



Environmental Analysis Program

Pomona College
Biogeochemistry Laboratory

SOP-L310

Chlorophyll *a* Extraction and Fluorometric Analysis

Standard Operating Procedure

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1. Purpose

This procedure describes the in vitro determination of chlorophyll *a* and pheophytin *a* concentrations in marine and freshwater phytoplankton by fluorescence. Chlorophyll-containing cells are concentrated on a glass fiber filter, extracted in 90% acetone, and measured on the Turner Designs Trilogy Fluorometer before and after acidification with 0.1 N HCl. The method is modified from US EPA Method 445.0 and APHA Standard Methods 10200 H (APHA et al., 2012; Arar & Collins, 1997).

2. Scope

- **Applies to:** Chlorophyll *a* and pheophytin *a* in surface water, freshwater, and marine water samples collected per [SOP-F300](#)
- **Does not apply to:** In vivo chlorophyll measurements with field multiprobes (see [SOP-F200](#)); benthic algae unless filtration volumes are adjusted; HPLC-based pigment analysis
- **Location:** EA Program laboratory, SGM 133

3. Responsibilities

Role	Responsibility
Lab PI	Approve SOP, ensure standards are current
Lab Manager	Maintain fluorometer, stock reagents and filters
Trained Personnel	Follow this SOP, report deviations
New Students	Complete training before independent use

4. Definitions

- **Chlorophyll a:** The primary photosynthetic pigment in phytoplankton, algae, and higher plants; absorbs red light (~665 nm) and blue light (~430 nm); the standard proxy for phytoplankton biomass in aquatic systems.
- **Chlorophyll b:** An accessory pigment found in green algae and higher plants; absorbs at ~647 nm; distinguishable from chlorophyll *a* by spectrophotometry or fluorometry.
- **Fluorometric method:** Measurement of chlorophyll *a* by fluorescence emission (~685 nm) after excitation (~430 nm); more sensitive than spectrophotometry; requires acidification to distinguish chlorophyll *a* from pheophytin *a*.
- **Pheophytin a:** The magnesium-free degradation product of chlorophyll *a*; retains similar absorption properties; distinguished from chlorophyll *a* by the acidification step, which converts chlorophyll *a* to pheophytin *a* and causes a characteristic decrease in fluorescence.

- **Trichromatic equation:** Spectrophotometric calculation of chlorophyll *a*, *b*, and *c* concentrations in an extract from absorbance readings at three wavelengths (665, 647, 630 nm); corrects for spectral overlap among pigments.
- **Turbid extract correction:** Subtraction of non-specific absorbance (measured at 750 nm, where chlorophyll does not absorb) from all pigment wavelength readings to correct for turbidity and colored dissolved compounds in the extract.

5. Safety and Hazards

Hazards

- **Chemical:** Acetone (HPLC grade) is highly flammable and an irritant. Work in the fume hood and away from ignition sources. Concentrated HCl is severely corrosive. 0.1 N HCl is a mild irritant.
- **Physical:** Glass filtration apparatus and cuvettes pose breakage risk. Sonicator tip generates heat and noise.
- **Noise:** The sonicator requires hearing protection (ear plugs or ear muffs) whenever it is in use.

5.1. Required PPE

- ☐ Safety glasses
- ☐ Lab coat
- ☐ Nitrile gloves
- ☐ Hearing protection (when using sonicator)

5.2. Emergency Procedures

For acetone spills, extinguish all ignition sources, ventilate the area, and clean up per [SOP-L003 Hazardous Materials Handling](#). For HCl splashes, flush with water for at least 15 minutes. Dispose of all acetone waste in the designated organic waste collection bottle.

6. Equipment and Materials

6.1. Equipment

Item	Notes
Turner Designs Trilogy Fluorometer (P/N 998-7210)	With Chlorophyll Acidification (Chlor-A) module; see SOP-L203
Millipore glass filtration unit	With vacuum pump and 47 mm fritted glass base
Sonicator	With probe tip; set to 70% amplitude

Item	Notes
Tissue grinder (alternative)	Teflon-tipped pestle with grooved surface, 30 mL glass grinding tube
Clinical benchtop centrifuge	Capable of 1,000 x g with fixed-angle rotor; see <i>SOP-L109</i>
Fume hood	Required for all acetone and HCl work

6.2. Consumables

Item	Notes
Whatman GF/F glass fiber filters (47 mm, 0.7 μ m)	For concentrating phytoplankton
Glass cuvettes (10 x 10 mm)	For fluorometer; see SOP-L203
15 mL glass or polypropylene centrifuge tubes	Screw-cap; for extraction
Plastic petri dishes	For storing filters before extraction
Aluminum foil	For wrapping samples to exclude light
Kimwipes	For blotting filters and cleaning cuvettes
Forceps (flat-tipped)	For handling filters
Pasteur pipettes or medicine droppers	For dispensing HCl
Class A volumetric pipettes	Assorted sizes
Class A volumetric flasks (50 mL, 100 mL, 1 L)	For standard preparation

6.3. Reagents and Standards

Item	Notes
Acetone, HPLC grade (CAS 67-64-1)	Stored in flammables cabinet
Concentrated HCl (sp. gr. 1.19; CAS 7647-01-0)	Stored in acid cabinet
Chlorophyll <i>a</i> standard (free of chlorophyll <i>b</i>)	Commercial source (e.g., Sigma); stored at –20 C
Milli-Q water (Type I)	From SOP-L11

7. Procedure

7.1. Reagent Preparation

0.1 N HCl: Add 0.85 mL of concentrated HCl to approximately 50 mL of Milli-Q water in a 100 mL volumetric flask. Bring to volume with Milli-Q water. Mix thoroughly.

90% Aqueous Acetone: Measure 100 mL of Milli-Q water into a 100 mL volumetric flask, then transfer to a 1 L volumetric flask. Fill to the 1 L mark with HPLC-grade acetone. Mix and transfer to a dark amber bottle. Label and store in the flammables cabinet.

Chlorophyll *a* Stock Standard Solution (SSS): Working in subdued light, transfer the contents of the chlorophyll *a* ampoule into a 50 mL volumetric flask and dilute to volume with 90% acetone. Determine the concentration spectrophotometrically. Wrap in foil and label with the lot number. Stable for at least six months in an airtight container at room temperature.

Primary Dilution Standard (PDS): Add 1 mL of SSS to a 100 mL volumetric flask and dilute to volume with 90% acetone. If the SSS was prepared from exactly 1 mg of pure chlorophyll *a*, the PDS concentration is 200 ug/L. Prepare fresh before use. Wrap in foil.

Quality Control Standards (QCs): Prepare from the PDS at the following concentrations: 5, 20, 50, and 200 ug/L. Wrap in foil.

! Important

All photosynthetic pigments are light- and temperature-sensitive. Perform all work in subdued light. Store all standards, QC materials, and filter samples in the dark at –20 C when not in use.

7.2. Filtration

1. Invert the sample bottle gently several times to suspend particles
2. Assemble the glass filtration unit: place the fritted glass base on the Erlenmeyer flask, position a GF/F filter on the frit using forceps, seat the glass funnel on top, and secure with the spring clamp
3. Connect the vacuum hose and turn on the vacuum
4. Working in subdued light, filter the sample in 20 mL increments using a graduated cylinder. The volume required depends on algal density:

Sample Type	Typical Volume
Benthic samples	approximately 5 mL
Freshwater	20–300 mL
Seawater	100–1000+ mL

5. Stop filtering when a brown or green tint is visible on the filter. Record the exact filtered volume. If the filter becomes too darkly colored, the sample may exceed the linear dynamic range and require dilution during analysis.
6. Using forceps and a micro spatula, fold the filter in half with the retained material on the inside
7. Gently blot excess water from the outside of the filter with a Kimwipe
8. Place the folded filter in a clean 15 mL centrifuge tube or a plastic petri dish
9. Include a laboratory reagent blank (100 mL Milli-Q water through a GF/F filter) and a field duplicate every 10–15 samples
10. Wrap the tubes or dishes in aluminum foil and store at –20 C. Filters may be stored frozen for up to 28 days before extraction.

7.3. Extraction – Sonication Method

1. Remove samples and QCs from the freezer and allow to reach room temperature
2. Add 25 mL of 90% aqueous acetone to each tube, ensuring the filter is fully submerged. Cap the tubes and invert several times.
3. Secure the sonicator probe in its clamp stand
4. **Put on hearing protection**
5. Set the sonicator to 70% amplitude
6. Submerge the tip halfway into the acetone solution and sonicate, moving the tip around the sample to break up the filter completely
7. After sonication, shake the tube vigorously, wrap in foil, and steep in the refrigerator (4 C) for a minimum of 2 hours

Important

Never operate the sonicator outside of solution. The tip will overheat and be damaged.

7.4. Extraction – Tissue Grinder Method (Alternative)

1. Using forceps, transfer the filter into a 30 mL glass grinding tube and push it to the bottom with the pestle or a glass rod
2. Add 4 mL of 90% acetone and grind with the pestle until the filter is converted to a slurry. Avoid overheating.
3. Pour the slurry into a 15 mL screw-cap centrifuge tube
4. Rinse the pestle and grinding tube with 6 mL of 90% acetone and add the rinse to the centrifuge tube
5. Cap, shake vigorously, wrap in foil, and steep at 4 C for 2 hours
6. Clean the grinder and pestle with water followed by acetone between samples; wipe dry with a Kimwipe

7.5. Post-Extraction Processing

1. Remove samples from the refrigerator and allow to reach room temperature (approximately 30 minutes)
2. Shake each tube vigorously
3. Centrifuge at 1,000 x g for 5 minutes using the fixed-angle rotor until all filter material is pelleted
4. Handle tubes carefully after centrifugation; do not resuspend the pellet

7.6. Fluorometric Analysis

1. Transfer 2.5 mL of clear supernatant from each tube into a clean glass cuvette, taking care not to disturb the pellet
2. Turn on the Trilogy Fluorometer and allow the 10-minute warm-up to complete (see [SOP-L203](#))

3. Select the **Chlor-A** (Chlorophyll Acidification) module on the touch screen
4. Calibrate using the QCs prepared from the PDS, or load a saved calibration. For a new calibration, follow the Direct Multipoint Calibration procedure in [SOP-L203](#), Section 7.3.
5. Run a 90% acetone blank
6. Wipe the outside of each cuvette with a Kimwipe before inserting into the fluorometer
7. Measure and record the pre-acidification fluorescence (F_b)

i Note

If the chlorophyll *a* reading exceeds 90% of the upper limit of the linear dynamic range (LDR = 1,000 ug/L), dilute the sample before proceeding. For a 1:2 dilution, add 1 mL of sample to 1 mL of 90% acetone. For a 1:100 dilution, add 50 uL of sample to 4.95 mL of 90% acetone.

8. Add 75 uL of 0.1 N HCl to the cuvette. Seal with parafilm and invert several times to mix. Wait 90 seconds.
9. Measure and record the post-acidification fluorescence (F_a)
10. Repeat for all samples, QCs, and blanks

8. Quality Control and Acceptance Criteria

8.1. Calculations

Variables:

- $[Std]$ = concentration of the calibration standard used (ug/L)
- S_b = standard fluorescence reading before acidification
- S_a = standard fluorescence reading after acidification
- F_b = sample fluorescence before acidification
- F_a = sample fluorescence after acidification
- V_e = volume of extract (mL)
- V_s = volume of sample filtered (mL)

Equations:

$$AR = \frac{S_b - \text{Blank}}{S_a - \text{Blank}}$$

$$\text{InterpB} = [Std] \times \frac{F_b - \text{Blank}}{S_b - \text{Blank}}$$

$$\text{InterpA} = [Std] \times \frac{F_a - \text{Blank}}{S_b - \text{Blank}}$$

$$\text{Chlorophyll } a \text{ (}\mu\text{g/L)} = \frac{AR}{AR - 1} \times (\text{InterpB} - \text{InterpA}) \times \frac{V_e}{V_s}$$

$$\text{Pheophytin } a \text{ (}\mu\text{g/L)} = (AR \times \text{InterpA} - \text{InterpB}) \times \frac{V_e}{V_s}$$

8.2. QC Requirements

- Run a laboratory reagent blank (LRB) every 10–15 samples. The LRB should read below the calculated lower detection limit.
- QC standards should recover within $\pm 20\%$ of the known value
- Relative standard deviation (RSD) of replicate measurements should be within $\pm 20\%$
- Field duplicates should agree within $\pm 20\%$ relative percent difference
- Samples, standards, LRBs, and QCs must all be at ambient room temperature during analysis. Fluorescence is temperature-dependent; cooler samples produce artificially high readings.

9. Waste Disposal

Waste Type	Disposal Method
Acetone extracts and rinses	Collect in the designated organic waste bottle in the fume hood; EH&S pickup
Acidified sample waste (HCl)	Add to the organic waste bottle (small volumes of dilute HCl in acetone matrix)
Used GF/F filters	Standard trash (if non-hazardous samples)
Used cuvettes	Rinse with acetone, then DI water; reuse or recycle

10. References

- USEPA. (1997). Method 445.0: *In Vitro Determination of Chlorophyll *a* and Pheophytin *a* in Marine and Freshwater Algae by Fluorescence*. Revision 1.2. National Exposure Research Laboratory, Cincinnati, OH.
- APHA, AWWA, WEF. (2012). Standard Methods 10200 H: Chlorophyll. In: *Standard Methods for Examination of Water and Wastewater*. 22nd ed. American Public Health Association (Eds.). Washington. 1360 pp.
- Turner Designs. *Trilogy Laboratory Fluorometer: User's Manual*.

APHA, AWWA, & WEF. (2012). *Standard methods for the examination of water and wastewater* (22nd ed., p. 1360). American Public Health Association.

Arar, E. J., & Collins, G. B. (1997). *Method 445.0: In vitro determination of chlorophyll *a* and pheophytin *a* in marine and freshwater algae by fluorescence*. U.S. Environmental Protection Agency, Office of Research; Development, National Exposure Research Laboratory. https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=309417

11. Training Record

Before performing chlorophyll analysis independently, personnel must:

1. Read this SOP, [SOP-L203 Trilogy Fluorometer](#), and [SOP-L003 Hazardous Materials Handling](#) in full
2. Review the SDS for acetone and HCl
3. Demonstrate proper filtration technique under supervision
4. Perform at least one complete extraction-through-analysis cycle on QC standards with acceptable recovery (+/- 20%)
5. Demonstrate proper fluorometer calibration
6. Be signed off by the PI or lab manager

12. Appendix A: Example Calculation

```
# Corrected chlorophyll a and pheophytin a calculation
# Per EPA Method 445.0

# Calibration standard
Std_conc <- 50          # ug/L (QC standard concentration)
S_b <- 485              # Standard fluorescence before acid
S_a <- 210              # Standard fluorescence after acid
blank <- 5              # Blank fluorescence

# Sample readings
F_b <- 320              # Sample fluorescence before acid
F_a <- 140              # Sample fluorescence after acid

# Volumes
V_e <- 25               # mL of extract (acetone)
V_s <- 100              # mL of sample filtered

# Calculations
AR <- (S_b - blank) / (S_a - blank)
InterpB <- Std_conc * (F_b - blank) / (S_b - blank)
InterpA <- Std_conc * (F_a - blank) / (S_b - blank)

chl_a <- (AR / (AR - 1)) * (InterpB - InterpA) * (V_e / V_s)
pheo_a <- (AR * InterpA - InterpB) * (V_e / V_s)

cat(sprintf("Acid ratio (AR):          %.3f\n", AR))
cat(sprintf("Chlorophyll a:           %.2f ug/L\n", chl_a))
cat(sprintf("Pheophytin a:           %.2f ug/L\n", pheo_a))
```

13. Appendix B: Interferences

Interference	Effect	Mitigation
Chlorophyll <i>b</i>	Overestimates chlorophyll <i>a</i>	Optical filters in the Chlor-A module minimize this; if severe, use HPLC
Pheophytin <i>a</i>	Overestimates chlorophyll <i>a</i> if not acidified	Acidification step corrects for this
Other fluorescent compounds	Positive bias	Laboratory reagent blanks detect this
Quenching (concentrated samples)	Underestimates concentration	Dilute samples approaching 90% of LDR
Temperature differences	Higher sensitivity at lower temperatures	Ensure all materials are at ambient temperature
Light exposure	Degrades pigments	Work in subdued light; wrap in foil
