Applicability of Chlorophyll *a* Methods
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For DEP purposes, four approved methods are discussed in this document for the measurement of chlorophyll *a.* These methods are published by the United States Environmental Protection Agency (EPA) as individual methods; or, by the American Public Health Association (APHA) as one method, which is found in the collection "Standard Methods for the Examination of Water and Wastewater" (SM). The approved methods are listed below according to the preferred terms for reporting the procedure used to account for pheophytin interferences. Each method describes procedures for sample preparation, which are here incorporated as part of the DEP-approved procedures.

1. Chlorophyll *a*, corrected for pheophytin: Spectrophotometric method with acidification, according to EPA 446.0 or SM 10200 H.2.b.

2. Chlorophyll *a*, corrected for pheophytin: Conventional fluorometric method with acidification, according to EPA 445.0 or SM 10200 H.3.

3. Chlorophyll *a*, free of pheophytin: Modified fluorometric method, using special, narrow-bandpass filters to eliminate spectral interference from pheophytin and chlorophyll *b*, according to EPA 445.0

4. Chlorophyll *a*, free of pheophytin: HPLC (High Performance Liquid Chromatography), pigment separation and analysis according to EPA 447.0 or SM 10200 H.4.

Recommendations for Evaluation of Data for Chlorophyll *a*

DEP-approved Methods
Chlorophyll *a* data generated using the methods listed above are approved for use for DEP purposes according to the cautions discussed in this document. Sample results must be reported as "corrected for pheophytin" or "free of pheophytin", as appropriate for the method used for analysis.

Sensitivity (Detection)
To achieve greater detection sensitivity than that obtainable by the spectrophotometric method, the fluorometric or HPLC methods may be used and may allow the collection of smaller sample volumes.

Selectivity (Interferences)
The trichromatic equations for the spectrophotometric method should not be used to generate chlorophyll *a* values unless pheophytin (and other pheopigment) interference is known to not be present or significant.

Fluorometric chlorophyll *a* values (including those corrected for pheophytin with acidification) must be evaluated carefully in freshwater, estuarine or coastal systems where higher levels of chlorophyll *b* (and in some cases, chlorophyll *c)* may cause considerable interference and render the conventional fluorometric methods unreliable. In typical freshwater samples, the conventional fluorometric methods may be unusable for the measurement of chlorophyll *a.*

Fluorometric chlorophyll *a* values using the narrow band pass filters may be used for samples collected in freshwater, estuarine and coastal systems without concern for significant interference from pheophytin, other pheopigments or chlorophyll *b*.

Analyzing, Comparing and Pooling Data
Evaluations of chlorophyll *a* data sets should take differences in method sensitivities into account when analyzing, pooling or comparing "non-detect" data.

Data for the same source (for example, the same sampling station or collection of stations in a waterbody, etc.) or other groupings of chlorophyll *a* data that have been generated using different analytical methods *may* need further evaluation in order to establish the comparability of results obtained from the associated methods.

Ideally, comparison or pooling of data using the same method is preferred.

Table 1 summarizes DEP recommendations for chlorophyll method applications.

A discussion and summary of method interferences can be found in Appendix A.

Table 1: Recommendations for chlorophyll methods by water type and sample concentration

Water type, sample chlorophyll concentration*	HPLC	Spectrophoto-metric	Fluorometric (Conventional)	Fluorometric (Modified, with Narrow Band Pass Filters)
Fresh, low	S	A	A	A
Fresh, high	S	A	NR	A
Estuarine, low	S	A	A	A
Estuarine, high	S	A	NR	A
Marine, low	S	A	A	S
Marine, high	S	A	A	A

A = Acceptable method
NR = Method not recommended
S = Superior method
* = Regulatory concentrations for chlorophyll currently range from 4 µg/L to >20 µg/L, and should be taken into consideration when choosing or evaluating methods.

Approved Method Revisions and Dates (includes those used for this document)
SM 10200 H, 17th – 20th editions & on-line (2001) edition, published by APHA
EPA 445.0, Rev. 1.2, September 1997
EPA 446.0, Rev. 1.2, September 1997
EPA 447.0, Version 1.0, September 1997

Additional Reference (also used for this document)
"Summary of Literature Comparing Methods for the Analysis of Chlorophyll in Water Samples", submitted 12/13/06 for EPA Contract# 68-C-04-006, prepared for USEPA, Office of Science and Technology, Health and Ecological Criteria Division

Appendix A

Considerations for Selection of Methods for Chlorophyll *a* Analysis

Due to variability in sample source taxonomic composition, trophic status and pigment spectral interferences, careful consideration must be given to determining which method for analysis of chlorophyll *a* is most appropriate for a sample or source.

Evaluation of the taxonomic composition of the sample source may provide information about the relative concentrations of pigments to be expected in the sample as an indication for the best choice of method. Literature sources may provide information about pigments associated with specific taxa.

In the absence of taxonomic information about the sample or sample source, the methods described in this document may be selectively used to estimate the apparent, relative concentrations of some of the chlorophyll pigments and chlorophyll degradation pigments to provide further information about the appropriate method choice for analyzing the sample for chlorophyll *a*.

Spectrophotometric method:

- This method provides less analytical sensitivity (higher detection limit) than either the fluorometric or HPLC methods.
- Pheophytin *a* and pheophorbide *a*, two common degradation products of chlorophyll *a*, can interfere with the determination of chlorophyll *a* because these pigments absorb light in the same spectral region as does chlorophyll *a*, and will result in errors in the measurement of chlorophyll *a* in relation to the concentration of interfering pigments present.
- Chlorophyll *a* is overestimated by the trichromatic equations (one of the spectrophotometric method options) when pheophytin *a* is present.
- The method option of using the monochromatic equation with the acidification step is required to correct for pheophytin interference, which will cause overestimation of the observed chlorophyll *a* absorbance and calculated concentration, if not corrected. During the acidification step, chlorophyll *b* converts to pheophytin *b*, which contributes to the observed pheophytin *a* absorbance, potentially causing an overestimation of the pheophytin *a* correction. Adequate mixing and controlled acid concentration and reaction time for the acidification are required in order to perform the correction according to the method.
- Pheophytin *a* is overestimated in the presence of certain carotenoid pigments.
- Chlorophyllide *a* is determined as chlorophyll *a* by this method.

Fluorometric methods:

- The greater sensitivity of the fluorometric methods provides lower detection limits than the spectrophotometric method.
- The conventional fluorometric methods (used without narrow-bandpass filters) are subject to spectral interferences in the presence of pheophytin *a*, pheophorbide *a*, chlorophyll *b* and chlorophyll *c*. Depending on the amounts present, chlorophylls *b* and *c* may significantly interefere with the measurement of chlorophyll *a*. Therefore, depending on the type of algae and the amounts of the various pigments present in the sample source, there are possible uncorrectable interferences using the fluorometric methods that may underestimate or overestimate chlorophyll *a*, and the measurement errors will vary with pigment ratios.

- The presence of pheophytin *a* in a sample requires the method acidification procedure to correct for its contribution to the observed chlorophyll *a* fluorescence. The pheophytin *a* measurement or correction includes any contribution from pheophorbides. Adequate mixing and controlled acid concentration and reaction time for the acidification are required in order to perform the pheophytin correction according to the method.

- The fluorometric method is unreliable because of inaccuracies in measurement if chlorophyll *b* is present. During the acidification step, chlorophyll *b* converts to pheophytin *b*, which contributes to the observed pheophytin *a* fluorescence, potentially causing an overestimation of the pheophytin *a* correction and understimation of chlorophyll *a*.

- Although not as pronounced in effect, chlorophyll *c* may contribute to the overestimation of chlorophyll *a* and underestimation of pheophytin *a*. As an example, *phaeodactylum* extract contains significant amounts of chlorophyll *c,* which results in overestimation of chlorophyll *a*.

- Chlorophyllide *a* is determined as chlorophyll *a* by this method.

- Where algal taxonomic classification is unavailable, the spectrophotometric or HPLC methods may provide more accurate results for chlorophyll *a* and pheophytin *a*.

- Special narrow band pass filters can be used to nearly eliminate the interferences of pheophytin *a*, other pheopigments and chlorophyll *b*. This modified fluorometric method only measures chlorophyll *a* values and does not allow determination of pheophytin *a*. Regardless, this method modification is appropriate when chlorophyll *b* or pheopigments are present in the sample. In this case, the method equations for calculation of chlorophyll *a* without performing the acidification step are used, with minimal overestimation of chlorophyll *a*.

- *In situ* fluorometric sensors (field testing meters), including those with temperature compensation, may yield inaccurate results if pheopigments are present. Results may also depend on the presence of other chlorophylls, dissolved organic matter, humic substances, turbidity; and, algae community composition, light exposure history, physiological status and cell morphology. Thus, environmental conditions at the time of the *in situ* measurement may significantly affect the measured cholorophyll *a* concentration. Measurements using *in situ* sensors are generally less sensitive (higher detection limit) than the laboratory-based fluorometric methods used to analyze filtered and extracted samples.

<u>HPLC (High Performance Liquid Chromatography) method:</u>

- This method can be used to quantify individual chlorophyll pigments and chlorophyll degradation pigments through the physical separation of the sample component pigments by chromatography. HPLC may provide more accurate values for chlorophyll *a* and pheophytin *a*.

- Results from HPLC analyses may be lower than those obtained for the same sample source by the spectrophotometric or fluorometric methods because of the ability to separate the interfering pigments by chromatography (and avoid positive interferences affecting the spectrophotometric and fluorometric methods).

Table A presents a summary of the above discussion

Table A: **Summary of Chlorophyll Method Interferences**

METHODS			***SAMPLE SOURCE***		***INTERFERENCES***		
Method Type	**Method Option**	**Method Number**	**Freshwater**	**Estuary or Coastal**	**Pheophytin or other degradation pigments**	**Chlorophyll *b***	**Other Interferences**
Spectrophotometric	Corrected for pheophytin (using acidification)	EPA 446.0, SM 10200 H.2.b.		Pheopigments may dominate and interfere.	Pheophytin *a* and pheophorbide *a* absorb in the same spectral band as chlorophyll *a*. Error is small as long as pheophytin *a* is the only degradation pigment.	Pheopigments overestimated. Chlorophyll *a* underestimated	Certain carotenoid pigments may interfere and overestimate pheophytin *a*. Chlorophyllide *a* is determined as chlorophyll *a.*
Fluorometric	Corrected for pheophytin (using acidification)	EPA 445.0, SM 10200 H.3.	Not generally usable and not recommended.	Pheopigments may dominate and interfere.	Pheophytin *a* and pheophorbide *a* fluoresce in the same spectral band as chlorophyll *a*. Error is small as long as pheophytin *a* is the only degradation pigment.	Pheopigments overestimated. Chlorophyll *a* underestimated	Chlorophyll *c* may also interfere with overestimation of chlorophyll *a* and underestimation of pheophytin *a.* Chlorophyllide *a* is determined as chlorophyll *a.*
Fluorometric	Free of pheophytin (using narrow-bandpass filters)	EPA 445.0			Minimal overestimation of chlorophyll *a*	Minimal overestimation of chlorophyll *a*	
HPLC	Free of pheophytin	EPA 447.0, SM 10200 H.4.					