A new calibration method for *in situ* fluorescence

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A new calibration method for *in situ* fluorescence

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1.0 Introduction

MWRA has revised its method for calibrating *in situ* chlorophyll fluorescence measurements for its effluent outfall ambient monitoring studies, which has incidentally resulted in a small effect on the Contingency Plan thresholds for chlorophyll.

In this report we review how chlorophyll is measured and how *in situ* probe measurements of fluorescence have been calibrated in the past to estimate chlorophyll. We describe a limitation of the method used in the past and a refined method that allows us to calibrate the fluorescence probe more accurately. We conclude by demonstrating that the recalibration has a small effect on vertically-integrated chlorophyll values on which Contingency Plan thresholds (MWRA, 2001) are based. There is no effect on the likelihood of a Contingency Plan threshold exceedance, neither did the recalibration affect whether the threshold was exceeded in previous years.

1.1 Background: fluorescence as a proxy for chlorophyll

In situ fluorescence is measured to learn about the fine-scale spatial distribution of phytoplankton biomass (carbon) in the water column. Direct measurement of phytoplankton biomass from a water sample involves separating phytoplankton from non-phytoplankton species, concentrating the cells, and then measuring the carbon content, which is a prohibitively expensive and labor-intensive procedure for routine analysis. In lieu of direct biomass measurements, biological oceanographers take advantage of the fact that all phytoplankton cells contain chlorophyll from which carbon content can be estimated based on the cell population carbon to chlorophyll ratio. However, laboratory measurement of chlorophyll extracted from seawater is itself relatively expensive, making it feasible to analyze only a handful of samples from each sampling station. For MWRA's studies, it is important to understand the finer-scale structure to accurately quantify the distribution of phytoplankton biomass. Measurement techniques for the continuous measurement of *in vivo* fluorescence (Lorenzen 1966) allow for high spatial resolution measurements of chlorophyll, which correlates to phytoplankton biomass.

The fluorescence technique exposes *in situ*, living phytoplankton cells to a flash of artificial light at one wavelength and measures the fluorescence response at a lower wavelength. The intensity of the response is approximately proportional to the chlorophyll concentration. Although the fluorometer is calibrated to a laboratory or secondary standard, the actual fluorescence per unit chlorophyll, or "quantum yield", in the field is influenced by cell size, temperature, turbidity, nutrient limitation, and irradiance; the fluorometer reading can differ from the actual chlorophyll concentration to a considerable degree. Calibration of the fluorometer response against laboratory measurements of chlorophyll extracted from grab samples of seawater is required to convert the fluorometer output to units of chlorophyll that match those from corresponding grab samples.

1.2 Calibration of fluorescence measurements from MWRA monitoring programs

Measurements of fluorescence, along with grab samples for calibration, are part of MWRA's monitoring programs in Massachusetts Bay and Cape Cod Bay (MWRA 2010, Libby *et al.*2011, Costa *et al.* 2010.) Grab samples are collected at three to five depths, and fluorescence measurements are made every 0.5 meters, and at each grab sample depth. A calibration derived from paired measurements of *in situ* fluorescence and extracted chlorophyll, is applied to all the raw fluorescence values to obtain calibrated fluorescence (as µg/L chlorophyll) at each depth. A new calibration is done for each survey. Until the end of 2011, MWRA used a linear regression of fluorescence against laboratory measurements from grab samples[1]. Not infrequently, the surface values appeared to follow a different relationship from the deeper samples in the survey; light can reduce the level of fluorescence observed. As in the example shown in Figure 1, those "too-low" surface values were therefore excluded from the regression (Figure 1b).

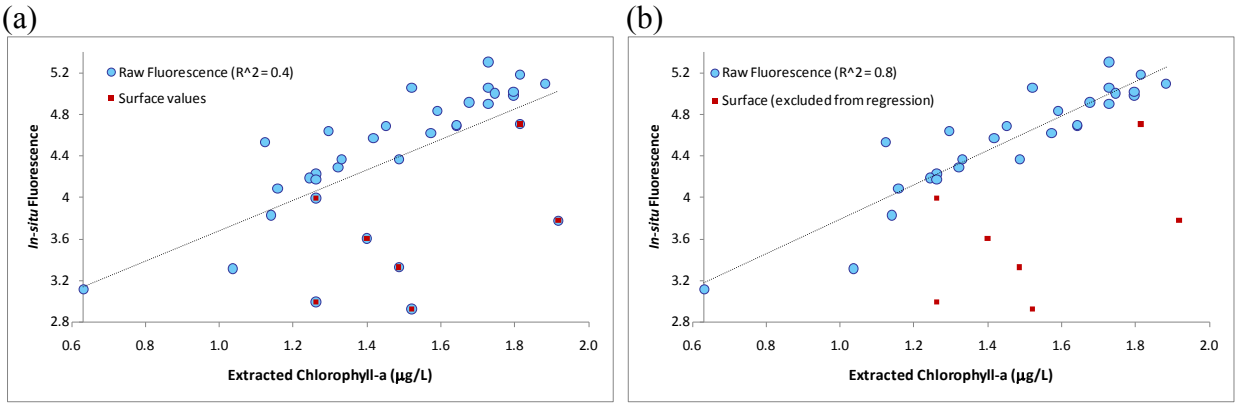

Figure 1. In-situ fluorescence of near-surface measurements quenched by light. Example from December 2003 survey. (a) linear regression including all data (b) linear regression excluding surface values.

In 2012 MWRA implemented an improved method for calibrating fluorescence, which includes the effect of light on fluorescence, and allows the inclusion of the surface samples in the calibration.

[1] In 1992-94 the calibration was done slightly differently as explained in a later section.

1.3 Non-photochemical quenching

From MWRA's surveys in Massachusetts Bay, Boston Harbor, and Cape Cod Bay, it has become apparent that the single greatest factor affecting fluorescence per unit chlorophyll is irradiance, measured as photosynthetically active radiation (PAR). Above a certain threshold, increases in PAR results in a decrease in quantum yield. This is due to an effect called "non-photochemical quenching," (Falkowski and Raven 1997) in which cells continue to fix carbon while displaying a reduction in fluorescence. The quenching effect can be quite pronounced, as shown in Figure 2.

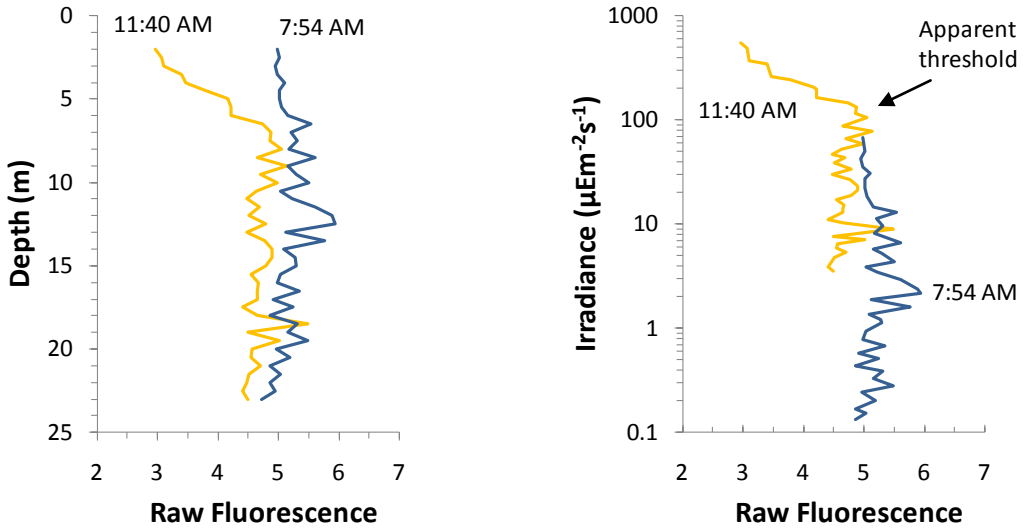

Figure 2. Example from December 2003 of fluorescence quenching due to irradiance. Downcast fluorescence from two nearfield sampling stations visited on the same day shows a surface quenching of raw fluorescence (left) at mid-day compared with early morning. Substituting *in situ* irradiance for depth (right) shows that the threshold for this is at irradiance levels near and above 100 $\mu Em^{-2}s^{-1}$ and that the magnitude of the quenching is proportional to log irradiance. We know from extracted chlorophyll measurements at five depths that chlorophyll was nearly uniform from surface to bottom that day.

This effect of high light levels must be eliminated or accounted for to accurately calibrate fluorescence to chlorophyll and especially to avoid under-estimation of chlorophyll from high-irradiance regions of the water column. We therefore developed a multiple regression model that included terms for both irradiance and chlorophyll.

2.0 New method: Inverse multiple regression model for calibration of fluorescence

Holm-Hansen *et al.* (2000) suggested that one could model the effect of irradiance on quantum yield for irradiance levels above a threshold. Below that light threshold, the quantum yield would be constant.

Following this approach, we chose the following multiple regression model:

$$\boldsymbol{F = \beta_0 + \beta_1 \times C + \beta_2 \times I_T + \varepsilon} \qquad \textbf{(1)}$$

where:

F = $\log_{10}$ fluorescence from probe

C = $\log_{10}$ extracted chlorophyll-a from grab sample

I_T = $\log_{10}$ irradiance above threshold irradiance value T, where:

- $I_T = 0$ when $I_Z \leq T$ and $I_T = I_Z - T$ when $I_Z > T$
- T is the threshold log irradiance at or above which non-photochemical quenching occurs
- I_Z = $\log_{10}$ (irradiance + 1) at depth z

β_0 is the intercept of the multiple regression

β_1 is the regression coefficient for extracted chlorophyll

β_2 is the regression coefficient for I_T

ε is the residual variation in F not explained by the regression

Prior to performing the multiple regression, a non-linear least-squares fit of the same model is used to determine the threshold irradiance value T. When this model fails to converge (no coefficients are estimated, or the threshold irradiance value is negative, or the coefficient β_2 for the irradiance term I_T is positive), we use = 1.5 = $\log_{10}$(30.6 $\mu Em^{-2}s^{-1}$) – a value representative of the low end of the range of threshold irradiance values typically seen in MWRA's data.

The improvement in fit by using the multiple regression model is shown for the example survey (December 2003) in Figure 3.

(a) (b)

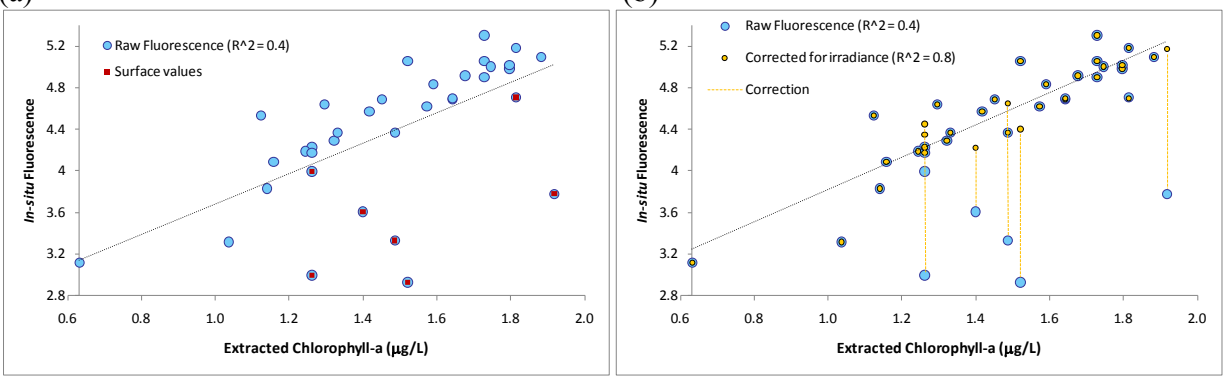

Figure 3. Improvement in calibration by correcting for the effect of irradiance using results from multiple regression model. (a) linear regression using all data. (b) linear regression using all data, after first correcting surface fluorescence values for the quenching effect of irradiance. Vertical lines in (b) show the magnitude of the correction.

The multiple regression model is used to recalibrate chlorophyll data from 1992-2011 (and going forward) with two types of exceptions:

1) We reject the multiple regression model when any of the following is true:

- coefficient for I_T >= 0 (*i.e.* high light increases rather than reduces quantum yield)
- p-value for overall F test > 0.05
- semi-partial R^2 for chlorophyll < 0.3

In those cases, we use a simple linear regression between fluorescence and chlorophyll (both log-transformed):

$$\boldsymbol{F = \beta_0 + \beta_1 \times C + \varepsilon} \qquad \textbf{(2)}$$

Where F, C, β_0, β_1, and ε are defined as for equation (1).

The simple linear regression is used for 42 of the 311 water column surveys from 1992-2011 (13%).

2) About 3% of surveys show no significant relationship between fluorescence and chlorophyll. In these cases, we reject the linear regression model in either of the following circumstances:

- p-value for overall F test > 0.05
- R^2 for chlorophyll < 0.3

and calculate calibrated fluorescence by scaling the data so that the median and inter-quartile range of the calibrated data match those of the extracted chlorophyll:

$$\textbf{calibrated fluorescence} = \boldsymbol{C_M + (F - F_M) \times (C_{IQR} / F_{IQR})} \qquad \textbf{(3)}$$

where:

F = Raw in-situ fluorescence (not log-transformed)
F_M = Median of raw in-situ fluorescence
C_M = Median of extracted chlorophyll-a
F_{IQR} = Inter-quartile range of in-situ fluorescence
C_{IQR} = Inter-quartile range of chlorophyll-a

This has the effect of scaling the calibrated fluorescence values so that they have the same median and interquartile range as the extracted chlorophyll.

3.0 Correction of error in 1992-1994 calibration

In the process of recalibrating fluorescence for all years, MWRA realized that in 1992-1994, chlorophyll rather than fluorescence was treated as the dependent variable, and that the regressions were forced through zero. When the regression is forced through zero, the regression line is steeper (Figure 4.) Thus, it predicts higher concentrations of chlorophyll for raw fluorescence values below the cross-over point in the regression lines of the two methods. Limiting calibrated fluorescence values to be above zero can lead to a high bias for depth-integrated chlorophyll, as shown in Figure 5. Forcing the regression through zero results in higher low values, and lower high values, for predicted chlorophyll (calibrated fluorescence). Integrated over the water column the net result is higher areal chlorophyll.

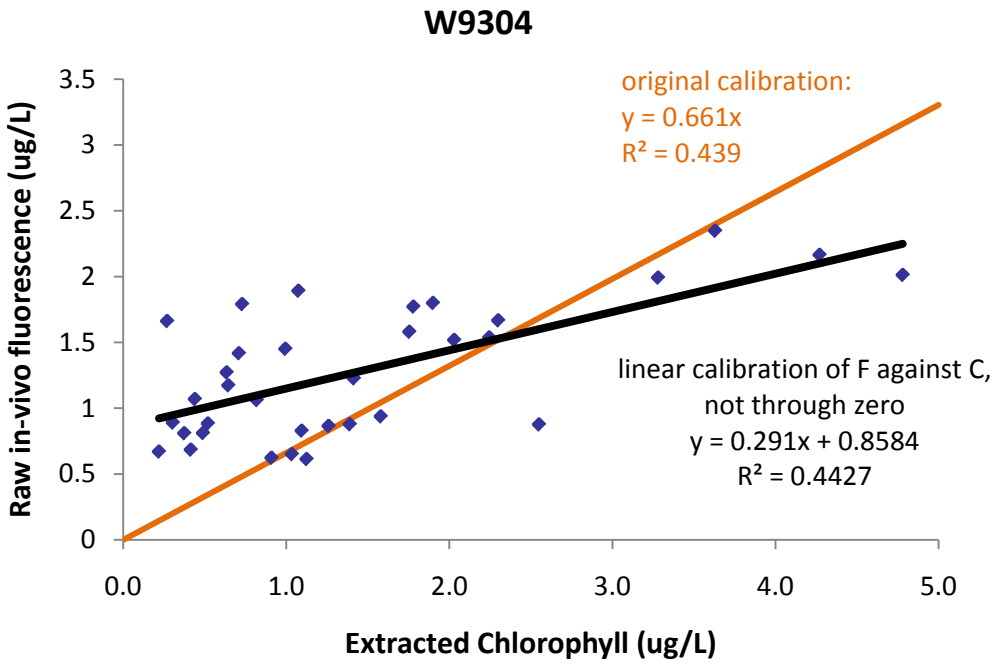

Figure 4. Example of the effect on the regression of forcing through zero (April 1993 survey.) Orange line is original calibration; black line is a linear regression of raw fluorescence against chlorophyll, not forced through zero. For simplicity we show the inverse of the original calibration regression which was done with fluorescence as the independent variable.

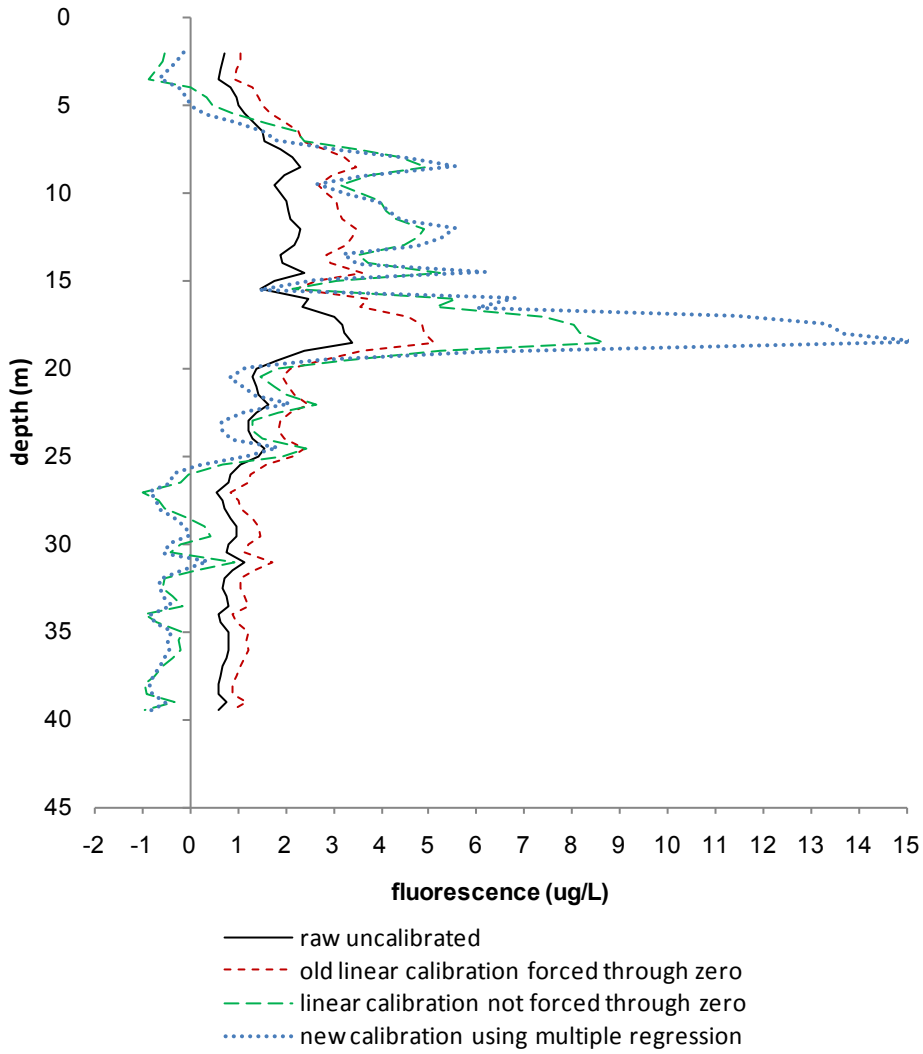

Figure 5. Example of the effect on the resulting calibrated data, of forcing the regression through zero (April 1993, station N16.) Black line is raw data, red line is old calibration (forced through zero), green is linear calibration not forced through zero, and blue is new multiple regression calibration.

Without forcing the calibration through zero, low fluorescence values may become small negative numbers after calibration. These are treated as nondetects and set to zero when vertically integrating fluorescence for the threshold calculation.

4.0 Effect on thresholds

The chlorophyll threshold is calculated by vertically integrating calibrated fluorescence (to obtain mg chlorophyll/m^2) at each nearfield station, averaging across the nearfield stations, and then averaging across all surveys during a season or year. The seasonal thresholds are based on the distribution of the pre-discharge (baseline) values, and equal the 95th percentile of the nine baseline values for winter-spring (February-April) and summer (May-August), and of the eight baseline values for autumn (September-October).

The calibrated fluorescence is used for threshold calculations because it is measured every 0.5 m from near the water surface to near the bottom, and thus its depth integral is a measure of the total chlorophyll in the water column. Basing the threshold on the depth-integrated value was recommended by the Outfall Monitoring Science Advisory Panel at its April 2001 meeting (see http://www.epa.gov/region1/omsap/omsap0401.html)

Starting with the raw fluorescence from all surveys to date, MWRA applied the new model to generate recalibrated fluorescence values. After recalibrating all surveys, we recalculated the baseline averages and determined the new 95th percentile values for each season as the new thresholds.

Although for individual hydrocasts, especially at mid-day during the darker months, the effect of light can be important (see Figure 2), the recalibration does not change the depth-integrated, averaged seasonal values by a large amount, and the difference in any season may be positive or negative, as shown in Figure 6.

A larger difference is seen in the early years, but this is due to the error in how the calibration was done in those years, as described above. This error has a larger, more systematic effect; correcting it tends to reduce the seasonal average chlorophyll as shown in Figure 7.

For the winter/spring and for the fall, the nearfield areal chlorophyll seasonal means are lognormally distributed for each season. Thus the baseline, that is the 95th percentile of the distribution, is calculated using:

$$\text{Threshold} = 10^{[\text{baseline log mean} + 1.648*(\text{baseline log std. dev.})]}.$$

For the summer, the nearfield areal chlorophyll seasonal means are normally distributed. Thus the 95th percentile is calculated as:

$$\text{Threshold} = \text{baseline mean} + 1.648*(\text{baseline std. dev.})$$

Because the seasonal thresholds depend on the variability (standard deviation) as well as on the mean, the threshold can increase even if the baseline values go down slightly, if they become more variable. This is the case for the fall threshold.

The baseline values for the annual threshold are calculated as 1.5 times (caution) or 2 times (warning) the average of the eight annual averages. The baseline years begin on September 6th. (Note that Figures 6 and 7 show annual averages of calendar years, so the 1992-2000 values are slightly different than those actually used in the threshold calculation.)

Table 1. Threshold values for areal chlorophyll, before and after recalibration

Time period	Threshold before recalibration (mg/m^2)	New threshold (mg/m^2)
winter/spring	226	199
summer	89	89
fall	218	239
annual (caution)	117	108
annual (warning)	157	144

Because the seasonal thresholds are based on the 95th percentile of the baseline seasonal means, the chance of exceeding a threshold is unchanged by the recalibration, even though the threshold values have decreased in most cases.

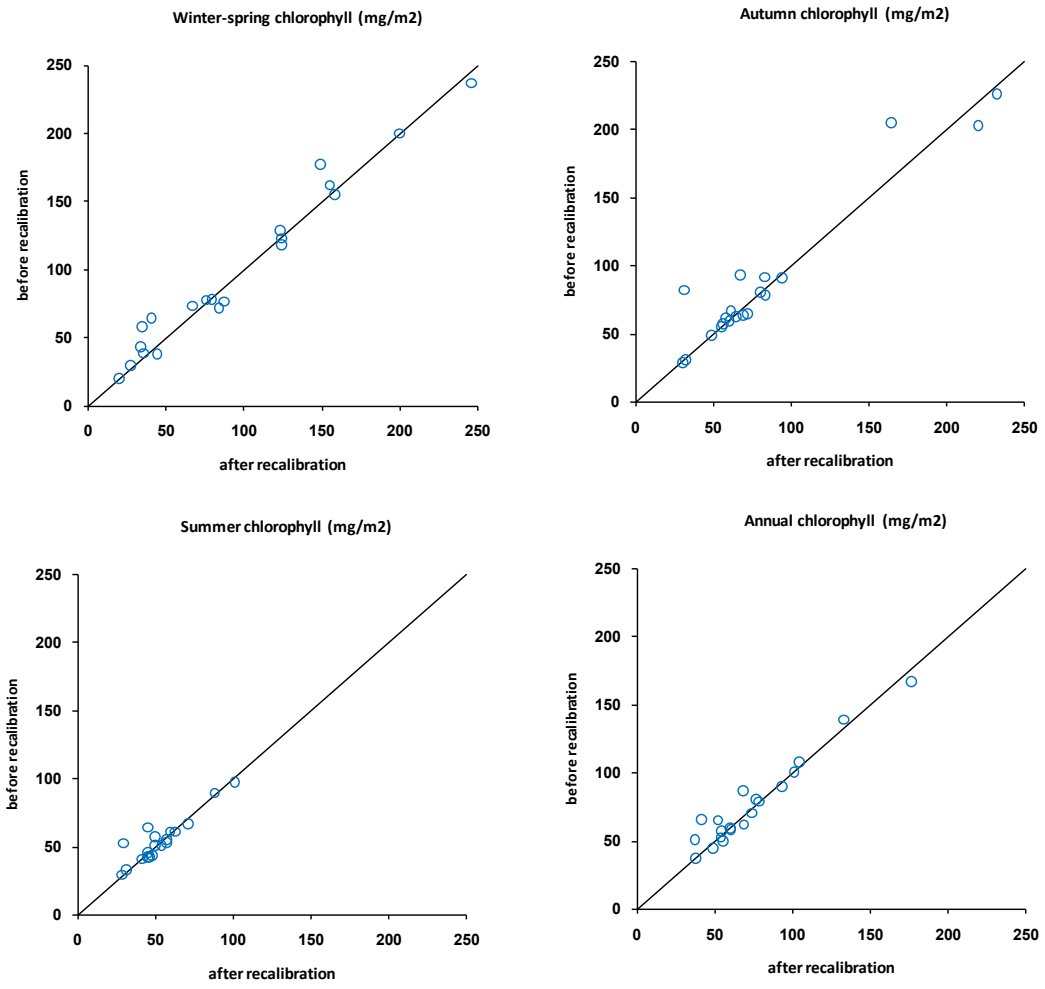

Figure 6. Effect of recalibration on seasonal and annual averages, of nearfield vertically integrated chlorophyll. Solid line is 1:1.

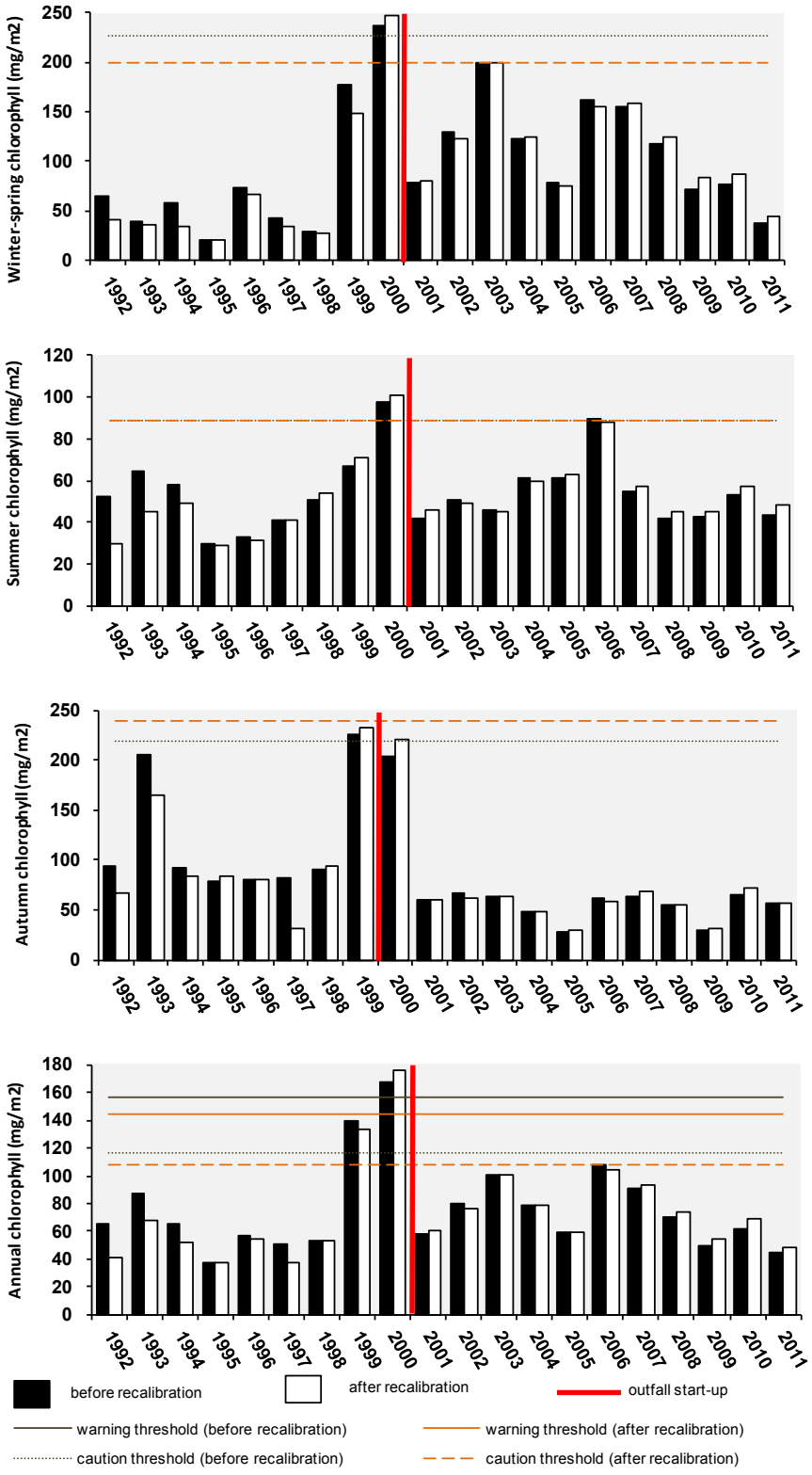

Figure 7. Time series of seasonal or annual averages, of survey averages, of vertically integrated fluorescence at nearfield stations. Outfall start-up occurred before the autumn 2000 season. Note: All the values plotted here reflect sampling according to the survey schedule as described in the Ambient Monitoring Plan Revision 2 (MWRA 2010) which was implemented in 2011.

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