

Washington State Department of Ecology

Environmental Assessment Program

Standard Operating Procedure for Chlorophyll *a* Analysis

Version 2.0

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Date – July 21, 2008

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EAP026

APPROVED

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Although Ecology follows the SOP in most instances, there may be instances in which Ecology uses an alternative methodology, procedure, or process.

Environmental Assessment Program

Standard Operating Procedure for Chlorophyll *a* Analysis

1.0 Scope and Application

1.1 This Standard Operating Procedure (SOP) is for the analysis of chlorophyll *a* samples collected during all seawater sampling events conducted by the Coastal and Estuarine Assessment Unit.

2.0 Summary of Method

2.1 Filter chlorophyll *a* samples

2.2 Sonicate samples

2.3 Centrifuge samples

2.4 Analyze chlorophyll *a* on Turner fluorometer

3.0 Interferences

3.1 The presence of chlorophyll *b* in all chlorophyll samples can lead to an underestimation of chlorophyll *a* concentration in all samples.

4.0 Sample Collection, Preservation, Storage and Holding Times

4.1 Once the chlorophyll samples have been collected in brown 65 ml polyethylene sample bottles, filtration should occur within 6 hours. Sample bottles should be kept in the dark and on ice until it is time for filtration. This will help to minimize degradation due to heat and light exposure. Once filtered, samples may be kept in the freezer for up to one month.

4.2 Phytoplankton pigments degrade when exposed to heat and light, so work with samples on ice and covered as much as possible.

5.0 Apparatus and Materials

5.1 Safety goggles

5.2 Nitrile exam gloves

5.3 De-ionized water (18 megohm) in a squirt bottle

5.4 Filtered seawater in a squirt bottle. Filtered seawater is made by filtering whole seawater through a 45um Whatman GF/F filter and stored in the fridge until use.

5.5 25 mm 0.45 um Whatman glass fiber filters (GF/F)

- 5.6 Filter forceps – stainless steel, straight, flat, smooth tip
- 5.7 Gast oil-less vacuum pump
- 5.8 Polycarbonate in-line filter holders and manifold
- 5.9 12 ml clear glass centrifuge tubes
- 5.10 Aluminum foil
- 5.11 Turner Designs 10-AU Fluorometer (analog or digital)
- 5.12 Sonicator – Vibra-Cell 500 Watt Ultrasonic Processor with probe
- 5.13 Centrifuge – IEC Centra CL2

6.0 Reagents

- 6.1 Filtered Seawater (FSW). Low chlorophyll a seawater that has been filtered through a 0.45 Whatman GF/F filter to remove any chlorophyll. Used during filtration and quality control procedures. Kept in a refrigerator and periodically re-filtered to remove any bacteria.
- 6.2 Magnesium Carbonate ($MgCO_3$), supersaturated in de-ionized water, in a squirt bottle. This chemical is stable at room temperature for 2 years. The MSDS can be found at <http://www.jtbaker.com/msds/englishhtml/M0143.htm>.
- 6.3 Certified ACS grade 90% acetone. Acetone is not known to be carcinogenic or teratogenic, but it can cause defatting of skin tissues on contact. The MSDS may be found at <http://jtbaker.com/msds/englishhtml/A0446.htm>. Dilute concentrated acetone to 90% with de-ionized water. Follow the SOP for Reagent Preparation to make 90% acetone.
- 6.4 10% Hydrochloric acid (HCl). HCl is a highly corrosive, caustic chemical. It may also have mutagenic and teratogenic properties. Extreme care must be taken when handling this chemical. The MSDS for HCl may be found at <https://fscimage.fishersci.com/msds/95551.htm>.

7.0 Procedure

7.1 Filtering Chlorophyll Samples

7.1.1 Notes:

- 7.1.1.1 **Phytoplankton pigments, including chl-a, degrade when exposed to heat and light, so work with samples on ice and covered as much as possible.*
- 7.1.1.2 **Keep sample bottles on ice and in the dark until filtration to minimize degradation due to heat and light exposure. (It is best to filter immediately; never store for >6 h).*
- 7.1.1.3 **Be sure to record bottle numbers as the volumes vary slightly and have all been pre-measured.*

- 7.1.2 **Label** centrifuge tubes. Be sure to use the 10 mL centrifuge tubes. Include 2 tubes for method blanks and 2 tubes for filtration blanks.
- 7.1.3 **Place** 0.45 um Whatman GF/F filters on all filtration rack frits using forceps. There is no up/down orientation to the filter. Attach the filter funnels and check to see that they are seated correctly. Leave the valves in the closed (horizontal) position.
- 7.1.4 **Add 3 drops of MgCO₃** solution (super-saturated in DI water) to each filter.
- 7.1.5 **Turn on** vacuum pump. Set pressure between 5-7 psi.
- 7.1.6 **Method Control Blank 1. Before sample filtration, filter** an equivalent volume of DI water to sample volumes through one filter. **Place** filter into the corresponding centrifuge tube.
- 7.1.7 **Filtration Control Blank 1. Before sample filtration, filter** an equivalent volume of FSW to rinse volume (~15 mLs.) through one filter. **Place** filter into the corresponding centrifuge tube.
- 7.1.8 **Place** sample bottles in front of the funnels. Double check the numbers on the sample bottle labels and the tubes to make sure they correspond.
- 7.1.9 **Pour** entire sample into filter cup.
- 7.1.10 **Rinse** sample bottle with filtered seawater and empty contents into filter cup. Open valve beneath filter cup to start filtration.
- 7.1.11 **Rinse** down filter cups with filtered seawater (FSW) after sample has filtered through. Turn valve off for each funnel as soon as all water has filtered through.
- 7.1.12 **Remove** filter from frit using forceps and fold it in half (pigment side in). Take care to not touch the pigments with the forceps.
- 7.1.13 **Place** in the corresponding centrifuge tube.
- 7.1.14 **Method Control Blank 2. After sample filtration, filter** an equivalent volume of DI water to sample volumes through one filter. **Place** filter into the corresponding centrifuge tube.

- 7.1.15 **Filtration Control Blank 2.** *After sample filtration, filter* an equivalent volume of FSW to rinse volume (~15 mLs.) through one filter. **Place** filter into the corresponding centrifuge tube.
- 7.1.16 **Prime** the acetone dispenser so that it is free of bubbles. Check that it is dispensing exactly 10 mL of acetone each time.
- 7.1.17 **Fill** centrifuge tubes with 10 ml of 90% acetone. Cap tubes. Be sure the filter is immersed in the acetone and tap tube gently to knock filter into acetone.
- 7.1.18 **Keep** tubes covered with foil as chl-*a* is degraded by exposure to light.
- 7.1.19 **Record** tube #s after chlorophyll sample bottle #s on field logs.
- 7.1.20 **Cover** test tube rack with foil and label with sampling date and project name. Store rack in freezer.
- 7.1.21 **For clean up:** Run hot water through the filtration rack. Run pump until cups are empty & tilt rack while pump is running so all water is emptied from rack. Remove filtration cups and rinse with hot water & DI. Replace cups on rack or store upside down on rack. Leave valves open on filtration rack to facilitate drying. Rinse off forceps with DI water.
- 7.2 Running Chlorophyll Samples
- 7.2.1 **Notes:**
- 7.2.1.1 **In all steps, try to avoid degradation of pigments by minimizing sample exposure to light and heat.*
- 7.2.1.2 **Chlorophyll Samples need to be run within 4 weeks to avoid expiration.*
- 7.2.2 **Set up** fluorometer and turn “on” (needs to warm up ~20 minutes). Fluorometers rely on the steady state of the internal light emitting bulb and therefore need time to warm-up.
- 7.3 Sonicator
- 7.3.1 **Fill** sonicator bath about 2/3 full with ice water.
- 7.3.2 **Take** centrifuge tubes from freezer and place in sonicator bath.
- 7.3.3 **Sonicate** tubes for 7 minutes at the following settings:
- 7.3.3.1 ***Amplitude = 60***
- 7.3.3.2 ***Pulser = ‘off’***
- 7.3.3.3 ***Timer = 7***
- 7.3.3.4 ***Tune to minimum frequency***
- 7.3.4 **Let** samples sit in sonicator for at least 10 minutes after sonicating (keep cold and dark).

7.4 Centrifuge

- 7.4.1 **Shake** the samples well – this is very important, otherwise the extract will remain concentrated around the filter and reading will not be accurate.
- 7.4.2 **Place** tubes in centrifuge for 5 minutes at 3600 rpm. Always make sure the centrifuge is balanced if you don't have a full load.
- 7.4.3 **Remove** tubes carefully, so as not to re-suspend particulates, and place tubes in rack – check to be sure particulates are settled out. Keep extracts in the cold and dark until use.

7.5 Analog Fluorometer

- 7.5.1 What about solid standard?
- 7.5.2 **Fill** a cuvette with 90% acetone and place in fluorometer. Reading should be very close to zero. If it is not, **DON'T** try to re-zero the instrument!
- 7.5.3 **In one motion**, carefully decant the extract of your first sample into a cuvette until the liquid is about ½-1 inch from the top. Keep the fold of the filter towards the cuvette as you are decanting from the centrifuge tube and the level of liquid above the filter, to minimize the amount of particulates in the cuvette.
- 7.5.4 **While holding the cuvette at the top**, wipe down the cuvette with a kimwipe so that no fingerprints will interfere when the sample is read by the fluorometer.
- 7.5.5 **Place** the cuvette in the fluorometer and the cap over the cuvette.
- 7.5.6 **Adjust** the broad scale (1x or 100x – the black lever to the right of where the cuvette is inserted) and the fine scale (min, 3.16x, 10x or 31.6x – the silver switch labeled “step”) until the reading falls between 3 and 9.5 on the top row of numbers.
- 7.5.7 You should read this number to the nearest 0.05. Each division on the scale represents a difference of 0.20 and there are 4 possible readings for each division. For example, if the needle falls between 5.20 and 5.40, your reading will be 5.20, 5.25, 5.30, 5.35 or 5.40. The scale should be at eye level for you to determine the exact reading.
- 7.5.8 **Record** this number as the **F_o**.
- 7.5.9 **Add** 2 drops of 1N HCl to the cuvette.
- 7.5.10 **Take** another reading and record this number as the **F_a**.
- 7.5.11 **Dump** the contents of the cuvette in a beaker or waste acetone jug, then rinse the
- 7.5.12 **Repeat** with the next sample.
- 7.5.13 When you are finished, rinse the cuvette 3 times with 90% acetone before storing.
- 7.5.14 Pour waste acetone into a labeled jug and store in the acetone cabinet.

- 7.6 Digital Turner 10-AU-005 Fluorometer
- 7.6.1 **Place** the solid standard in the fluorometer. Read the low and high standard and record values.
- 7.6.2 **Fill** a cuvette with 90% acetone and place in fluorometer. Reading should be a minimal value on the 'low' concentration scale. Typically blank value falls in the range from .100 to .400. Record value on analysis log sheet. Repeat with a second blank.
- 7.6.3 **In one motion**, carefully decant the extract of your first sample into a cuvette until the liquid is about ½-1 inch from the top. Keep the fold of the filter towards the cuvette as you are decanting from the centrifuge tube and the level of liquid above the filter, to minimize the amount of particulates in the cuvette.
- 7.6.4 **While holding the cuvette at the top**, wipe down the cuvette with a kimwipe so that no fingerprints will interfere when the sample is read by the fluorometer.
- 7.6.5 **Place** the cuvette in the fluorometer and the cap over the cuvette.
- 7.6.6 The fluorometer has been set to the AUTO-RANGE mode. Allow the fluorometer to self-adjust the concentration range (HIGH, MED or LOW). Once the fluorometer has detected the sample concentration range it will generate a reading in raw fluorescence units (upper right-hand corner of home screen). Press the '*' key and wait until the fluorometer shows 'DONE!' beside the concentration value. Immediately record this value as the F_o .
- 7.6.7 **NOTE:** for very low readings (<10.0), the instrument will fluctuate more. Since readings are of 3 significant digits for all ranges, this fluctuation is not an issue as long as it occurs in the decimal places of the reading. Record the value shown when the instrument shows 'DONE!' beside the value.
- 7.6.8 **Add** 2-3 drops of 1N HCl to the cuvette.
- 7.6.9 **Allow** the fluorometer to self-adjust and press the '*' key to obtain the correct value. Take another reading of the raw fluorescence and record this number as the F_a .
- 7.6.10 **Dump** the contents of the cuvette in a beaker or waste acetone jug, then rinse the cuvette 3 times with 90% acetone in order to get rid of all acid residues.
- 7.6.11 **Repeat** with the next sample.

- 7.6.12 When you are finished, rinse the cuvette 3 times with 90% acetone before storing.
- 7.6.13 After running samples:
- 7.6.13.1 **Place** the solid standard in the fluorometer. Read the low and high standard and record values.
- 7.6.13.2 **Pour** waste acetone into a labeled jug and store in the acetone cabinet.
- 7.6.13.3 Centrifuge labels must be recorded with the original bottle numbers to guarantee proper transfer of data. Data should be entered into chlorophyll spreadsheets as soon as possible after samples are run.

8.0 Calibration and Standardization

- 8.1 The fluorometer must be calibrated once a year using a known chlorophyll *a* standard, preferably one prepared from phytoplankton algae. A dilution series of known concentrations is made to adequately cover the full range of the instrument. The concentration read on the fluorometer is compared to the known concentration of the standard and a regression line generated. The r^2 value of this regression should be 1.000.
- 8.2 At the beginning of each run, the solid standard must be run in the digital fluorometer to ensure the instrument has not drifted significantly. In addition, two 90% acetone blanks must also be run to ensure insignificant drift. These must also be run at the end of each run to verify stability during the entire run.
- 8.3 Filtered seawater blanks must be made every day that chlorophyll samples are filtered to ensure clean filtration equipment. Data sheets should be QA'd by a second person after the data has been entered into spreadsheets.

9.0 Calculations

- 9.1 The equations to calculate concentrations of Chl *a* and Pheo *a* (in ug/L) for field samples using the determined calibration factors are:

$$\text{Chl } a = F_s * \tau / (\tau - 1) * (F_o - F_a) * (V_{\text{ext}} * \text{Dilution Factor}) / V_{\text{filt}}$$

$$\text{Pheo } a = F_s * \tau / (\tau - 1) * (\tau * F_a - F_o) * (V_{\text{ext}} * \text{Dilution Factor}) / V_{\text{filt}}$$

where V_{ext} is the extract volume, V_{filt} is the sample volume (both in same units) and τ is the average ratio of F_o/F_a for all samples (derived from the samples used when calibrating the instrument). τ will remain the same until the next time the instrument is calibrated.

10.0 Quality Control

10.1 See sections 8.2 and 8.3 for run-based quality procedures. Duplicate and replicate samples may be included in sampling regimes based on quality requirements.

11.0 Precision and Accuracy

11.1 Duplicate chlorophyll samples should have a coefficient of variance (CV) of $\leq 5.0\%$. This also applies to 90% acetone blank and filtered seawater blanks.

12.0 Safety

12.1 Follow general procedures for safety found in the *Environmental Assessment Program Safety Manual*.

12.2 Always wear gloves when handling acetone because it may cause defatting of skin tissues on contact.

13.0 Hazardous Waste Disposal

13.1 Acetone waste should be disposed of in a clearly marked vessel (usually an empty acetone jug). Once full, this jug is return to Ecology's HQ building for disposal by the Spills team.

14.0 Bibliography

14.1 Environmental Assessment Program, 2006. Environmental Assessment Program Safety Manual. March 2006. Washington State Department of Ecology. Olympia, WA.

14.2 UNESCO. (1994). Protocols for the joint global ocean flux study (JGOFS) core measurements. pp. 104-118.