DEPARTMENT OF
ECOLOGY
State of Washington

Standard Operating Procedure EAP026 Version 4.1

Chlorophyll *a* Analysis

Jan 2023
Publication 23-03-231
[Recertified 2021]

Purpose of this Document

The Washington State Department of Ecology develops Standard Operating Procedures (SOPs) to document agency practices related to sampling, field and laboratory analysis, and other aspects of the agency's technical operations.

Publication Information

This SOP is available on the Department of Ecology's website at https://apps.ecology.wa.gov/publications/SummaryPages/2303231.html.

Ecology's Activity Tracker Code for this SOP is 15-051.

***Recommended citation*:**
Bos, Julia and Mya Keyzers. 2021. Standard Operating Procedure EA026, Version 6.0: Standard Operating Procedure EAP026, version 6.0 Chlorophyll a Analysis. 23-03-231. Washington State Department of Ecology, Olympia.
https://apps.ecology.wa.gov/publications/SummaryPages/2303231.html. (Recertified 2021.)

Contact Information

Publications Coordinator
Environmental Assessment Program
Washington State Department of Ecology
P.O. Box 47600
Olympia, WA 98504-7600
Phone: 360-407-6764

Washington State Department of Ecology – https://ecology.wa.gov

- Headquarters, Olympia 360-407-6000
- Northwest Regional Office, Shoreline 206-594-0000
- Southwest Regional Office, Olympia 360-407-6300
- Central Regional Office, Union Gap 509-575-2490
- Eastern Regional Office, Spokane 509-329-3400

Original Author – Julia Bos
Date – July 21, 2008

Original Reviewer – Simone Hoffer
Date – July 21, 2008

Current Author – Mya Keyzers
Date – May 15, 2021

Current Reviewer – Grace McKenney
Date – May 15, 2021

QA Approval – Arati Kaza, Ecology Quality Assurance Officer

Recertification Date – August 26, 2021

Signatures Available Upon Request

The Washington State Department of Ecology's (Ecology's) Standard Operating Procedures (SOPs) are adapted from published methods, or developed by in-house technical and administrative experts. Their primary purpose is for internal Ecology use, although sampling and administrative SOPs may have a wider utility. Our SOPs do not supplant official published methods. Distribution of these SOPs does not constitute an endorsement of a particular procedure or method.

Any reference to specific equipment, manufacturer, or supplies is for descriptive purposes only and does not constitute an endorsement of a particular product or service by the author or by Ecology.

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

SOP Revision History

Revision Date	Revision History	Summary of Changes	Sections	Reviser(s)
2/28/2007	1.0	Final editorial changes	Title page	Bill Kammin
7/21/08	2.0	Added QC procedures	7.1	Julia Bos
2/17/2012	3.0	Updated equipment, added pictures	5.0,7.0	Mya Keyzers
3/10/2015	4.0	Revised rinsing method, added section with preservation information, updated calibration and standardization, added table of QAQC procedures & instructions for lab level calculation & data checks, added more information on Precision and Accuracy, changed Bibliography section to References & added more references.	7.0, 7.2, 8.0, 10.0, 11.0, 14.0	Julia Bos, Mya Keyzers
1/24/2018	4.0	None		Mya Keyzers
3/30/2018	4.0	Certified		Tom Gries
10/30/2019	4.1	Updated to current template. Revised section 8.8 and 8.9- Analysis and QC Blanks to reflect current methods. Added definitions. Removed analogue fluorometer instructions.	8.8, 8.9 2, 3,6	Laura Hermanson
5/14/2021	4.1	Updated to new template. Revised Equipment and Summary of Procedure sections.	5, 6	Grace McKenney

1.0 Purpose and Scope

1.1 This document is the Environmental Assessment Program (EAP) Standard Operating Procedure (SOP) for the analysis of chlorophyll *a* samples collected during all seawater sampling events conducted by the Marine Monitoring Unit.

1.2 This SOP is not suitable for the analysis of chlorophyll *a* in freshwater samples.

2.0 Applicability

2.1 This procedure is to be followed during chlorophyll *a* analysis of seawater samples. Samples should be run within one month of collection.

3.0 Definitions

3.1 DI Water – Deionized Water.

3.2 FRB – Filtration Reagent Blank.

3.3 FSW – Filtered Seawater.

3.4 LRB – Lab Reagent Blank.

3.5 SDS – Safety Data Sheet.

4.0 Personnel Qualifications/Responsibilities

4.1 This SOP is to be used by qualified users of the marine waters laboratory. All personnel must be properly trained by qualified staff on methods and safety protocol before conducting this work. Staff competency is deemed satisfactory when protocol can be completed independently.

4.2 Job classes that will use this SOP are NRS1, NRS2, NRS3, and WCC Interns.

5.0 Equipment, Reagents, and Supplies

5.1 Equipment

5.1.1 Turner Designs 10-AU Fluorometer (analog or digital).

5.1.2 Sonicator – Vibra-Cell 500 Watt Ultrasonic Processor with probe.

5.1.3 Centrifuge – Clinical 200 VWR.

5.2 Reagents

5.2.1 Filtered Seawater (FSW). Low chlorophyll *a* seawater that has been filtered through a 47-mm diameter, 0.45 µm pore size Whatman GF/F filter to remove any cells or organisms containing chlorophyll. FSW is used during filtration and quality control procedures. It is kept in a refrigerator and periodically re-filtered to remove any bacteria.

5.2.2 Magnesium Carbonate (MgCO3), supersaturated in deionized water and in a squirt bottle. This chemical is stable at room temperature for 2 years. More information can be found in the magnesium carbonate Safety Data Sheet (SDS).

5.2.3 Certified ACS grade 90% acetone. Acetone is not known to be carcinogenic or teratogenic, but it can cause defatting of skin tissues on contact. More information can be found in the acetone SDS. Dilute concentrated acetone to 90% with deionized water. Follow the SOP for Reagent Preparation to make 90% acetone.

5.2.4 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. More information can be found in the hydrochloric acid SDS

5.3 Supplies

5.3.1 Safety goggles

5.3.2 Nitrile laboratory gloves

5.3.3 Deionized water (18 megohm) in a squirt bottle – Vibra-Cell 500 Watt Ultrasonic Processor with probe.

5.3.4 Filtered seawater in a squirt bottle. Filtered seawater is made by filtering whole seawater through a 47 mm diameter, 45 µm pore size Whatman GF/F filter and refrigerated until use.

5.3.5 25-mm diameter, 0.45-µm pore size Whatman glass fiber filters (GF/F)

5.3.6 Filter forceps – stainless steel, straight, flat, smooth tip

5.3.7 Gast oil-less vacuum pump

5.3.8 Polycarbonate in-line filter holders and manifold

5.3.9 15 ml clear plastic centrifuge tubes

5.3.10 Aluminum foil

6.0 Summary of Procedure

6.1 Filter Chlorophyll Samples.

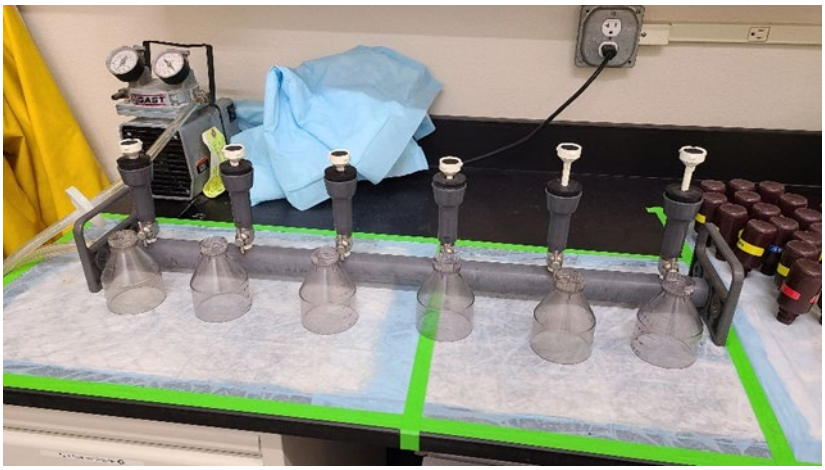

Figure 1. Chlorophyll a filtration rack with six cups and vacuum pump.

6.1.1 Label centrifuge tubes. Be sure to use 15 mL centrifuge tubes. Include 2 tubes for method blanks and 2 tubes for filtration blanks.

6.1.2 Place 0.45 µm 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.

6.1.3 Add 2-3 drops of MgCO3 solution (super-saturated in DI water) to each filter.

6.1.4 Turn on vacuum pump. Make sure pressure is set between 5-7 psi.

6.1.5 Lab (Method Control) Blank 1. Before sample filtration, filter an equivalent volume of DI water to sample volumes (~65mL) through one filter. Place filter into the corresponding centrifuge tube labeled LRB1.

6.1.6 Filtration Reagent Blank 1. Before sample filtration, filter an equivalent volume of FSW to rinse volume (~65 mL) through one filter. Place filter into the corresponding centrifuge tube labeled FB1.

6.1.7 Place sample bottles in front of the funnels. Double check the numbers on the sample bottle labels and the centrifuge tubes to make sure they correspond.

6.1.8 Pour entire sample into filter cup.

6.1.9 Rinse sample bottle and lid with filtered seawater and empty contents into corresponding filter cup. Open valve beneath filter cup to start filtration.

6.1.10 Rinse down filter cups with filtered seawater (FSW) after sample has filtered through. As soon as all water has filtered through, turn valve off for each funnel.

6.1.11 Remove filter from frit using forceps and fold it in half (pigment side in). Take care to not touch the pigments with the forceps.

6.1.12 Place filter in the corresponding centrifuge tube.

6.1.13 Rinse the filter cups with FSW after each sample.

6.1.14 Repeat filtering for all samples.

6.1.15 Lab (Method Control) Blank 2. After sample filtration, filter an equivalent volume of DI water to sample volumes (~65 mL) through one filter. Place filter into the corresponding centrifuge tube labeled LRB2.

6.1.16 Filtration Reagent Blank 2. After sample filtration, filter an equivalent volume of FSW to rinse volume (~65 mL) through one filter. Place filter into the corresponding centrifuge tube labeled FB2.

6.1.17 Prime the acetone dispenser so that it is free of bubbles. Check that it is dispensing exactly 10 mL of acetone each time.

6.1.18 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.

6.1.19 Keep tubes covered with foil as chlorophyll *a* is degraded by exposure to light.

6.1.20 Cover test tube rack with foil and label with project name, sampling date, and number of tubes used. Store rack in freezer.

6.1.21 Clean filter cups by running first hot tap water, and then DI water through the filtration rack. Run pump until cups are empty and tilt rack while pump is running so all water is emptied from rack. Remove filtration cups and rinse with hot water and DI water. Store cups upside down on rack. Leave valves open on filtration rack to facilitate drying. Rinse off forceps with DI water.

6.1.22 Clean sample bottles and lids by rinsing 3x with warm tap water, and then 3x with DI water.

6.1.23 Empty carboy of sample waste..

6.2 Preserve Samples

6.2.1 Samples (filters in 90% acetone extraction) should be stored frozen (-20°C to 70°C) in the dark until analysis.

6.2.2 Filters can be stored frozen at -20° to -70°C for as long as 4 weeks without significant loss of chlorophyll a.

6.3 Process Chlorophyll Samples for Analysis

Figure 2. Benchtop Fluorometer with 90% acetone bottle, cap beaker, vial rack, kimwipes, and sonicator.

6.3.1 In all steps, try to avoid degradation of pigments by minimizing sample exposure to light and heat.

6.3.2 Chlorophyll samples need to be run within 4 weeks to avoid expiration.

6.3.3 Set up fluorometer and turn on (needs to warm up ~20 minutes). Fluorometers rely on a steady state of an internal light-emitting bulb and, therefore need time to warm up.

6.3.4 Fill sonicator bath about ⅔ full with ice water.

6.3.5 Take centrifuge tubes from freezer and place in sonicator bath.

6.4 Sonicate Samples

Figure 3. Sonicator control box and wooden insulated box where samples go.

6.4.1 Sonicate tubes for 7 minutes at the following settings:

Amplitude = 60

Pulser = off

Timer = 7

Tune to minimum frequency

6.4.2 Let samples sit in sonicator for at least 10 minutes after sonicating (keep cold and dark).

6.5 Centrifuge Samples

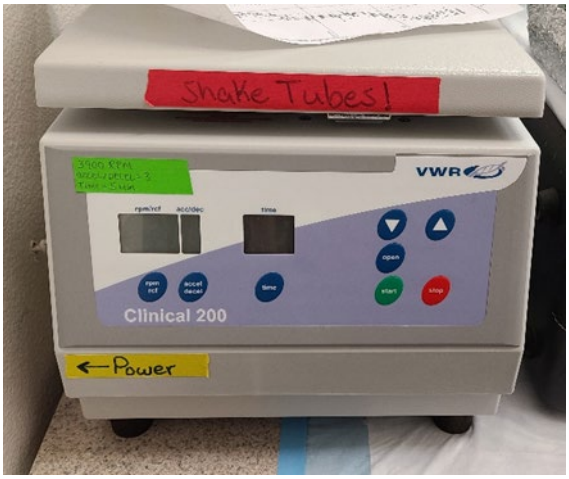

Figure 4. Centrifuge controls and settings.

6.5.1 Check that all tubes still read 10mL of sample, and make sure caps are fully screwed on. Make note if any tubes have less than 10mL of sample in them.

6.5.2 Shake the samples well – this is very important, otherwise, the extract will remain concentrated around the filter and reading will not be accurate.

6.5.3 Place tubes in centrifuge for 5 minutes at 3900 rpm. Always make sure the centrifuge is balanced if it is not fully loaded. To balance it, use a tube with 10 ml DI water.

6.5.4 Remove tubes carefully, so as not to re-suspend particulates, and place tubes in rack. Check to be sure particulates are settled out. Keep samples in the cold and dark until they are being measured.

6.6 Analyze Samples Using the Digital Turner 10-AU-005 Fluorometer

Figure 5. Benchtop Fluorometer.

6.6.1 Place the solid standard in the fluorometer. Read the low and high standard and record values. Check to see that standard values are within the correct range, and make note if they are out of range.

6.6.2 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 0.100 to 0.400. Record value on laboratory analysis log sheet. Repeat with two more blanks.

6.6.3 In one motion, carefully decant the extract of the first sample into a cuvette until the liquid is about ½ to 1 inch from the top of the cuvette. Keep the fold of the filter toward the cuvette while decanting from the centrifuge tube and the level of liquid above the filter, to prevent dispensing particulates in the cuvette.

6.6.4 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.

6.6.5 Place the cuvette in the fluorometer and the cap over the cuvette.

6.6.6 The fluorometer has been set to the AUTO-RANGE mode. Allow the fluorometer to self-adjust the concentration range (HIGH, MED or LOW). It will read "OVER" while it self-adjusts. 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 Fo.

NOTE: For very low readings (<10.0), the instrument will fluctuate more. Since readings are 3 significant digits for all ranges, this fluctuation is not an issue as long as it occurs in the decimal places (0.1 or 0.01) of the reading. Record the value displayed when the instrument shows 'DONE!' beside the value.

6.6.7 Add 2-3 drops of 1N HCl to the cuvette.

6.6.8 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 Fa.

6.6.9 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 remove all acid residues.

6.6.10 Repeat with the next sample.

6.6.11 When finished, rinse the cuvette 3 times with 90% acetone before storing.

6.6.12 After running samples:

6.6.12.1 Place the solid standard in the fluorometer. Read the low and high standard and record values.

6.6.12.2 Pour waste acetone into a labeled jug and store in the acetone cabinet.

6.6.13 Centrifuge labels must be recorded with the original bottle numbers to guarantee proper transfer of data. Data should be transferred to digital storage as soon as possible after samples are run.

7.0 Records Management

7.1 Enter all F_o/F_a readings into the EAPMW database in order to calculate chlorophyll *a* and pheophytin concentrations.

7.2 Save paper log in designated binder.

8.0 Quality Control and Quality Assurance

8.1 Calibration and Standardization

8.1.1 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 is generated. The r2 value of this regression should be close to 1.000. The slope and offset of this independent calibration is used to calculate the final concentration of samples analyzed on the instrument.

8.1.2 At the beginning of each sample analysis, the solid standard must be run in the digital fluorometer for high and low readings to ensure the instrument has not drifted significantly. These readings are documented and tracked via standard control charting procedures. 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.

8.1.3 Method blanks and filtered seawater (reagent) blanks must be included with every set of filtered chlorophyll samples to ensure clean sampling and filtration equipment. This also ensures no addition of chlorophyll to the samples from the FSW rinse and assures proper laboratory handling and analysis. Data sheets are reviewed by a second technician after the data has been entered into electronic format.

8.2 Calculations

8.2.1 The equations to calculate concentrations of chlorophyll *a* and pheophytin *a* (in ug/L) for field samples using the determined calibration factors are:

Chl *a* = Fs * τ/(τ -1) * (Fo-Fa) * (Vext * Dilution Factor)/Vfilt

Pheo *a* = Fs * τ /(τ -1) * (τ *Fa-Fo) * (Vext * Dilution Factor)/Vfilt

8.2.1.1 Where:

- V_{ext} is the extract volume
- V_{filt} is the sample volume (both in same units) and
- τ (tau) is the average ratio of Fo/Fa 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.

8.3 Quality Control

8.3.1 See sections 8.1.2 and 8.1.3 for run-based quality assurance procedures. See Table 1 for typical QA/QC procedures used with this method.

8.3.2 Replicate samples may be included in various sampling programs based on quality assurance requirements. These procedures are documented in Quality Assurance Project or Monitoring Plans under the Sampling Design section.

8.4 Laboratory Processing and Analysis Checks

8.4.1 Ensure all information for each sample is recorded completely and correctly, along with internal laboratory quality control (QC) information. Following is a list of elements to review:

- Chain of custody info
- Volume filtered
- Volume of extraction
- Dilutions
- Tube/bottle number
- Replicate information
- Filtration/processing notes or exceptions
- Lab controls – blanks, standards
- Instrument readings before and after acidification (Fo, Fa)
- Date analyzed
- Analyst
- Instrument used
- Instrument calibration info

8.5 Data Entry QC Review

8.5.1 Check data tools (spreadsheet, database, software tools) for integrity:

- Cell references and all links are working correctly
- Calculation equations are correct
- Formatting and data types are correct (i.e. date/time, volumes - mL vs. liters, etc.)

8.5.2 Check calibration coefficients:

- Appropriate calibration used (check date of last fluorometer calibration
- Correct coefficients used

8.5.3 Check data entry:

- Auto-generated data as appropriate by project. Examples: date, station, depth, niskin, bottle number, bottle volumes, dilution factor
- Manually entered data: Fo and Fa readings, dilution factor, comments and exceptions (refer to analysis sheet)

8.5.4 Check result calculation:

8.5.4.1 Verify equations:

$$[Chla] = \{(Fs * (\tau / \tau - 1)*(Fo - Fa))*(Vext*DilutionFactor)\}/Vfilt$$

[Pheo] = {(Fs * (τ / τ - 1)*(τ *Fa - Fo)))*(Vext*DilutionFactor)}/Vfilt

[Fo/Fa ratio] = Fo/Fa

Where:

Fs is the calibration factor for the fluorometer. Fs will remain the same until the next time the instrument is calibrated.

Fo is the initial reading of the sample.

Fa is the reading after acidification of the sample.

DilutionFactor is the dilution of the extracted sample (usually 1, 2 or 4 – 100%, 50% or 25% strength).

V_{ext} is the extract volume (in liters).

V_{filt} is the sample volume (in liters).

τ (tau) is the average ratio of Fo/Fa derived during instrument calibration (typically close to 2.0). **τ** will remain the same until the next time the instrument is calibrated.

8.6 Post Data Entry QC Tests

8.6.1 Check chlorophyll and pheophytin concentrations for obvious problems – spurious data and outliers such as:

- Negative values
- Very high values; results >20 ug/L for chlorophyll a, >3 ug/L for pheophytin
  (Is dilution factor correct?)

8.6.1.1 Check Fo/Fa results not close to 2.0 (i.e. Fo/Fa < 1.5 or >2.1)

8.6.1.2 Check pheophytin results as follows:

If pheo is <0.00 (negative), check Fo:Fa ratio. If result is > **τ** ("tau" – the theoretical Fo:Fa ratio generated during calibration), this means that the pheophytin results are lower than the instrument calibrated method limit; report result as 0.00 and flag with the "UJ" code. This result is indicative of a fresh algal bloom with very little degraded chlorophyll.

8.7 Analysis and QC of Blanks

8.7.1 Calculate acetone blank readings as a % of the lowest sample Fo reading. These should be less than 1%. (Typically, the results should be in the 0.150 - 0.300 range. If higher, the cuvette was damaged or acetone had impurities.) Replace cuvettes if damaged or old.

8.7.2 If method blank (DIW) results are above 0.1ug/L, examine how contamination may have occurred:

- If only the LRB1 OR LRB2 fails → contamination is random and we cannot definitively trace contamination to reagents, equipment, or improper rinsing.
- If the LRB1 and LRB2 fail together → contamination is random and we cannot definitively trace contamination to *reagents, equipment, or improper rinsing. ***very unlikely to be DIW.***
- If LRB2 and FB2 fail together → contamination is likely being introduced by improper rinsing. Follow up by comparing contamination levels to the lowest chlorophyll values in the data set. Flag samples that are affected by more than 3% with the "JB" code to indicate that significant contamination was introduced during processing.

8.7.3 If reagent blank (FSW) results are above 0.1ug/L, examine how contamination may have occurred:

- If only the FB1 OR FB2 fails → contamination is random and we cannot definitively trace contamination to reagents, equipment, or improper rinsing (LRB2 would catch improper rinsing).
- If the FB1 and FB2 fail together → contamination is likely being introduced by the reagent (unfiltered seawater)
- If FB2 and LRB2 fail together → contamination is likely being introduced by improper rinsing. Follow up by comparing contamination levels to the lowest chlorophyll values in the data set. Flag samples that are affected by more than 5% with the "JB" code to indicate that significant contamination was introduced during processing

8.7.4 If more than a third of the samples fail blank criteria, flag the entire data set and investigate how contamination may have been introduced. If concurrent blank failures occur regularly, check reagents, lab equipment, and consistency in training.

Table 1. Chlorophyll *a* Quality Control Measures

	QA/QC Sample	Why/What it Measures	Current Method?
1.	**Acetone blanks** "Background" ***Run 2-3 during analyses***	Measure Instrument Detection Limit (IDL) + * cuvette quality * acetone quality	**Y**
2.	**Lab (method control) Blanks** (LBs) Blank matrix = GF/F filter + DI rinse + $MgCO_3$ in acetone ***Run 1 ea. before and after filtration***	Measures lab/filtration environment (contamination)	**Y**
3.	**Filtration Reagent Blanks** (FRBs) FSW rinse onto GF/F filter of random sample bottle & filtration cup ***Run 1 ea. before and after filtration***	Measures filtered seawater (FSW) quality and any chlorophyll *a* addition to samples	**Y** (***before and after*** filtration)
4.	**Quality Control Sample** **1** - dilution of stock standard ***Run every quarter*** **2** - solid (2°) standard ***Run before & after every set of samples.***	Measures & verifies instrument performance + * analyst performance Measures & verifies instrument performance.	**N** (could be a "blind" or "spiked" samples) **Y**
5.	**Lab replicates** Replicate samples (e.g., from same Niskin) collected every event.	Measures sampling proficiency & replication; variability in lab processing	**Y** (take replicates on every event)
6.	**Field replicates** Replicate samples (from same depth, different Niskin *or*	Measures field method & sampling variability	**Y** (take replicates on every event)

8.8 Precision and Accuracy

8.8.1 Precision and accuracy of chlorophyll *a* sampling and analyses are determined according to sampling design needs of each program or project. These will be stated in the "Measurement Quality Objectives" section of each project's Quality Assurance Project or Monitoring Plan.

8.8.2 Otherwise, according to the methods used, typical precision and accuracy are as follows:

8.8.2.1 Replicate chlorophyll samples should have a coefficient of variance (CV) of ≤ 10.0%. This also applies to 90% acetone blank and filtered seawater blanks. For chlorophyll *a* concentrations exceeding 0.5 µg/L, a precision of ±8% is possible, if samples have been allowed to extract in acetone for 24 hours or longer.

8.8.2.2 The sensitivity of detection has been estimated as 0.01 µg/L for a 2-liter sample (Parsons et al., 1984). Chlorophyll *a* concentrations should be reported as mg/m3 or µg/L to the nearest 0.01 units.

8.8.2.3 Accuracy (or bias) is reported as Percent (%) Recovery according to EPA Method 445.0 (Arar and Collins, 1997). According to this method, depending on the species composition of natural waters, the method can yield % recoveries close to 100%.

9.0 Safety

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

9.2 Always wear laboratory gloves when handling acetone because it may cause defatting of skin tissues on contact. Also wear appropriate gloves and use extreme care when handling HCl. It is a highly corrosive caustic chemical that may have mutagenic and teratogenic properties.

9.3 Chemical Safety Data Sheets (SDSs) for all chemicals used in the procedures outlined in this SOP can be found on the EAP SharePoint site. Also, binders containing SDSs can be found in all field vehicles, vessels, Ecology buildings, or other locations where potentially hazardous chemicals may be handled. EAP staff that follow Ecology SOPs are required to familiarize themselves with these SDSs and take the appropriate safety measures for these chemicals.

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

9.5 Hazardous Waste Disposal

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

10.0 References

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