series of experiments to establish the sensitivities of the measurement system. Experience with the controversy concerning the measurement of dissolved organic carbon and nitrogen in natural waters (Benner and Strom 1993) amply demonstrates that blanks must be carefully monitored to ensure accuracy (i.e., to minimize systematic error).

For a variety of reasons, it is sometimes difficult or impossible to measure appropriate blanks concurrently with the oceanographic measurements. Deployments of *in situ* sensors can be particularly problematic. Changes in the blank due to fouling and instrument drift can confound the interpretation of long-term records from optical instruments that have internal light sources (Davis et al. 2000). Radiometric measurements of solar radiation and ocean color should be corrected for the signal generated in the absence of light: a dark correction. Generally, the signal in the dark is sensitive to temperature, so the correction from a laboratory calibration cannot be assumed to hold in the field (Cullen and Davis 2002). Uncertainty in the dark signal can strongly influence estimates of irradiance at depths corresponding to the limit of detection, leading to large errors in the estimation of attenuation coefficients (Morrow and Booth 1997). Fortunately, it is often possible to assess the uncertainty in the blank to determine if it compromises the measurement (see Laney et al. 2001 for an optical application). If the measured signal is much larger than the blank, nitpicking about blank corrections is unwarranted.

Introduced by Carl Lorenzen in 1966, *in vivo* fluorometry is used widely in limnology and oceanography to estimate phytoplankton biomass in terms of chlorophyll. Consistent with basic principles of analysis, measurements of light emitted by phytoplankton should be corrected for the signal from the same water, with phytoplankton removed by filtration (potential effects of scattering must also be assessed). Lorenzen did this and then showed through experimentation that purified water was an acceptable substitute for filtered sea water because the signals from both types of blanks were small, compared to his samples, and not sufficiently different from each other to influence his results. Our experience with standard fluorometers such as the Turner Designs series shows that the difference between filtered sea water and purified freshwater blanks is small compared to the fluorescence of phytoplankton in all but the clearest oceanic waters. However, much of the ocean has very clear water.

We discuss here the measurement of variable chlorophyll fluorescence, used as a diagnostic of nutrient limitation in phytoplankton (Kolber et al. 1988; Parkhill et al. 2001). A simulation, guided by real measurements in the Pacific Ocean, demonstrates that for measurements of variable fluorescence in ultra-oligotrophic waters, the blank can make a big difference.

VARIABLE FLUORESCENCE AS A DIAGNOSTIC OF NUTRIENT LIMITATION

The measurement and interpretation of phytoplankton fluorescence has been reviewed many times (Cullen 1982; Falkowski and Kolber 1995), and only a brief overview of variable fluorescence is presented here. Light absorbed by

The Limnology and Oceanography
Bulletin

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The *L&O Bulletin* is published quarterly by the American Society of Limnology and Oceanography, 5400 Bosque Blvd., Suite 680, Waco, TX 76710 USA. Postage paid at Waco, Texas. POSTMASTER: Send address changes to ASLO Business Office, 5400 Bosque Blvd., Suite 680, Waco, TX 76710 USA.

Subscription price to regular members is included in annual dues. Information on institutional subscriptions is available upon request from the ASLO Business Office.

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photosynthetic pigments has only three fates: photosynthesis, fluorescence, or the radiationless production of heat. The competitive relationship between photosynthesis and fluorescence is illustrated by the measurement of fluorescence during manipulation of photosynthetic reactions (i.e., modulated or active fluorescence techniques). When cells are dark-adapted so that functional reaction centers of photosystem II (PSII) are open (i.e., the likelihood for photosynthetic reactions is maximal), measured fluorescence yield is low (F_0); when the centers are closed (reduced) by saturating flashes of light, or by adding an inhibitor of noncyclic photosynthetic electron flow such as DCMU, fluorescence is maximal (F_m). Variable fluorescence, F_v (i.e., $F_m - F_0$) is thus a measure of the capacity for noncyclic photosynthetic electron flow. The ratio, F_v / F_m, can be related directly to maximum photochemical quantum efficiency.

Fluorescence parameters vary in response to environmental factors, such as light history, nutrient stress and other environmental insults. Consequently, F_v / F_m is used as a diagnostic of nutrient limitation in the ocean (e.g., Geider et al. 1993; Behrenfeld et al. 1996). For marine phytoplankton, values lower than about 0.65 are consistent with physiological stress. Variable fluorescence techniques include fluorescence +/- DCMU (e.g., references in Parkhill et al. 2001), Pulse-Amplitude-Modulated (PAM) fluorometry (Schreiber et al. 1986), Pump-and-Probe fluorometry (Kolber et al. 1988), and Fast-Repetition-Rate fluorometry (FRRF, Kolber and Falkowski 1993). Arguments about the validity and intercomparability of each method are not relevant here; we are exploring the degree to which patterns in the measurements can be associated with uncertainty in the blank.

Here we assess a potential problem in the use of an inappropriate blank (fresh water purified via deionization, dissolved organic matter adsorption, and frequently UV oxidation in Milli-Q or Nanopure water systems; hereafter referred to as "DI water") rather than filtered sea water (FSW) for shipboard measurements of variable fluorescence (e.g., Cullen and Renger 1979; Falkowski and Kolber 1995). Note that in the ratio, F_v / F_m (i.e., $(F_m - F_0) / F_m$), fluorescence from the blank affects only the denominator so that a blank with unnaturally high fluorescence leads to an underestimate of F_m, hence an overestimate of F_v / F_m. Conversely, a blank with less fluorescence than sea water with the phytoplankton removed leads to an underestimate of F_v / F_m. We show that for measurements of variable *in vivo* fluorescence in the oligotrophic open ocean, results are strongly sensitive to uncertainties in the blank, perhaps leading to erroneous conclusions about the fundamental controls of primary productivity in vast oligotrophic regions of the sea. Our message reinforces the long-standing, but sometimes weakly implemented requirement, that analysts explicitly evaluate analytical blanks when reporting oceanographic measurements. The importance of blanks has been recognized before, but we believe it bears repeating.

SPATIAL PATTERN OF VARIABLE FLUORESCENCE IN THE PACIFIC OCEAN

A transect of the Pacific Ocean along 150°W, roughly from Hawaii to Tahiti, encounters oligotrophic waters to the north and south with higher chlorophyll in the surface layer of the equatorial Pacific (Landry et al. 1997). Fluorescence at the surface, measured on discrete samples near midday and corrected for the FSW blank, is also higher in equatorial waters (Figure 1A). In oligotrophic waters at the extremes of the transect, the fluorescence signal from FSW (the appropriate blank) is nearly as high as that from whole sea water.

On one occasion, we measured the fluorescence of the ship's DI water, widely used as a blank for the measurement of fluorescence in discrete and flow-through systems. This DI had higher fluorescence than FSW (Figure 1B). The result seems surprising at first, but the system had no module for UV oxidation, and its water would have needed further treatment to serve as a good blank for dissolved organic carbon. Measurement of a DI blank was discontinued; stability of the instrument is reflected in the record of acetone blanks for the determination of extracted chlorophyll using the same fluorometer.

The effects of using different blanks are shown in Figure 1C, where F_v / F_m was calculated using either the proper FSW blank or the FSW blank increased by 50% of the observed fluorescence increment from DI water (from Figure 1B). Results are significantly different: $(F_v / F_m)_{DI}$ (using the DI blank) shows a strong pattern with latitude, suggesting

high values of about 0.65 in the blue waters north and south of the iron-limited equatorial region, and $(F_v / F_m)_{FSW}$ (using the FSW blank) shows little change with latitude. We conclude that, unless proven otherwise through direct comparison with FSW blanks, DI water is an inappropriate blank for the measurement of F_v / F_m. Nonetheless, DI water from a source with UV oxidation and new cartridges is very useful for monitoring instrument response and for detecting fouling in cuvettes. We now recommend routine measurements of DI along with FSW blanks.

TEMPORAL PATTERN OF VARIABLE FLUORESCENCE

Because the magnitude of both F_0 and F_m vary through the day, generally decreasing in bright light near the surface due to non-photochemical quenching (Dandonneau and Neveux 1997), diel patterns of F_v / F_m can be subject to a bias similar to that in Figure 1C, but with respect to time, not location. Simply, F_v / F_m will be overestimated if the blank is artifactually high, and the error will increase as F_m decreases, as occurs in bright light. Our interest in the temporal effects of blanks was spurred by the results of Behrenfeld and Kolber

Figure 1. Influence of blanks on discrete determinations of F_v / F_m during cruise WEC88 along 150°W, starting at 15°N and ending at 15°S, Feb - Mar 1988 aboard the R/V Wecoma (plotted versus day of year, with days 62 - 67 on the equator). Fluorescence of dark-adapted samples was measured with a Turner Designs 10-005R fluorometer before (F_0) and after (F_m) addition of DCMU. A) Midday measurements of F_0 and F_m for the surface sample are plotted as they would be measured before subtracting the FSW blank, which is shown in black. Freshly filtered (GF/F) sea water in a scrupulously cleaned cuvette, generally from a combined sample from four depths, served as the blank for fluorescence measurements (+/- s.e.; n = 4 to 17, depending on the day). Blanks were tested +/- DCMU, which had no significant influence on the blank. B) Three blanks were run on the same fluorometer during the cruise: FSW and DI (Milli-Q) for *in vivo* fluorescence, and acetone for extracted chlorophyll using the same instrument (measurements reflect stability of the instrument). C) The fluorescence-based measure of photosynthetic efficiency, F_v / F_m (where $F_v = F_m - F_0$). Black crosses are $(F_v / F_m)_{FSW}$ calculated using FSW as the correct blank (data from 1A). A locally weighted least-squares fit (solid line) indicates the trend. Filled red circles show $(F_v / F_m)_{DI}$ that would be obtained by consistently overestimating the measured FSW blank by 0.043V, less than half the measured increment from DI water in 1B (0.087 V). Data from J.J. Cullen.

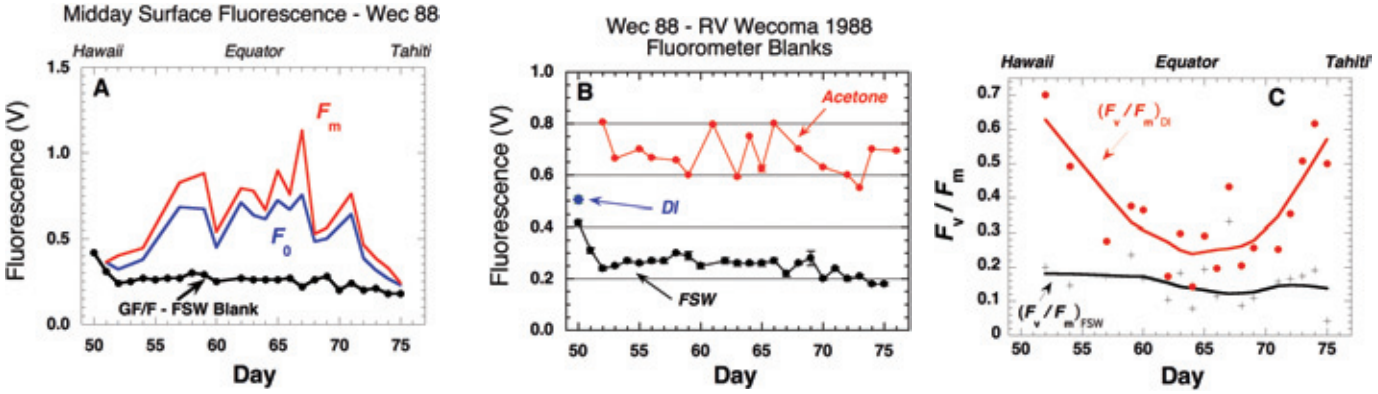
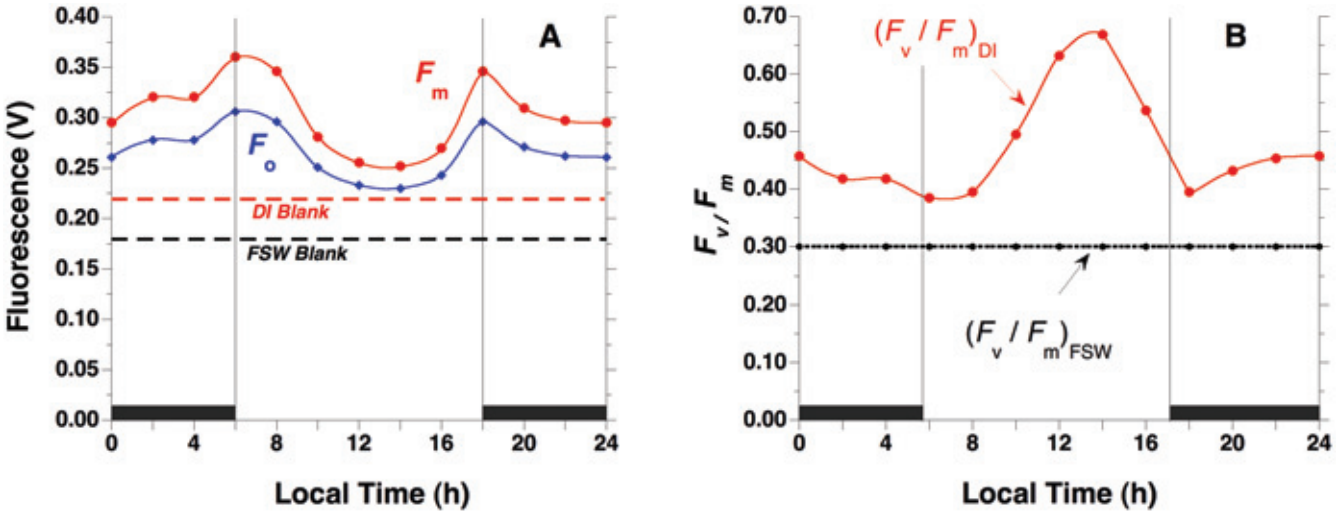


Figure 2. Hypothetical model showing a diel pattern of calculated photochemical quantum efficiency, F_v / F_m, generated using two different blanks. (A) Modeled diel pattern of F_0 and F_m scaled to be consistent with surface waters in the South Pacific. Assumptions are described in the text. The black dashed line is the FSW blank measured at midday; the dashed red line is an artifactually high blank consistent with an offset of less than 50% of the measured increment associated with using DI water, rather than FSW, at an oligotrophic station. B) The accurate diel pattern of $(F_v / F_m)_{FSW}$ is the flat line, calculated using the FSW blank; the red line, with a strong diel pattern in $(F_v / F_m)_{DI}$, was generated by using the DI blank to calculate F_v / F_m.



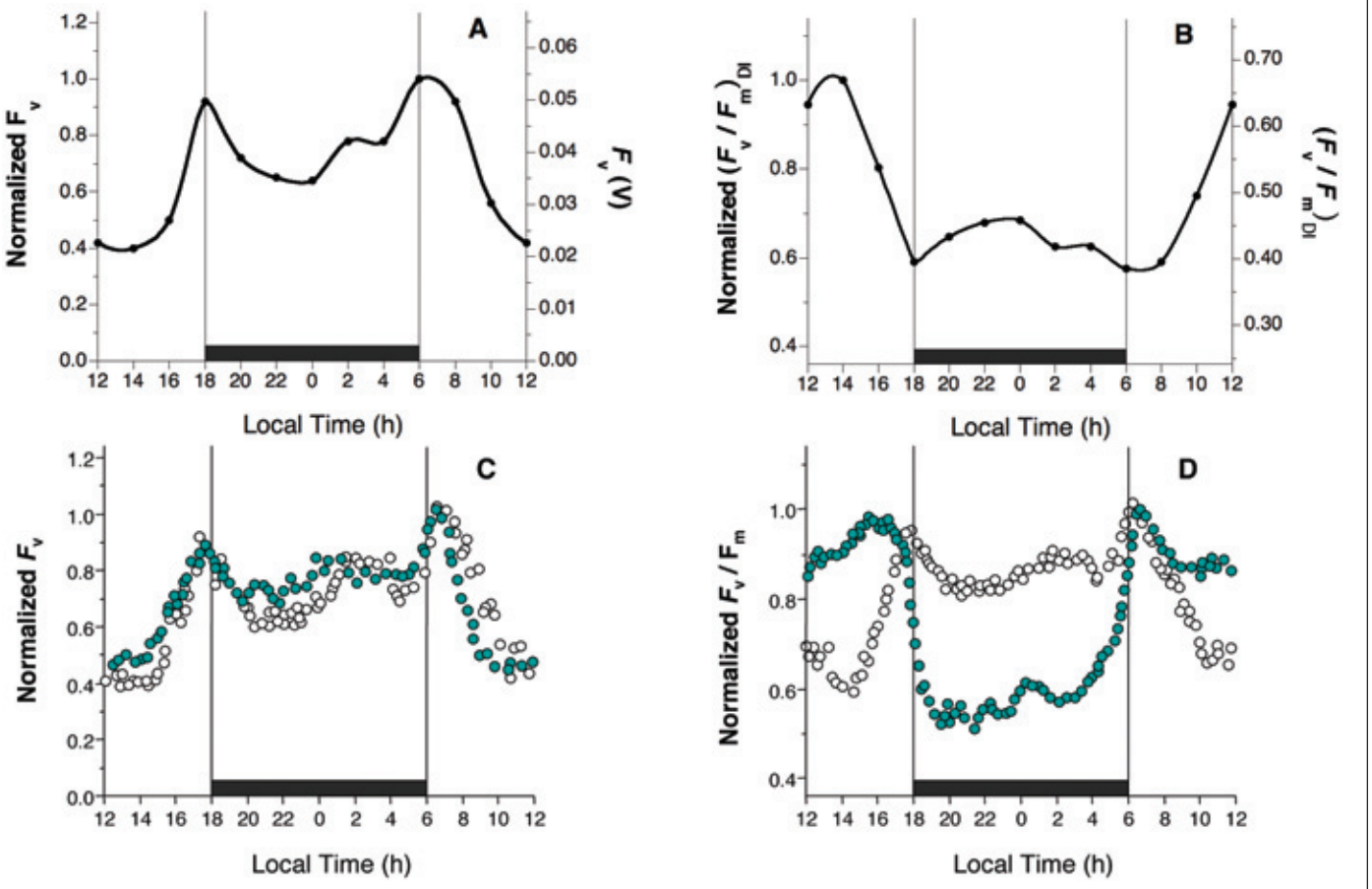
(1999). In the South Pacific, they found high values of F_v / F_m during the day, when fluorescence was low, and low values at night, when fluorescence was high. This report drew our attention because other studies (e.g., Greene et al. 1994), as well as measurements from our laboratory over more than 20 years, observe a depression of F_v / F_m at the surface during the day. Like many others, Behrenfeld and Kolber used DI water for their blank and reported no comparison with FSW. Would their reported diel pattern of F_v / F_m have been significantly different if FSW had been used for blanks, and those blanks were lower?

To examine the potential influence of blanks on the determination of F_v / F_m in the South Pacific gyre (where measurements should be most sensitive to the blank), we constructed a simple model of oligotrophic waters where both F_0 and F_m vary through the day, but F_v / F_m remains constant. The model is based on a diel record of F_v that is generally consistent with the average diel cycle reported for the South Pacific by Behrenfeld and Kolber (1999); it is constrained using our measurements of fluorescence and blanks in the Pacific Ocean. The hypothetical simulation, illustrated in Figure 2 and compared with the results of Behrenfeld and Kolber in Figure 3, follows these constraints:

- The absolute values, in volts, for F_0 (Figure 2A) are scaled to match our data for midday at 15°S, corrected for the FSW blank (from day 75 in Figure 1A).
- F_m (Figure 2A) is calculated from the blank-corrected F_0 assuming F_v / F_m is constant, night and day, at a value consistent with nutrient stress, 0.3. By definition, F_v / F_m calculated with the FSW blank, $(F_v / F_m)_{FSW}$ (Figure 2B), is equal to 0.3, the accurate result.
- $(F_v / F_m)_{DI}$ (Figure 2B) is calculated assuming the use of a blank which is 0.04 V higher than the FSW blank. This is less than 50% of the measured offset from DI water as compared to FSW in blue water of the north Pacific (from Figure 1B).

The modeling exercise clearly shows that in oligotrophic waters a strong but artificial diel pattern of F_v / F_m, high in the day and low at night, can be generated by the use of an inappropriate blank (Figure 2B, red line). As shown in Figure 3, our modeled values of F_v are similar to those of Behrenfeld and Kolber (1999). However, in our model, the accurate $(F_v / F_m)_{FSW}$ is invariant at 0.3; Behrenfeld and Kolber show a

Figure 3. Comparison of our simulation (A and B) with results published by Behrenfeld and Kolber (1999) (replotted from their Figure 2). The data from our simulation are normalized to daytime maxima and plotted with nighttime in the middle of the record for compatibility with their presentation. A.) Our simulated pattern in normalized F_v (F_m - F_0, which is insensitive to variations in the blank) is similar to their data for the South Pacific (filled symbols in C; open symbols are for central Atlantic gyres). B.) The diel pattern of $(F_v / F_m)_{DI}$, generated in our simulation by an assumed artifact, is similar in amplitude and phase, but not all details, to the pattern of F_v / F_m reported by Behrenfeld and Kolber (D), who used DI water for blanks.



pattern of normalized F_v / F_m that is similar in amplitude and phase, but not in all details, to our $(F_v / F_m)_{DI}$ which was based on an assumed artifact.

Behrenfeld and Kolber (1999) interpreted the diel pattern of F_v / F_m, particularly the decrease at night, as evidence for iron limitation of primary productivity in the oligotrophic central South Pacific, as well as in the equatorial Pacific, where, during IronExII, the diel pattern disappeared in the iron-fertilized patch while persisting in control waters outside the patch. It may be relevant that the artifactual diel pattern of F_v / F_m associated with a high blank (Figure 2) is dampened when both F_v / F_m and F_m increase, as occurs when iron limitation is relieved. We have modeled this result for a hypothetical enrichment experiment like IronExII, but the simulation involves much speculation about the value of the blank in the equatorial Pacific, reinforcing our conclusion that results cannot be interpreted with confidence if the value of the blank is not constrained. Regardless, even if the diagnostic diel variation of fluorescence was accurately recorded in the equatorial Pacific, it could not be taken as evidence that the same pattern exists somewhere else. In turn, fundamentally different diel variability reported for the Pacific vs. Atlantic Oceans (Figure 3D) might be due to physiology or, possibly, to different DI water supplies as compared to natural water. The question is difficult to resolve when measurements of FSW blanks are lacking.

We do not assert that the measurements of Behrenfeld and Kolber (1999) from the South Pacific gyre are compromised by the artifact simulated in our model. Their instrument system, and many other aspects of the measurements, were substantially different from ours, so there is no way to know if their measurements were significantly influenced by an artifactually high blank associated with DI water. That is exactly our point: if the blanks are not reported, there is no way to know.

Our simulation, based on published data from the South Pacific and direct measurements in the same region, illustrates why an appropriate blank must be measured and shown rigorously to have an insignificant influence on an analysis. Considering the importance of determining what controls primary production in the open ocean, we feel that when it comes to the measurement and interpretation of variable fluorescence, blanks do matter.

CONCLUSIONS

Researchers are measuring and interpreting F_v / F_m in many parts of the ocean, but few are reporting the measurement of appropriate blanks. Many are using a commercially available submersible instrument, and some use the readings from well below the photic zone for blanks. The appropriate blank cannot be measured directly with an unmodified instrument: it is sea water from which phytoplankton have been removed, under the same ambient irradiance as encountered through the vertical profile. The magnitude of potential errors can be assessed if the signals from deep water are compared to those from FSW (both F_0 and F_m) at the same site under a range of irradiances, with an evaluation of temperature effects and the influence of scattering. This is tractable, and we understand that some researchers have made the measurements. For flow-through and discrete-sample fluorometers, measurement of FSW blanks and comparison with DI water should be part of the calibration routine.

Problems with blanks, no matter the analyte being measured, are insignificant when the sample signal is much greater than uncertainty in the blank. Nevertheless, this should be demonstrated and reported in scientific communications or dealt with explicitly and reported when it is not true. However, inspection of the literature indicates that such measures are neither universally demanded nor routinely undertaken. Rigorous assessment of an analytical blank may not always seem worth the effort, but it is necessary, like it or not.

Acknowledgments: Supported by NOAA, NSERC and the Office of Naval Research. Comments by Ricardo Letelier, Catherine Brown and Yannick Huot on earlier versions, reviews by Heidi Sosik and three others, and excellent suggestions from the Editor were helpful.

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