Approved by:_____Effective Date:_____Revision #_____

1. **Method:** 10200 H. Spectrophotometric Determination of Chlorophyll - *Standard Methods for the Examination of Water and Wastewater* - 20th edition.

2. **Matrix:** Surface Water

3. **Detection Limits:**
   3.1 Ambient water reporting limits: chlorophyll *a* - 5.0 µg/L, pheophytin - 3.0 µg/L
   3.2 Laboratory reporting limits: chlorophyll *a* - 5.0 µg/L, pheophytin - 3.0 µg/L

4. **Scope and Application:** This method is used to determine chlorophyll *a* and pheophytin in natural water bodies using visible wavelength spectrometry.

5. **Summary of Method:** Phytoplankton containing chlorophyll *a* in a measured volume of sample is concentrated by filtration through a glass fiber filter. The photo-pigments are extracted from the phytoplankton by grinding the filter with a tissue grinder and steeping the filter slurry in 90% aqueous acetone solution overnight. The filter slurry is then centrifuged to clarify the solution and then the supernatant is transferred to a glass spectrophotometric cell. For the pheophytin corrected chlorophyll *a*, the sample's absorbance is measured at 750 and 664 nm before acidification and 750 and 665 nm after acidification with .1 N HCl. No calibration of the instrument is required. Absorbance values are entered into a set of equations in the computer that utilize the extinction coefficients of the pure pigments in 90% acetone. Concentrations are reported in ug/L.

6. **Definitions:**

   6.1 Field split - A field split is a single sample subdivided by field staff immediately following collection and submitted to the laboratory as two separately identified samples. Split samples are preserved, handled, shipped, and analyzed identically. Analysis of field splits give a measure of the precision associated with sample handling, preservation and storage, as well as with laboratory procedures.

   6.2 Ambient Water Reporting Limit (AWRL) - Established reporting limits established by the TCEQ surface water quality monitoring programs so that data may be screened for the Texas Water Quality Inventory. For individual parameters, data must be reported at or below the AWRL. The AWRL for chlorophyll a is 5 ug/L and the AWRL for pheophytin is 3 ug/L

   6.3 Laboratory Reporting Limit (RL) – The lowest concentration that the laboratory chooses to report quantitative data at within an accuracy limit of 75-125%. The laboratory must demonstrate and document on an ongoing basis its ability to quantitate at the reporting limit. To meet this requirement for chlorophyll a analyses, the laboratory purchases pure

chlorophyll *a* extract in 90% acetone and analyses a reporting limit check standard. The certified standard is used to meet the reporting limit requirement by confirming recovery of a .5 mg/L standard of chlorophyll *a* in 90% acetone. This concentration in the extract would result if 1000 mL of ambient water containing 5 ug/L of chlorophyll *a* are extracted into a final 10 mL volume of 90% acetone.

6.4 Laboratory control standard duplicate (LCS) - An LCS duplicate is prepared in the laboratory by splitting aliquots of the chlorophyll *a* extract described in 6.3. Both samples are carried through the analytical process. LCS duplicates are used to assess precision of only the analytical process and are performed at a rate of one per batch. (Note: For the purpose of this analysis the LCS duplicate is a split of the RL check standard and is not prepared at the mid-range of the analysis)

6.5 Laboratory sample duplicate - A laboratory sample duplicate is prepared in the laboratory by splitting a 2 L sample into sample aliquots and filtering 1 liter each and otherwise put each filter through the entire preparation and analytical process. Laboratory sample duplicates are used to assess precision of the entire preparation and analytical process and are performed at a rate of 5 % or once per batch whichever is greater.

6.7 Method blank (also known as a laboratory reagent blank) – A 1 L aliquot of reagent water that is filtered, extracted, and analyzed exactly like a sample. It is used to determine if interferences are present in the analytical system.

6.8 Linear Dynamic Range (LDR) – The range over which the instrument response is linear. The LDR for this test has been determined to be between 0.1 and 1.0 absorbance units.

7. **Interferences:**

7.1 Spectrophotometric – Other photo pigments, degradation products and/or any extracted compounds that absorb light between 630 and 665 nm can interfere with the determination of chlorophyll *a* because they absorb light in the same region of the spectrum as does chlorophyll *a*. A number of procedural steps (to include sample steeping and acidification time) affect light absorption and thus the determination of chlorophyll *a*. A spectrophotometer with a narrow band (path) is needed, samples should be kept out of sunlight as much as possible to avoid degradation, and the steps in this SOP should be followed very closely. This method does not withstand even slight changes in technique.

7.2 Turbidity – An absorbance measurement is made at 750 nm to assess turbidity in the sample. This value is subtracted from the 664 and 665 nm readings. A 750 nm absorbance value of >.005 indicates a poorly clarified sample and causes measurement error.

8. **Safety:**

8.1 Always wear a lab coat, gloves and safety glasses while preparing reagents and performing the method.

8.2 Acetone is considered to be a chemical hazard and may be toxic to biological systems if ingested or inhaled. The grinding of filters should be performed in a fume hood due to the volatilization of acetone. Special handling procedures are listed in the Material Safety Data Sheet (MSDS).

8.3 Hydrochloric acid is also a chemical hazard and care should be taken when preparing the solution. The reactivity of HCL with water or with tissue through contact may cause injury. Acid solutions should be prepared in a fume hood to avoid inhalation. Special handling procedures are also listed in the Material Safety Data Sheet (MSDS).

9. **Equipment and Supplies:**

9.1 Filtration equipment
- 9.1.1 Whatman glass fiber filters GF/F (47-mm diameter)
- 9.1.2 Vacuum pump
- 9.1.3 47-mm fritted disk base and glass filter tower
- 9.1.4 Side arm filter flask
- 9.1.5 Aluminum weighting dish with handle (Fisher 08-732)
- 9.1.6 Disposable solvent resistant syringe (BD 20 mL syringe)
- 9.1.7 Disposable solvent resistant syringe filter (.45 *u*m)
- 9.1.8 Fluorepolymer resin policeman (used to manipulate the sample during the maceration process)

9.2 Wheaton grinding unit.
- 9.2.1 TFE glass grinder
- 9.2.2 Pyrex glass grinder tube

9.3 Centrifuge, capable of 675 g and calibrated centrifuge tubes
9.4 Spectrophotometer, with a resolution not to exceed 2 nm
9.5 Cuvettes (1 cm pathlength matching cuvettes, with caps)
9.6 5 ml pipettor, 1 mL graduated disposable pipet, 3 mL volumetric pipet
9.7 Tweezers
9.8 Rack for holding centrifuge tubes
9.9 Brown polyethylene sample collection bottles, 2 liters
9.10 Volumetric flask, 10 mL
9.11 Aluminum foil

10. Reagents and Standards:

10.1 Chlorophyll *a* certified chlorophyll stock standard 10mg/L ± 1 mg/L: Obtained from Turner Designs (408) 212-4050. To prepare a .5 mg/L reporting limit check standard, add .5 mL of standard with a .5mL volumetric pipet to a 10 mL volumetric flask that has been rinsed with 90% aqueous acetone solution. Bring to volume with 90 % aqueous acetone solution and cap. Wrap flask in aluminum foil and store in refrigerator before use. Make fresh from stock each time right before samples are analyzed. Store stock solution in the freezer between uses.

Calculate the exact concentration based on the certified concentration provided by Turner.
(Note: .5 mg/L is the concentration in the extract which would result if 1000 mL of ambient water containing 5 ug/L of chlorophyll *a* is extracted into a final 10 mL volume of 90% acetone.)

10.2 HCl, .1N Add 8.5 mL of concentrated HCL to approximately 500 mL water and dilute to 1 liter.

10.3 Aqueous Acetone solution: Measure 100 mL of saturated magnesium carbonate solution with a 100 mL graduated cylinder into a 1 L graduated cylinder and transfer into a storage bottle. Measure 900 mL of HPLC grade acetone into a 1000 mL graduated cylinder and transfer into the storage bottle containing the acetone. Mix well.

10.4 Saturated Magnesium Carbonate Solution: Weigh 1.00 gram magnesium carbonate with the top loading balance and transfer into a 100 mL volumetric flask. Bring to volume with deionized water.

11. Sample Collection, Preservation, Shipment, Filtration and Storage:

11.1 Two liter samples are collected as grab samples in brown polyethylene bottles and delivered to the lab in coolers on ice. Samples are stored in the refrigerator at 4°C until processing. The holding time is 24-48 hours at 4° C, until filtration, and after filtration, 28 days, frozen, until analysis. Normally, 1 L or less will be filtered. However, a 2 L sample is collected so that additional water from water bodies with very low turbidity and very low chlorophyll *a* concentrations can be filtered. This will result in adequate concentration of phytoplankton on the filter to obtain absorbance units within the linear dynamic range of the analysis. (OD 664 between 0.1 and 1.0 AU with a 1 cm path length cell)

11.2 Filter samples in subdued light. Remove samples one at a time from the refrigerator. Place a 47 mm glass fiber filter on the filter funnel. Pour an appropriate volume of sample on top of filter. Both the amount of suspended solids and chlorophyll concentration in the sample will determine the amount of sample filtered. Filter enough sample to yield chlorophyll concentrations in the extract that are within the linear dynamic range of the analysis. Typically, enough sample is filtered when a visible green color is apparent on the filter. Filtering more than 1 L of sample and/or using multiple filters may be necessary to concentrate enough phytoplankton on the filter. Apply vacuum at no more than 2/3 atm (atmospheres) and continue until all water is removed. Release the vacuum as the last bit of water is drawn down, being careful not to suck the filter dry. Split one sample into two 1 L aliquots and filter and run as sample duplicates. Filter 1 L of deionized water as a method blank after all samples are filtered and otherwise treat it as a sample throughout the preparation and analytical process.

11.3 Carefully remove the filter tower and the filter from the base and fold in half and in half again. Blot the outside of the filter dry, place in a petri dish, tape closed, wrap in aluminum foil and store frozen in ziplock bags for at least 24 hours but no more than 28 days. Be sure to mark volume filtered, sample date, filtration date and sample ID (lab number or site name) on the individual samples.

12. Quality Control: Section 1020 B of *Standard Methods* applies to this method. To perform this method initially, each analyst will perform, and have on record, a determination of method detection limit and an initial demonstration of capability. In addition, precision and bias are controlled on an on-going basis through the analyses of samples in Table 12.

Table 12. QC Analyses, Method Performance, and Corrective Action

Parameter	Description	Frequency	Performance and Acceptance Criteria	Corrective Action for Out of Control Data
Method Blank	1 liter aliquot of reagent water that is filtered, extracted, and analyzed exactly like a sample.	1 per batch	<RL, or for high-level analyses, <5% of the lowest value of the batch	Do not precede with analysis: 1. Rezero instrument 2. Check cuvettes to ensure they match 3. Check for turbidity in extract 4. Trouble shoot instrument 5. Report to supervisor 6. Provide notations on bench sheet
RL Check Standard	Certified standard used to meet the reporting limit requirement by confirming recovery of a .5 mg/L standard of Chl *a* in 90% acetone.	1 per batch	75-125%	Do not precede with analysis: 1. Rezero instrument 2. Check cuvettes 3. Rerun the sample being careful with acidification technique and time 4. Prepare another RL check standard 5. Report to supervisor 6. Provide notations on bench sheet
LCS/LCS dup	Sample used to assess precision by splitting aliquots of the RL check standard and analyzing separately.	1 per batch	<20%RPD	Do not precede with analysis: 1. Rezero instrument 2. Check cuvettes 3. Rerun the sample being careful with acidification technique and time 4. Prepare another RL check standard 5. Report to supervisor 6. Provide notations on bench sheet
Laboratory sample duplicate	Sample prepared in the laboratory by splitting a 2L sample into 2 1L aliquots and run as separate samples.	20% or 1 per batch, whichever is greater	Evaluated against control charts	1. Try to determine cause (i.e., low level of chl *a* in sample, not enough sample filtered, etc.) 2. Report to supervisor 3. Provide notations on bench sheet 4. QAO is responsible for determining impact of batch and verifying and validating data
Field Split	Single sample subdivided by field staff and submitted to lab as 2 separate samples.	10% or 1 per batch, whichever is greater	Screening level of <30% RPD	1. Try to determine cause (i.e., field or lab issue) 2. Report to supervisor 3. Provide notations on bench sheet 4. QAO is responsible for determining impact of batch and verifying and validating data

13. Calibration and Standardization:

13.1 No calibration is required.
13.2 Standardization is based on the Turner Chlorophyll *a* reporting limit check standard in an intermediate stock solution of 90% acetone
13.3 Cuvettes used to zero the instrument and measure sample absorbances are matched

14. Procedure:

14.1 To grind the samples, remove filters from freezer, one sample at a time.
14.2 Roll and fold filter. Place into grinding tube.
14.3 Add 2-3 mL of 90% aqueous acetone solution to grinding tube.
14.4 Remove stopper and begin grinding with Wheaton grinding unit and TFE glass grinder. Grind for 1- 2 minutes. The grinder speed should be set at approximately level four. The speed is set low enough to prevent splatter of the acetone but fast enough to grind the filter sufficiently.
14.5 After the grinding step is completed, transfer contents of grinding tube into a centrifuge tube. The fluorepolymer resin policeman can be used to scrape the side of the tubes. With aqueous acetone, rinse all filter residue into centrifuge tube, and bring final volume up to exactly 10.1 mL. (This volume yields an extract volume of 10.0 mL when accounting for the displacement volume of the 47 mm glass fiber filter).
14.6 Stopper the centrifuge tube, label, and place in the dark @4 C to steep for 14-18 hours. Rinse the grinding apparatus with tap water prior to a final rinse with DI water and then 90% acetone. Repeat process for each sample. Do not leave samples out of the refrigerator when they are not being worked on.
14.7 First thing the following morning, turn on the spectrophotometer to let it warm up while clarifying the samples and preparing to run the analysis. To clarify the samples remove tubes from refrigerator and place tubes in centrifuge and spin for 5 minutes at 500 g. Be sure the centrifuge is balanced and covered. Put tubes back in the refrigerator.
14.8 To further clarify the extract volume to ensure 750 nm readings are <.005 AU, put the centrifuged extract volume in a syringe and push through the solvent resistant disposable filter (0.45 um). Minimize sample retention time in syringe and filter. Analyze the samples in the order in which they were extracted so that all samples are steeped for approximately the same amount of time. Measure volume of extract. *(See footnote #1 for an alternative procedure using the same tube for both grinding and centrifugation. This method does not involve pouring the sample from a grinding tube to a centrifuge tube and does not require additional clarification using a syringe filter.)*
14.9 To begin running the samples, start by running first the reporting limit check sample in duplicate. (The duplicate analysis serves as the LCS duplicate. Transfer <u>exactly</u> 3 mL of extract (sample) to one of a set of matched cuvettes using a volumetric l pipet, and 3 mL of 90% aqueous

acetone solution into the other cuvette ("blank"). Cap cuvettes with cap. Use 1 cm cuvettes at all times.

14.10 Use the "blank" to zero the instrument at all the selected wavelengths. Read absorbance of "sample" at same wavelengths.

14.11 Add exactly 0.1 mL of 0.1N HCl with a 1 mL graduated pipet to the sample cuvettes. Gently invert to mix for exactly 90 seconds. Read the acidified extract volumes at 665 and 750 nanometers.

14.12 Calculate chlorophyll a, and pheophytin, using the formulas in Section 15.

14.13 After evaluation of the reporting limit check standard and the LCS duplicate sample, repeat steps 14.1 through 14.11 for each sample.

15. Calculations:

Using the following formula, calculate chlorophyll *a* and pheophytin concentrations from the turbidity corrected $664_{\text{before acidification}}$ and $665_{\text{after acidification}}$ readings by subtracting the 750 Abs reading from its respective 664 and 665 reading and applying the following formula:

$$\text{Chlorophyll ug/L} = \frac{26.7\,(\text{corr. } 664 - \text{corr. } 665) \times \text{volume of extract in L}}{[\text{volume of sample in L}] \times 1\text{cm}} \times 1000$$

$$\text{Pheophytin, ug/L} = \frac{26.7\,[1.7(\text{corr. } 665) - \text{corr. } 664] \times \text{volume of extract in L}}{[\text{volume of sample in L}] \times 1\text{ cm}} \times 1000$$

16. Method Performance: See Table 12

17. Pollution Prevention:

17.1 Minimal amounts of acetone solvent are used for this analysis. Glassware and equipment are rinsed first rinsed with tap and then deionized water and finally acetone to prevent excessive use of solvent.

18. Data Assessment and Acceptance Criteria for Quality Control Procedures: See Table 12

19. Corrective Action for Out of Control Data: See Table 12

20. Contingencies for Handling Out of Control or Unacceptable Data:

20.1 The impact of out of control data will be determined during data verification and/validation. Determination of acceptability will be made by the QAO and the customer will be notified.

20.2 The customer will be notified if a sample does not meet holding time criteria or other sampling criteria.

21. Waste Management:

Samples and rinse solvent need to be disposed in the container marked "Waste Solvent" located in the fume hood. No other special requirements apply.

22. References:

Standard Methods for the Examination of Water and Wastewater 20th edition" 10200 H. Chlorophyll

23. Tables, Diagrams, Flow Charts:

23.1 Bench sheet
23.2 Spreadsheet used to perform calculations
23.3 MSDS Sheets for Acetone and hydrochloric acid

Footnote #1 – Alternative maceration/centrifugation technique: *Macerate the filter inside the centrifuge tube with a tissue homogenizer using an exact amount of acetone solution. After centrifugation, pipet 3 mL of the clarified extract from the centrifuge tube directly to the cuvette. The amount of acetone solution added (e.g., 10 mL) is used as the extract volume in the calculations.*