Warren Lab Chlorophyll a extraction/analysis methods

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We use a modified method from EPA method 445.

Summary From EPA 445:

***2.1*** Chlorophyll-containing phytoplankton in a

measured volume of sample water are concentrated by

filtering at low vacuum through a glass fiber filter. The

pigments are extracted from the phytoplankton in 90%

acetone with the aid of a mechanical tissue grinder and

allowed to steep for a minimum of 2 h, but not to exceed

24 h, to ensure thorough extraction of the chlorophyll *a*.

The filter slurry is centrifuged at 675 g for 15 min (or at

1000 g for 5 min) to clarify the solution. An aliquot of the

supernatant is transferred to a glass cuvette and

fluorescence is measured before and after acidification to

0.003 N HCl with 0.1 N HCl. Sensitivity calibration factors,

which have been previously determined on solutions of pure chlorophyll *a* of known concentration, are used to interferences are present in the laboratory environment,

calculate the concentration of chlorophyll *a* and

pheophytin *a* in the sample extract. The concentration in

the natural water sample is reported in μg/L.

Method Steps From EPA 445:

***11.1* Extraction of Filter Samples**

*11.1.1* If sampled filters have been frozen, remove them

from the freezer but keep them in the dark. Set up the

tissue grinder and have on hand tissues and squirt bottles

containing water and acetone. Workspace lighting should

be the minimum that is necessary to read instructions and

operate instrumentation. Remove a filter from its

container and place it in the glass grinding tube. The filter

may be torn into smaller pieces to facilitate extraction.

Push it to the bottom of the tube with a glass rod. With a

volumetric pipet, add 4 mL of the aqueous acetone

solution (Sect. 7.6) to the grinding tube. Grind the filter

until it has been converted to a slurry. (**NOTE:** Although

grinding is required, care must be taken not to overheat

the sample. Good judgement and common sense will

help you in deciding when the sample has been

sufficiently macerated.) Pour the slurry into a 15-mL

screw-cap centrifuge tube and, using a 6-mL volumetric

pipet, rinse the pestle and the grinding tube with 90%

acetone. Add the rinse to the centrifuge tube containing

the filter slurry. Cap the tube and shake it vigorously.

Place it in the dark before proceeding to the next filter

extraction. Before placing another filter in the grinding

tube, use the acetone and water squirt bottles to

thoroughly rinse the pestle, grinding tube and glass rod.

The last rinse should be with acetone. Use a clean tissue

to remove any filter residue that adheres to the pestle or

to the steel rod of the pestle. Proceed to the next filter

and repeat the steps above. The entire extraction with

transferring and rinsing steps takes 5 min. Approximately

500 mL of acetone and water waste are generated per 20

samples from the rinsing of glassware and apparatus.

*11.1.2* Shake each tube vigorously before placing them

to steep in the dark at 4oC. Samples should be allowed

to steep for a minimum of 2 h but not to exceed 24 h.

The tubes should be shaken at least once during the

steeping period.

*11.1.3* After steeping is complete, shake the tubes

vigorously and centrifuge samples for 15 min at 675 g or

for 5 min at 1000 g. Samples should be allowed to come

to ambient temperature before analysis. This can be

done by placing the tubes in a constant temperature

water bath or by letting them stand at room temperature

for 30 min. Recalibrate the fluorometer if the room

temperature fluctuated ± 3EC from the last calibration

date.

*11.2.1* After the fluorometer has warmed up for at least

15 min, use the 90% acetone solution to zero the

instrument on the sensitivity setting that will be used for

sample analysis.

*11.2.2* Pour or pipet the supernatant of the extracted

sample into a sample cuvette. The volume of sample

required in your instrument's cuvette should be known so

that the correct amount of acid can be added in the

pheophytin *a* determinative step. For a cuvette that holds

5 mL of extraction solution, 0.15 mL of the 0.1 N HCl

solution should be used. Choose a sensitivity setting that

yields a midscale reading when possible and avoid the

minimum sensitivity setting. If the concentration of

chlorophyll *a* in the sample is $ 90% of the upper limit of

the LDR, then dilute the sample with the 90% acetone

solution and reanalyze. Record the fluorescence

measurement and sensitivity setting used for the sample.

Remove the cuvette from the fluorometer and acidify the

extract to a final concentration of 0.003 N HCl using the

0.1 N HCl solution. Use a pasteur type pipet to

thoroughly mix the sample by aspirating and dispensing

the sample into the cuvette, keeping the pipet tip below

the surface of the liquid to avoid aerating the sample.

Wait 90 sec before measuring fluorescence again.

NOTE: Proper mixing is critical for precise and accurate

results. Twenty-five to thirty-five samples can be

extracted and analyzed in one 8 hr day.

**NOTE:** If you are using special narrow bandpass filters

for chl *a* determination, **DO NOT** acidify samples. Use

the “uncorrected” chl *a* calculations described in Section

12.1.

**Our Modification**s

We do not use a centrifuge, nor do we grind our samples. If we are analyzing natural stream substrates (rocks) we filter our samples through Wattman GFF filters. We assume that the scraping process (scraping periphyton off rocks) grinds the samples sufficiently.

Use the template Chlorophyll a workbook but make sure to save a new name to it so you don’t save over the template.

**Chl a Extraction**

Filters need to be frozen for at least 24 hours prior to extraction to break apart chloroplasts. If we are analyzing NDS glass fritted discs, we put the discs in a 20 ml plastic cup, and directly add 10-15 ml of acetone to extract chl. a. GFF Filters are placed in 20 ml glass scintillation vials and extracted with 15 ml of acetone. I like to use 15 ml because then you have to dilute less later. Filters should be extracted in acetone for 2-4 hours IN THE DARK. Samples should be room temperature for fluorometer readings. I normally just store the samples for 2 hours in the dark. Shake samples at least once during this time period.

**Fluorometer procedure**

Measure fluorescence under the fume hood and make sure the room lights are off. You will have to keep the fume hood lights on. Only have the sample you are analyzing in the light and keep all other samples in the dark.

1. Turn on fluorometer and make sure it is on the chl a channel. Then take the solid standard and read it in the fluorometer. The calibration value of the solid standard should be written on the box for the solid standard (~155 I think). If the fluorometer reading is within 3 or so of the calibration value, you are ready to measure chl a. If not, you need to calibrate the fluorometer. To do this, go to CALIBRATION on the fluorometer. When asked to insert a blank, use a cuvette filled with acetone. Then when asked to add the calibration solution, add the solid standard. Then accept the calibration. Lastly check it again to make sure the solid standard now reads within the correct range.
2. For a single scintillation vial, take 3 ml of extraction solution using a 1 ml pipette and add the solution to a cylindrical cuvette. Take a fluorescence reading. If this reading is below the solid standard calibration values (~155), you are good. Record the value. If not, dispose of the solution in the cuvette, as you will need to dilute the solution.
3. To dilute the solution, use 1 ml of sample solution and then acetone to dilute. The dilution ratio will be recorded in the spread sheet (1 ml solution to 2 ml acetone = .333). I often mix 1 ml of solution and 2 ml acetone directly in a glass cuvette as this equals the 3 ml needed in the cuvette. If more acetone than this is needed, you need to use a small (~30 ml) beaker to mix the solution. Once mixed, add 3 ml of solution to the cuvette and measure. If less than the calibration solution, you are good. If still greater than the solution, you need to dilute even more.
4. Once a good first reading is achieved, record this value as Fo in the excel workbook. Then add 0.15 ml of 0.1 M HCL per 5 ml of solution in the cuvette (our cuvettes hold 3 ml).\*\*\*\*\*\*\*\* Change of methods \*\*\*\* See Parker et al. 2016 about acidification strength and the time needed to wait. We should change our methods to inject 0.26mL HCl to the 3 mL sample to reach 0.008 mol HCL/L instead of the 0.15mL of to reach 0.003 mol HCL/L we have been doing. Check out Parker 2016 before doing Chl a. It is key that after adding the HCL, a pipette is used to mix the solution. Pull in 1 ml of solution and then push it back into the cuvette and repeat until sample is thoroughly mixed. Start a timer and after 90 seconds, measure fluorescence again. Record this value as Fa in the workbook.

M1V1=M2V2

(0.1M HCl)(V1)=(0.008 M HCl)(0.00324)

V1 = 0.26mL

We assume that the 0.1M HCl solution we have is still 0.1M. This may have varied and the easiest way to tell is to do a titration. Contact Julie Pett Ridge for more information.