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Chapter A4

METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

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LABORATORY ANALYSIS

Biomass/chlorophyll ratio for phytoplankton

(B-6560-85)

Parameter and Code:
Biomass-chlorophyll ratio, phytoplankton: 70949

Plankton and periphyton communities normally are dominated by algae. As degradable, nontoxic organic materials enter a body of water, a frequent result is that a greater percentage of the total biomass is heterotrophic (non-chlorophyll-containing) organisms, such as bacteria and fungi. This change can be observed in the biomass to chlorophyll *a* ratio (or autotrophic index). Periphyton ratios for unpolluted water have been reported in the range of 50 to 100 (Weber, 1973); whereas, values greater than 100 may result from organic pollution (Weber and McFarland, 1969; Weber, 1973).

1. Applications

The method is suitable for the determination of chlorophylls *a* and *b* in concentrations of 0.1 μg/L and greater.

2. Summary of method

A filtered phytoplankton sample is ruptured mechanically, and the chlorophyll pigments are separated from each other and degradation products by high-pressure liquid chromatography and are determined by fluorescence spectroscopy (Shoaf and Lium, 1976, 1977). The dry weight and ash weight of the phytoplankton are determined to obtain the weight of organic matter (biomass). The biomass/chlorophyll *a* ratio is calculated from these values.

3. Interferences

3.1 A substantial quantity of sediment may affect the chlorophyll extraction process. Inorganic matter in the sample will cause erroneously large dry and ash weights; nonliving organic matter in the sample will cause erroneously large dry (and thus organic) weights.

3.2 Exposure of the sample to heat, light, or acid can result in photochemical or chemical degradation of the chlorophylls. Large values will result from the presence of fragments of tree leaves or other plant materials that contain chlorophyll. Large populations of photosynthetic bacteria also will result in large values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 *Analytical balance*, capable of weighing to at least 0.1 mg.

4.2 *Auto-injector* (recommended, but not required).

4.3 *Centrifuge.*

4.4 *Centrifuge tubes*, 15 and 50 mL, conical, screwcap, graduated.

4.5 *Desiccator*, containing anhydrous calcium sulfate.

4.6 *Drying oven*, thermostatically controlled for use at 105 °C.

4.7 *Evaporation device.*

4.8 *Filters*, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 μm.

4.9 *Filter funnel*, nonmetallic, that has vacuum or pressure apparatus.

4.10 *Fluorometer*, equipped with excitation and emission filters.

4.11 *Forceps or tongs.*

4.12 *Glass bottles*, screwcap, smallest appropriate size for the sample.

4.13 *Glass funnels.*

4.14 *Gloves*, long-service latex.

4.15 *High-pressure liquid chromatograph* (HPLC), consisting of a solvent programmer, an isochromatic pump, an oven, and a column. (The column oven needs to be capable of maintaining a constant temperature in the 25 to 35 °C range.)

4.16 *High-vacuum pump*, capable of providing an absolute pressure of less than 1 torr.

4.17 *Muffle furnace*, for use at 500 °C.

4.18 *Pasteur pipets*, disposable.

4.19 *Porcelain crucibles.*

4.20 *Separatory funnels*, 125 mL.

4.21 *Spectrometer* (spectrophotometer; fig. 57), that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.22 *Tissue homogenizer*, 30-mL homogenizing flasks, and blades.

4.23 *Vacuum flasks*, stoppers, glass tubing, vacuum tubing, and a sintered glass tube.

4.24 *Vacuum desiccator.*

4.25 *Vacuum oven.*

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Acetone*, 90 percent. Add nine volumes of acetone to one volume of distilled water and mix.

5.2 *Chlorophyll* a *stock solution.* Transfer 1 mg chlorophyll *a* to a 100-mL volumetric flask and fill to capacity using 90-percent acetone (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2, 5.3, 5.4, and 5.5 are wrapped with aluminum foil as an added precaution.

5.3 *Chlorophyll* b *stock solution.* Transfer 1 mg chlorophyll *b* to a 100-mL volumetric flask and fill to capacity using 90-percent acetone.

5.4 *Chlorophyll standard solution.* Mix 25 mL chlorophyll *a* stock solution with 25 mL chlorophyll *b* stock solution in a 50-mL centrifuge tube.

5.5 *Chlorophyll working standard solutions.* Use a 5-mL pipet to prepare the following mixtures.

5.5.1 *High standard solution*, chlorophylls *a* and *b*. Add 5 mL chlorophyll standard solution to 5 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.2 *Mid-range standard solution*, chlorophylls *a* and *b*. Add 3 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.3 *Low standard solution*, chlorophylls *a* and *b*. Add 1 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.6 *Distilled or deionized water.*

5.7 *Diethyl ether*, distilled in glass, unpreserved.

5.8 *Dimethyl sulfoxide* (DMSO).

5.9 *Methyl alcohol*, 96-percent. Pour 960 mL methyl alcohol, distilled in glass, into a 1-L graduated cylinder. Add distilled water to the mark and mix.

5.10 *Nitrogen gas*, prepurified.

6. Analysis

6.1 *Sample preparation.* Analyze only samples on glass-fiber filters. Record the volume of water filtered for the phytoplankton sample. [If a biomass determination is required, save the DMSO layer (see 6.1.7).]

6.1.1 Allow the frozen filter to thaw 2 to 3 minutes at room temperature.

CAUTION.—Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.1.2 Place the filter in a 30-mL tissue homogenizing flask. Add 15 mL DMSO and homogenize until the sample has been ruptured.

6.1.3 Transfer the sample to a 50-mL graduated centrifuge tube, and rinse the homogenizing flask and blade using 5 mL DMSO. Add the rinse to the centrifuge tube.

6.1.4 Add 20 mL diethyl ether to the centrifuge tube, screw on the cap, and shake vigorously for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.5 Remove the cap and slowly add, almost dropwise, 10 mL distilled water to the centrifuge tube. Secure the cap and shake gently. Vent, then shake for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.6 Centrifuge at 1,000 r/min for 10 minutes.

6.1.7 Transfer the top diethyl ether layer, using a disposable pipet, to a 125-mL separatory funnel. (If the DMSO layer appears green after diethyl ether extraction, repeat 6.1.4 through 6.1.7. There are, however, some green chlorophyll derivatives not extractable using diethyl ether.)

6.1.8 Add 15 mL distilled water to the separatory funnel and shake vigorously for 10 seconds, venting often. Allow the layers to separate. (Break emulsions by adding 1 to 2 mL acetone and swirling the funnel gently.)

6.1.9 Drain and discard the bottom layer.

6.1.10 Rinse the upper part of the separatory funnel using 2 to 3 mL acetone. Remove the bottom layer that forms in the funnel and discard.

6.1.11 Decant the diethyl ether layer through the top of the separatory funnel into a centrifuge tube. Rinse the funnel using 5 mL diethyl ether, and add the rinse to the centrifuge tube.

6.1.12 Place the centrifuge tube on the evaporation device, and evaporate to 0.2 to 0.4 mL using a gentle stream of nitrogen gas.

6.1.13 Add sufficient acetone to the sample extract so the color intensity is between the color intensities of the high and low standard solutions. If the color of the sample extract is not within the specified range after the addition of 20 mL acetone, take a 1-mL aliquot of the 20 mL extract, and dilute volumetrically until the desired color intensity is obtained.

6.2 *High-pressure liquid-chromatographic analysis.*

6.2.1 Measure the absorbance of the chlorophyll stock solutions using a spectrometer. Measure the absorbance at 664 nm for chlorophyll *a* and at 647 nm for chlorophyll *b*. Record the absorbance for three replicates of chlorophylls *a* and *b*. Average the three values for chlorophyll *a* and the three values for chlorophyll *b* separately, and record each average separately for subsequent calculations.

6.2.2 Operate the HPLC system using 96-percent methyl alcohol as the mobile phase at a flow of 1.5 mL/min until the pressure stabilizes.

6.2.3 Calibrate the instrument by injecting 10 μL of the mid-range standard solution, and record the peaks of chlorophylls *a* and *b*.

6.2.4 Verify that the response of the fluorometer is linear by injecting the high and low standard solutions.

6.2.5 Analyze the sample by injecting 10 μL of the sample extract into the HPLC. Record the peaks of chlorophylls *a* and *b*, if any.

6.3 *Dry weight and ash weight of organic matter.*

6.3.1 Bake a porcelain crucible at 500 °C for 20 minutes. Cool to room temperature in a desiccator. Silica gel is not recommended. Measure the tare weight to the nearest 0.1 mg.

6.3.2 Remove the DMSO supernatant (6.1.7) using a disposable pipet. If biomass particles are visible in the supernatant, centrifuge first and then remove the supernatant. If the supernatant is still murky, filter through a

tared glass-fiber filter, burn at 500 °C, and add filter ashes to sediment in crucible.

6.3.3 Quantitatively transfer the sediment to a 30-mL porcelain crucible using a microspoon or microspatula and rinses of distilled water.

6.3.4 Place the crucible in a 105 °C oven overnight to evaporate the water.

6.3.5 Place the crucible in a desiccated (preheated to 105 °C) vacuum oven. Lower the pressure in the oven to approximately 20 torr. Leave the crucible in the oven for 2 hours. Approximately every one-half hour or hour, redraw the vacuum (without reaching atmospheric pressure in the oven) to remove the DMSO fumes from the oven.

6.3.6 Cool crucible in a vacuum desiccator to room temperature.

6.3.7 Weigh crucible to the nearest 1 mg in a desiccated balance.

6.3.8 Reheat crucible in the vacuum oven for 1 hour.

6.3.9 Cool crucible in a vacuum desiccator and weigh. If the weight is not constant, reheat until constant weight within 5 percent is obtained. This value is used to calculate the dry weight.

6.3.10 Place the crucible containing the dried residue in a muffle furnace at 500 °C for 1 hour until a constant weight is obtained. This value is used to calculate the ash weight (Note 2).

Note 2: The ash is wetted to reintroduce the water of hydration of the clay and other minerals that, though not evaporated at 105 °C, is lost at 500 °C. This water loss may be as much as 10 percent of the weight lost during ignition and, if not corrected, will be interpreted as organic matter (American Public Health Association and others, 1985).

7. Calculations

7.1 *Chlorophyll.*

7.1.1 Calculate the exact concentrations of the chlorophyll stock solutions from the equation:

$$C_s = \frac{A}{ab},$$

where

C_s = concentration of chlorophyll stock solution, in milligrams per liter;
A = average absorbance obtained in 6.2.1;
a = specific absorptivity [0.0877 L/mg×cm for chlorophyll *a* and 0.0514 L/mg×cm for chlorophyll *b* (Jeffrey and Humphrey, 1975)]; and
b = path length, in centimeters.

7.1.2 Verify and correct the concentrations of the chlorophyll working standard solutions in 5.5 by using the chlorophyll stock solutions determined in 7.1.1.

7.1.3 Calculate the response factor for chlorophylls *a* and *b* in the chlorophyll working standard solution:

$$RF = \frac{V \times C_m}{I_s},$$

where

RF = response factor of chlorophyll *a*, in milligrams per unit area;
V = volume of mid-range standard solution injected, in milliliters;
C_m = concentration of chlorophyll *a* or *b* in the mid-range standard solution, in milligrams per liter; and
I_s = integrated area of the component peak.

7.1.4 Use the data from 6.2.5 to calculate the concentration of chlorophyll *a* or *b* in the original sample from the equation:

$$\text{Concentration (micrograms per liter)} = \frac{RF \times IV_e}{A_s \times V_i},$$

where

RF = response factor of chlorophyll *a* or *b* from 7.1.3, in milligrams per unit area;
I = integrated area of the chlorophyll *a* or *b* peak in the sample as determined in 6.2.5;
V_e = final volume of the sample extract from 6.1.13, in milliliters;
A_s = volume of water filtered in 6.1, in liters; and
V_i = volume of sample extract injected in 6.2.5, in microliters.

7.2 *Biomass.*

$$\text{Organic weight (milligrams per liter)} = \frac{\text{Dry weight (milligrams)} - \text{Ash weight (milligrams)}}{\text{Volume filtered onsite (liters)}}.$$

7.3 Ratio

$$= \frac{\text{Biomass (milligrams per liter)} \times 1{,}000}{\text{Chlorophyll } a \text{ or } b \text{ (micrograms per liter)}}.$$

8. Reporting of results

8.1 Report concentrations of chlorophylls *a* and *b* as follows: less than 1 μg/L, one decimal; 1 μg/L and greater, two significant figures.

8.2 Report biomass as follows: less than 1 mg/L, one decimal; 1 mg/L and greater, two significant figures.

8.3 Report ratio to three significant figures.

9. Precision

No precision data are available.

10. Sources of information

American Public Health Association, American Water Works Association, and Water Pollution Control Federation, 1985, Standard methods for the examination of water and wastewater (16th ed.): Washington, D.C. American Public Health Association, 1,268 p.

Jeffrey, S.W., and Humphrey, G.F., 1975, New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Shoaf, W.T., and Lium, B.W., 1976, Improved extraction of chlorophyll *a* and *b* from algae using dimethyl sulfoxide: Limnology and Oceanography, v. 21, no. 6, p. 926-928.

——— 1977, The quantitative determination of chlorophyll *a* and *b* from fresh water algae without interference from degradation products: Journal of Research of the U.S. Geological Survey, v. 5, no. 2, p. 263-264.

Weber, C.I., 1973, Recent developments in the measurement of the response of plankton and periphyton to changes in their environment, *in* Glass, G., ed., Bioassay techniques and environmental chemistry: Ann Arbor Science, p. 119-138.

Weber, C.I., and McFarland, B., 1969, Periphyton biomass-chlorophyll ratio as an index of water quality: Cincinnati, Ohio, Federal Water Pollution Control Administration, Analytical Quality Laboratory, 19 p.

Chlorophyll in periphyton by spectroscopy

(B-6601-85)

Parameters and Codes:
Chlorophyll *a*, periphyton, spectrometric, uncorrected (mg/m^2): 32228
Chlorophyll *b*, periphyton, spectrometric, (mg/m^2): 32226
Chlorophyll *c*, periphyton, spectrometric, (mg/m^2): 32227
Chlorophyll, total, periphyton, spectrometric, uncorrected (mg/m^2): 32225

1. Applications

The method is suitable for all water and may be used for periphyton from natural or artificial substrates.

2. Summary of method

Chlorophyll pigments are determined simultaneously without detailed separation. The periphyton is scraped from a known area, suspended in water, and concentrated on a membrane filter. A water sample is filtered, and the periphyton cells retained on the filter are ruptured mechanically, using 90-percent acetone, to facilitate extraction of pigments. Concentrations of chlorophylls are calculated from measurements of absorbance of the extract at four wavelengths, corrected for a 90-percent acetone blank.

3. Interferences

Erroneously large values may result from the presence of fragments of tree leaves and other plant materials. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 *Artificial substrates*, made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 *Centrifuge*, swing-out type, 3,000 to 4,000 r/min, and 15-mL graduated centrifuge tubes.

4.3 *Collecting devices*, for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.4 *Filters*, metricel, alpha-6, 0.45 μm, 25-mm diameter.

4.5 *Filter flask*, 1 or 2 L. Onsite, a polypropylene flask is used.

4.6 *Filter funnel*, vacuum, 1.2 L, stainless steel.

4.7 *Filter holder*, Pyrex microanalysis, frit support, 25 mm.

4.8 *Glass pan*, smallest appropriate size for scraping substrate.

4.9 *Manostat*, that has mercury and calibration equipment to regulate the filtration suction to not more than 250 mm of mercury when filtering, using an aspirator or an electric vacuum pump.

4.10 *Membrane filter*, white, plain, 0.45-μm mean pore size, 47-mm diameter.

4.11 *Pasteur pipets*, disposable.

4.12 *Sample containers*, suitable for the type of sample. Glass bottles are useful containers for artificial substrates or for pieces of natural substrates.

4.13 *Scraping device*, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.14 *Source of vacuum for filtration.* A water-aspirator pump or an electric vacuum pump for laboratory use; a hand-held vacuum pump and gauge for onsite use.

4.15 *Spectrometer* (spectrophotometer; fig. 57), that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.16 *Tissue grinder*, glass, pestle-type, 15-mL capacity. Homogenizer should be motor driven at about 500 r/min.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Acetone*, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 *Distilled or deionized water.*

6. Analysis

6.1 If filter was frozen, allow it to thaw for 5 minutes at room temperature.

6.2 If an artificial substrate is used, scrape the periphyton off the substrate, using the scraping device, into a glass pan. Transfer all solid material to the tissue grinder.

6.3 Rinse the scraping device and substrate using 90-percent acetone. Store for 10 minutes in the dark at room temperature.

6.4 Grind at 400 r/min for 3 minutes.

6.5 Transfer the sample to a 15-mL graduated centrifuge

tube, and wash the pestle and grinder two or three times using 90-percent acetone. Adjust to some convenient volume, such as 10 ± 0.1 mL.

6.6 Centrifuge at 3,000 to 4,000 r/min for 10 minutes.

6.7 Carefully pour or pipet the supernatant into the spectrometer cell. Do not disturb the precipitate. If the extract is turbid, clear by making a twofold dilution using 90-percent acetone, or by filtering through an acetone-resistant filter.

6.8 Read the absorbances at 750, 664, 647, and 630 nm and compare to a 90-percent acetone blank. (Dilute the extract using 90-percent acetone if the absorbance is greater than 0.8.) If the 750-nm reading is greater than 0.005 absorbance unit per centimeter of light path, decrease the turbidity as in 6.7.

7. Calculations

7.1 Subtract the absorbance at 750 nm from the absorbance at each of the other wavelengths (that is, 664, 647, and 630 nm). Divide the differences by the light path of the spectrometer cell, in centimeters. The concentrations of chlorophylls in the extract, in micrograms per milliliter, are calculated by the following equations (Jeffrey and Humphrey, 1975):

$$\text{Chlorophyll } a, \text{ in micrograms per milliliter} = 11.85e_{664} - 1.54e_{647} - 0.08e_{630};$$

$$\text{Chlorophyll } b, \text{ in micrograms per milliliter} = -5.43e_{664} + 21.03e_{647} - 2.66e_{630};$$

and

$$\text{Chlorophyll } c, \text{ in micrograms per milliliter} = -1.67e_{664} - 7.60e_{647} + 24.52e_{630};$$

where

$$e_{664} = \frac{\text{Absorbance at 664 nm} - \text{Absorbance at 750 nm}}{\text{Light path (centimeters)}};$$

$$e_{647} = \frac{\text{Absorbance at 647 nm} - \text{Absorbance at 750 nm}}{\text{Light path (centimeters)}};$$

and

$$e_{630} = \frac{\text{Absorbance at 630 nm} - \text{Absorbance at 750 nm}}{\text{Light path (centimeters)}}.$$

7.2 Convert the values derived in 7.1 to the concentrations of chlorophylls, in milligrams per square meter, in the originally collected sample. For example:

$$\text{Chlorophyll } a \text{ (milligrams per square meter)} = \frac{\text{Derived value (micrograms per milliliter)} \times \text{Extract volume (milliliters)}}{\text{Area of scraped surface (square meters)} \times 1{,}000}.$$

8. Reporting of results

Report concentrations of chlorophyll *a*, *b*, or *c*, in milligrams per square meter, to three significant figures.

9. Precision

9.1 The precision of chlorophyll determinations is affected by the area scraped, the range of chlorophyll values calculated, the volume of extraction solvent, and the light path of the spectrometer cells.

9.2 Tilley and Haushild (1975a and b) reported that 21 glass microscope slides exposed for 2 weeks at a single site in the Duwamish River, Wash., had chlorophyll *a* concentrations that ranged from 1.33 to 2.81 mg/m^2 and had a mean of 1.97 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 7.4 mg/m^2. Twenty-two slides exposed for 3 weeks at a single site had chlorophyll *a* concentrations that ranged from 1.89 to 4.86 mg/m^2 and had a mean of 3.44 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

10. Sources of information

Jeffrey, S.W., and Humphrey, G.F., 1975, New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Tilley, L.J., 1972, A method for rapid and reliable scraping of periphyton slides, *in* Geological Survey Research 1972: U.S.Geological Survey Professional Paper 800-D, p. D221-D222.

Tilley, L.J., and Haushild, W.L., 1975a, Net primary productivity of periphytic algae in the intertidal zone, Duwamish River Estuary, Washington: Journal of Research of the U.S. Geological Survey, v. 3, no. 3, p. 253-259.

——— 1975b, Use of productivity of periphyton to estimate water quality: Water Pollution Control Federation Journal, v. 47, no. 8, p. 2157-2171.

Chlorophyll in periphyton by chromatography and spectroscopy

(B-6620-85)

Parameters and Codes:
Chlorophyll *a*, periphyton, chromatographic/spectrometric (mg/m^2): 70955
Chlorophyll *b*, periphyton, chromatographic/spectrometric (mg/m^2): 70956

1. Applications

The method is suitable for all water. The method is not suitable for the determination of chlorophyll *c*.

2. Summary of method

A periphyton sample is obtained, and the chlorophylls are extracted from the algal cells. The chlorophylls are separated from each other and from chlorophyll degradation products by thin-layer chromatography. Chlorophylls are eluted and measured using a spectrometer.

3. Interferences

A substantial quantity of sediment may affect the extraction process. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 *Air dryer.*

4.2 *Artificial substrates*, made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.3 *Centrifuge.*

4.4 *Centrifuge tubes*, graduated, screwcap, 15-mL capacity.

4.5 *Chromatography sheet*, thin-layer cellulose, 5×20 cm, 80-μm thick cellulose.

4.6 *Collecting devices*, for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.7 *Developing tank and rack.*

4.8 *Evaporation device.*

4.9 *Filters*, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 μm.

4.10 *Glass bottles*, screwcap, smallest appropriate size for the sample.

4.11 *Glass pan*, smallest appropriate size for scraping substrate.

4.12 *Gloves*, long-service latex.

4.13 *Grinding motor*, that has 0.1 horsepower.

4.14 *Microdoser*, and 50-μL syringe.

4.15 *Pasteur pipets*, disposable.

4.16 *Scraping device*, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.17 *Solvent-saturation pads*, 13.4×22 cm.

4.18 *Spectrometer* (spectrophotometer; fig. 57), that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.19 *Tissue grinder*, glass, pestle-type, 15-mL capacity. Homogenizer should be motor dirven at about 500 r/min.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Acetone*, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 *Chlorophyll* a *stock solution*. Add 1 mL 90-percent acetone to 1 mg chlorophyll *a* (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2 and 5.3 are wrapped with aluminum foil as an added precaution.

5.3 *Chlorophyll* b *stock solution*. Add 1 mL 90-percent acetone to 1 mg chlorophyll *b*.

5.4 *Dimethyl sulfoxide* (DMSO).

5.5 *Distilled or deionized water.*

5.6 *Ethyl ether.*

5.7 *Methyl alcohol.*

5.8 *Nitrogen gas*, prepurified.

5.9 *Petroleum ether*, 30 to 60 °C.

6. Analysis

6.1 If filter was frozen, allow it to thaw 2 to 3 minutes at room temperature.

6.2 If an artificial substrate is used, scrape the periphyton off the substrate, using the scraping device, into a glass pan. Transfer all solid material into the tissue grinder.

6.3 Rinse the scraping device and substrate using DMSO.

CAUTION.—Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.4 Grind at 400 r/min for 3 minutes.

6.5 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder twice using DMSO.

6.6 Add an equal volume of ethyl ether. Screw on cap

and shake vigorously for 10 seconds. Wait 10 seconds and repeat shaking for 10 seconds more.

6.7 Remove cap and add slowly, almost dropwise, a volume of distilled water equal to 25 percent of the total volume of extractant (DMSO).

6.8 Cap and shake as in 6.6.

6.9 Centrifuge at 1,000 r/min for 10 minutes.

6.10 During centrifugation, prepare chromatography tank by pouring 294 mL petroleum ether and 6 mL methyl alcohol into the tank. Mix well. Prepare fresh before each use. Use two solvent-saturation pads and the developing rack to dry the chromatography sheet.

6.11 Remove the top ethyl ether layer containing chlorophyll using a pipet, and place in another 15-mL graduated centrifuge tube.

6.12 Add an equal volume of distilled water, and shake as in 6.6.

6.13 Centrifuge at 1,000 r/min for 5 minutes.

6.14 Remove the top ethyl ether layer using a pipet, and place in conical tube in evaporation device. Evaporate to dryness by blowing nitrogen gas over the ethyl ether surface.

6.15 Immediately add 0.5 mL acetone. Mix. Wait 30 seconds and mix again. If all chlorophyll is not in solution, then repeat procedure.

6.16 Using microdoser, streak 25 μL of the acetone-chlorophyll solution on the chromatography sheet, 15 mm from the bottom and 6 mm from each side, using the air dryer to speed evaporation of the solvent. If excessive trailing occurs during chromatography, the volume of the solvent should be decreased.

6.17 Develop chromatograph in the dark, using chlorophyll solution(s). Use enough chlorophyll (about 5 μL of the solutions as in 5.2 or 5.3, or both) to visually locate the spot of pigment. The time required for development is about 30 minutes. Remove strips when solvent has traveled within 2 to 3 cm from top of strip.

6.18 Determine R_f values (Note 2) for pure chlorophylls.

Note 2: R_f value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

6.19 Locate the R_f value on the chromatography sheet; and, using a razor blade, scrape the cellulose off the sheet at the spot of the R_f value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × R_f. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 mL. This step should be done immediately after the chromatograph is removed from the tank. Shake the scraped cellulose and acetone vigorously for 10 seconds. Wait 1 minute and shake again vigorously for 10 seconds more.

6.20 Centrifuge at 1,000 r/min for 5 minutes.

6.21 Remove supernatant and read the absorbance on the spectrometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*.

7. Calculations

7.1 If the absorbance is greater than 0.01, determine concentrations using the specific absorptivities of 0.0877 L/mg×cm for chlorophyll *a* and 0.0514 L/mg×cm for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$C = \frac{A}{ab},$$

where

C = concentration of chlorophyll, in milligrams per liter;
A = absorbance;
a = specific absorptivity; and
b = path length, in centimeters.

If the absorbance is less than 0.01, use the fluorescence technique.

7.2 The concentration of chlorophyll obtained in 7.1 is corrected for the concentration step onsite and in the determination:

$$\text{Original sample (milligrams chlorophyll per square meter)} = \frac{\text{Micrograms chlorophyll per milliliter [as in 6.21 × (3 mL)]} \times \frac{500\ \mu L}{25\ \mu L}}{\text{Area of surface scraped (square meters)} \times 1{,}000}.$$

8. Reporting of results

Report concentrations of chlorophyll *a* or *b*, in milligrams per square meter, to three significant figures.

9. Precision

Tilley and Haushild (1975a and b) reported that 21 glass microscope slides exposed for 2 weeks at a single site in the Duwamish River, Wash., had chlorophyll *a* concentrations that ranged from 1.33 to 2.81 mg/m^2 and had a mean of 1.97 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 7.4 mg/m^2. Twenty-two slides exposed for 3 weeks at a single site had chlorophyll *a* concentrations that ranged from 1.89 to 4.86 mg/m^2 and had a mean of 3.44 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

No other precision data are available.

10. Sources of information

Jeffrey, S.W., and Humphrey, G.F., 1975, New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Tilley, L.J., and Haushild, W.L., 1975a, Net primary productivity of periphytic algae in the intertidal zone, Duwamish River Estuary, Washington: Journal of Research of the U.S. Geological Survey, v. 3, no. 3, p. 253-259.

_____ 1975b, Use of productivity of periphyton to estimate water quality: Water Pollution Control Federation Journal, v. 47, no. 8, p. 2157-2171.

Chlorophyll in periphyton by high-pressure liquid chromatography

(B-6630-85)

Parameters and Codes:
Chlorophyll *a*, periphyton, chromatographic/fluorometric (mg/m^2): 70957
Chlorophyll *b*, periphyton, chromatographic/fluorometric (mg/m^2): 70958

1. Applications

The method is suitable for the determination of chlorophylls *a* and *b* in periphyton in concentrations of 0.1 mg/m^2 and greater and is suitable for all water.

2. Summary of method

A periphyton sample is ruptured mechanically, and the chlorophyll pigments are separated from each other and degradation products by high-pressure liquid chromatography and determined by fluorescence spectroscopy (Shoaf and Lium, 1976, 1977).

3. Interferences

Exposure of the sample to heat, light, or acid can result in photochemical or chemical degradation of the chlorophylls. Large values will result from the presence of fragments of tree leaves or other plant materials that contain chlorophyll. Large populations of photosynthetic bacteria also will result in large values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 *Artificial substrates*, made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.2 *Auto-injector* (recommended, but not required).

4.3 *Centrifuge.*

4.4 *Centrifuge tubes*, 15 and 50 mL, conical, screwcap, graduated.

4.5 *Centrifuge tubes*, 50 mL, conical, pennyhead stopper, graduated.

4.6 *Collecting devices*, for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.7 *Evaporation device.*

4.8 *Fluorometer*, equipped with excitation and emission filters.

4.9 *Glass pan*, smallest appropriate size for scraping substrate.

4.10 *Gloves*, long-service latex.

4.11 *High-pressure liquid chromatograph* (HPLC), consisting of a solvent programmer, an isochromatic pump, an oven, and a column. (The column oven needs to be capable of maintaining a constant temperature in the 25 to 35 °C range.)

4.12 *Pasteur pipet*, disposable.

4.13 *Scraping device*, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.14 *Separatory funnels*, 125 mL.

4.15 *Spectrometer* (spectrophotometer; fig. 57), that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.16 *Tissue homogenizer*, 30-mL homogenizing flasks, and blades.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Acetone*, 90 percent. Add nine volumes of acetone to one volume of distilled water and mix.

5.2 *Chlorophyll* a *stock solution*. Transfer 1 mg chlorophyll *a* to a 100-mL volumetric flask and fill to capacity using 90-percent acetone (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2, 5.3, 5.4, and 5.5 are wrapped with aluminum foil as an added precaution.

5.3 *Chlorophyll* b *stock solution*. Transfer 1 mg chlorophyll *b* to a 100-mL volumetric flask and fill to capacity using 90-percent acetone.

5.4 *Chlorophyll standard solution*. Mix 25 mL chlorophyll *a* stock solution with 25 mL chlorophyll *b* stock solution in a 50-mL centrifuge tube.

5.5 *Chlorophyll working standard solutions*. Use a 5-mL pipet to prepare the following mixtures.

5.5.1 *High standard solution*, chlorophylls *a* and *b*. Add 5 mL chlorophyll standard solution to 5 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.2 *Mid-range standard solution*, chlorophylls *a* and *b*. Add 3 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.3 *Low standard solution*, chlorophylls *a* and *b*. Add

1 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.6 *Distilled or deionized water.*

5.7 *Diethyl ether*, distilled in glass, unpreserved.

5.8 *Dimethyl sulfoxide* (DMSO).

5.9 *Methyl alcohol*, 96 percent. Pour 960 mL methyl alcohol, distilled in glass, into a 1-L graduated cylinder. Add distilled water to the mark and mix.

5.10 *Nitrogen gas*, prepurified.

6. Analysis

6.1 *Sample preparation.*

6.1.1 Allow the frozen sample to thaw 2 to 3 minutes at room temperature.

6.1.2 Scrape the periphyton off the substrate into a glass pan.

6.1.3 Use 15 mL DMSO to rinse the solid material into a 30-mL homogenizing flask. Homogenize the sample until the cells have been ruptured.

CAUTION.—Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.1.4 Transfer the sample to a 50-mL graduated centrifuge tube, and rinse the homogenizing flask and blade using 5 mL DMSO. Add the rinse to the centrifuge tube.

6.1.5 Add 20 mL diethyl ether to the centrifuge tube, screw on the cap, and shake vigorously for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.6 Remove the cap and slowly add, almost dropwise, 10 mL distilled water to the centrifuge tube. Secure the cap and shake gently. Vent, then shake for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.7 Centrifuge at 1,000 r/min for 10 minutes.

6.1.8 Transfer the top diethyl ether layer, using a disposable pipet, to a 125-mL separatory funnel. (If the DMSO layer appears green after diethyl ether extraction, repeat 6.1.5 through 6.1.8. There are, however, some green chlorophyll derivatives not extractable using diethyl ether.)

6.1.9 Add 15 mL distilled water to the separatory funnel, and shake vigorously for 10 seconds, venting often. Allow the layers to separate. (Break emulsions by adding 1 to 2 mL acetone and swirling the funnel gently.)

6.1.10 Drain and discard the bottom layer.

6.1.11 Rinse the upper part of the separatory funnel using 2 to 3 mL acetone. Remove the bottom layer that forms in the funnel and discard.

6.1.12 Decant the diethyl ether layer through the top of the separatory funnel into a centrifuge tube. Rinse the funnel using 5 mL diethyl ether, and add the rinse to the centrifuge tube.

6.1.13 Place the centrifuge tube on the evaporation device, and evaporate to 0.2 to 0.4 mL using a gentle stream of nitrogen gas.

6.1.14 Add sufficient acetone to the sample extract so the color intensity is between the color intensities of the high and low standards. If the color of the sample extract is not within the specified range after the addition of 20 mL acetone, take a 1-mL aliquot of the 20 mL extract, and dilute volumetrically until the desired color intensity is obtained.

6.2 *High-pressure liquid-chromatographic analysis.*

6.2.1 Measure the absorbance of the chlorophyll stock solutions using a spectrometer. Measure the absorbance at 664 nm for chlorophyll *a* and at 647 nm for chlorophyll *b*. Record the absorbance for three replicates of chlorophylls *a* and *b*. Average the three values for chlorophyll *a* and the three values for chlorophyll *b* separately, and record each average separately for subsequent calculations.

6.2.2 Operate the HPLC system using 96-percent methyl alcohol as the mobile phase at a flow of 1.5 mL/min until the pressure stabilizes.

6.2.3 Calibrate the instrument by injecting 10 μL of the mid-range standard solution, and record the peaks of chlorophylls *a* and *b*.

6.2.4 Verify that the response of the fluorometer is linear by injecting the high and low standard solutions.

6.2.5 Analyze the sample by injecting 10 μL of the sample extract into the HPLC. Record the peaks of chlorophylls *a* and *b*, if any.

7. Calculations

7.1 Calculate the exact concentrations of the chlorophyll stock solutions from the equation:

$$C_s = \frac{A}{ab},$$

where

C_s = concentration of chlorophyll stock solution, in milligrams per liter;

A = average absorbance obtained in 6.2.1;

a = specific absorptivity [0.0877 L/mg×cm for chlorophyll *a* and 0.0514 L/mg×cm for chlorophyll *b* (Jeffrey and Humphrey, 1975)]; and

b = path length, in centimeters.

7.2 Verify and correct the concentrations of the chlorophyll working standard solutions in 5.5 by using the chlorophyll stock solutions determined in 7.1.

7.3 Calculate the response factor for chlorophylls *a* and *b* in the chlorophyll working standard solution:

$$RF = \frac{V \times C_m}{I_s},$$

where

RF = response factor of chlorophyll *a*, in milligrams per unit area;

V = volume of mid-range standard solution injected, in milliliters;

C_m = concentration of chlorophyll *a* or *b* in the mid-range standard solution, in milligrams per liter; and

I_s = integrated area of the component peak.

7.4 Use the data from 6.2.5 to calculate the concentration of chlorophyll *a* or *b* on the original substrate:

$$\text{Concentration (milligrams per square meter)} = \frac{RF \times IV_e}{A_s \times V_i \times 1{,}000},$$

where

RF = response factor of chlorophyll *a* or *b*, in milligrams per unit area;
I = integrated area of the chlorophyll *a* or *b* peak in the sample as determined in 6.2.5;
V_e = final volume of the sample extract from 6.1.14, in milliliters;
A_s = area of substrate, in square meters; and
V_i = volume of sample extract injected in 6.2.5, in microliters.

8. Reporting of results

Report concentrations of chlorophyll *a* or *b* as follows: less than 1 mg/m^2, one decimal; 1 mg/m^2 and greater, two significant figures.

9. Precision

No precision data are available.

10. Sources of information

Jeffrey, S.W., and Humphrey, G.F., 1975, New spectrophotometric equations for determining chlorophylls *a*, *b*, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Shoaf, W.T., and Lium, B.W., 1976, Improved extraction of chlorophyll *a* and *b* from algae using dimethyl sulfoxide: Limnology and Oceanography, v. 21, no. 6, p. 926-928.

——— 1977, The quantitative determination of chlorophyll *a* and *b* from fresh water algae without interference from degradation products: Journal of Research of the U.S. Geological Survey, v. 5, no. 2, p. 263-264.

Chlorophyll in periphyton by chromatography and fluorometry

(B-6640-85)

Parameters and Codes:
Chlorophyll *a*, periphyton, chromatographic/fluorometric (mg/m^2): 70957
Chlorophyll *b*, periphyton, chromatographic/fluorometric (mg/m^2): 70958

1. Applications

The method is suitable for all water. The method is not suitable for the determination of chlorophyll *c*.

2. Summary of method

A periphyton sample is obtained and the chlorophylls are extracted from the algal cells. The chlorophylls are separated from each other and chlorophyll degradation products by thin-layer chromatography. Chlorophylls are eluted and measured using a spectrofluorometer.

3. Interferences

A substantial quantity of sediment may affect the extraction process. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 *Air dryer.*

4.2 *Artificial substrates*, made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.3 *Centrifuge.*

4.4 *Centrifuge tubes*, graduated, screwcap, 15-mL capacity.

4.5 *Chromatography sheet*, thin-layer cellulose, 5×20 cm, 80-μm thick cellulose.

4.6 *Collecting devices*, for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.7 *Developing tank and rack.*

4.8 *Evaporation device.*

4.9 *Filters*, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 μm.

4.10 *Glass bottles*, screwcap, smallest appropriate size for the sample.

4.11 *Glass pan*, smallest appropriate size for scraping substrate.

4.12 *Gloves*, long-service latex.

4.13 *Grinding motor*, that has 0.1 horsepower.

4.14 *Microdoser*, and 50-mL syringe.

4.15 *Pasteur pipets*, disposable.

4.16 *Scraping device*, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.17 *Solvent-saturation pads*, 13.4×22 cm.

4.18 *Spectrofluorometer* (fig. 58), that has red-sensitive R446S photomultiplier, or equivalent. Use cells that have a light path of 1 cm.

4.19 *Spectrometer* (spectrophotometer; fig. 57), that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.20 *Tissue grinder.*

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Acetone*, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 *Chlorophyll* a *stock solution*. Add 1 mL 90-percent acetone to 1 mg chlorophyll *a* (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2 and 5.3 are wrapped with aluminum foil as an added precaution.

5.3 *Chlorophyll* b *stock solution*. Add 1 mL 90-percent acetone to 1 mg chlorophyll *b*.

5.4 *Dimethyl sulfoxide* (DMSO).

5.5 *Distilled or deionized water.*

5.6 *Ethyl ether.*

5.7 *Methyl alcohol.*

5.8 *Nitrogen gas*, prepurified.

5.9 *Petroleum ether*, 30 to 60 °C.

6. Analysis

6.1 If sample was frozen, allow it to thaw 2 to 3 minutes at room temperature.

6.2 If an artificial substrate is used, scrape the periphyton off the substrate, using the scraping device, into a glass pan. Transfer all solid material into the tissue grinder.

6.3 Rinse the scraping device and substrate using DMSO.

CAUTION.—Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.4 Grind at 400 r/min for 3 minutes.

6.5 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder twice using DMSO.

6.6 Add an equal volume of ethyl ether. Screw on cap and shake vigorously for 10 seconds. Wait 10 seconds and repeat shaking for 10 seconds more.

6.7 Remove cap and add slowly, almost dropwise, a volume of distilled water equal to 25 percent of the total volume of extractant (DMSO).

6.8 Invert the centrifuge tube gently, vent (to prevent tube from breaking from excess pressure), and then shake vigorously.

6.9 Centrifuge at 1,000 r/min for 10 minutes.

6.10 During centrifugation, prepare chromatography tank by pouring 294 mL petroleum ether and 6 mL methyl alcohol into tank. Mix well. Prepare fresh before each use. Use two solvent-saturation pads and the developing rack to dry the chromotography sheet.

6.11 Remove the top ethyl ether layer containing chlorophyll using a pipet, and place in another 15-mL graduated centrifuge tube.

6.12 Add an equal volume of distilled water, and shake as in 6.6.

6.13 Centrifuge at 1,000 r/min for 5 minutes.

6.14 Remove the top ethyl ether layer, using a pipet, and place in the conical tube in the evaporation device. Evaporate to dryness by blowing nitrogen gas over the ethyl ether surface.

6.15 Immediately add 0.5 mL acetone. Mix. Wait 30 seconds and mix again. If all chlorophyll is not in solution, then repeat procedure.

6.16 Using the microdoser, streak 25 μL of the acetone-chlorophyll solution on the chromotography sheet, 15 mm from the bottom and 6 mm from each side, using the air dryer to speed evaporation of the solvent. If excessive trailing occurs during chromatography, the volume of the solvent should be decreased.

6.17 Develop chromatograph in the dark, using chlorophyll solution(s). Use enough chlorophyll (about 5 μL of the solutions as in 5.2 or 5.3, or both) to visually locate the spot of pigment. The time required for development is about 30 minutes. Remove strips when solvent has traveled within 2 to 3 cm from top of strip.

6.18 Determine R_f values (Note 2) for pure chlorophylls.

Note 2: R_f value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

6.19 Locate the R_f value on the chromatography sheet; and, using a razor blade, scrape the cellulose off the sheet at the spot of the R_f value minus 0.07 for chlorophyll *a* (0.14 for chlorophyll *b*) × R_f. Place the cellulose into a graduated centrifuge tube, and add acetone to a volume of 3 mL. This step should be done immediately after the chromatograph is removed from the tank. Shake the scraped cellulose and acetone vigorously for 10 seconds. Wait 1 minute and shake again vigorously for 10 seconds more.

6.20 Centrifuge at 1,000 r/min for 5 minutes.

6.21 Determine the concentration of chlorophyll *a* or *b* using the spectrofluorometer as follows. Curves are prepared daily to standardize the spectrofluorometer. Five standard solutions of each chlorophyll should be prepared at the concentrations of 0.5, 1, 2, 3, and 4 mg/L. These are prepared from the chlorophyll stock solutions by an appropriate dilution using 90-percent acetone.

6.22 For chlorophyll *a*, set the spectrofluororneter for an excitation wavelength of 430 nm and an emission wavelength of 670 nm. For chlorophyll *b*, the excitation wavelength is 460 nm and the emission wavelength is 650 nm. Set entrance and exit slits at 2 mm. Plot chlorophyll concentration versus relative fluorescence intensity. Determine unknown concentrations from the appropriate standard solution curve.

7. Calculations

7.1 The absorbance then is read on a spectrometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of solutions and samples using the specific absorptivities of 0.0877 L/mg×cm for chlorophyll *a* and 0.0514 L/mg×cm for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

$$C = \frac{A}{\alpha b},$$

where

C = concentration of chlorophyll, in milligrams per liter;
A = absorbance;
α = specific absorptivity; and
b = path length, in centimeters.

7.2 The concentration of chlorophyll obtained in 6.22 is corrected for the concentration step onsite and in the determination:

$$\text{Original sample (milligrams chlorophyll per square meter)} = \frac{\text{Micrograms chlorophyll per milliliter (as in 6.22)} \times 3\ \text{mL}}{\text{Area of surface scraped (square meters)} \times 1{,}000} \times \frac{500\ \mu\text{L}}{25\ \mu\text{L}}.$$

8. Reporting of results

Report concentrations of chlorophyll *a* or *b*, in milligrams per square meter, to three significant figures.

9. Precision

Tilley and Haushild (1975a and b) reported that 21 glass microscope slides exposed for 2 weeks at a single site in the Duwamish River, Wash., had chlorophyll *a* concentrations that ranged from 1.33 to 2.81 mg/m^2 and had a mean of 1.97 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 7.4 mg/m^2. Twenty-two slides exposed for 3 weeks at a single site had chlorophyll *a* concentrations that ranged from 1.89 to 4.86 mg/m^2 and had a mean of 3.44 mg/m^2. The 95-percent confidence limit (approximated by two standard deviations) was 14.4 mg/m^2.

No other precision data are available.

10. Sources of information

Jeffrey, S.W., and Humphrey, G.F., 1975, New spectrophotometric equations for determining chlorophylls a, b, c_1, and c_2 in higher plants, algae, and natural phytoplankton: Biochemie und Physiologie der Pflanzen, v. 167, p. 191-194.

Tilley, L.J., and Haushild, W.L., 1975a, Net primary productivity of periphytic algae in the intertidal zone, Duwamish River Estuary, Washington: Journal of Research of the U.S. Geological Survey, v. 3, no. 3, p. 253-259.

——— 1975b, Use of productivity of periphyton to estimate water quality: Water Pollution Control Federation Journal, v. 47, no. 8, p. 2157-2171.

Biomass/chlorophyll ratio for periphyton

(B-6660-85)

Parameter and Code:
Biomass/chlorophyll ratio, periphyton: 70950

Plankton and periphyton communities normally are dominated by algae. As degradable, nontoxic organic materials enter a body of water, a frequent result is that a greater percentage of the total biomass is heterotrophic (nonchlorophyll-containing) organisms, such as bacteria and fungi. This change can be observed in the biomass to chlorophyll *a* ratio (or autotrophic index). Periphyton ratios for unpolluted water have been reported in the range of 50 to 100 (Weber, 1973); whereas, values greater than 100 may result from organic pollution (Weber and McFarland, 1969; Weber, 1973).

1. Applications

The method is suitable for the determination of chlorophylls *a* and *b* in concentrations of 0.1 mg/m^2 and greater.

2. Summary of method

A periphyton sample is ruptured mechanically, and the chlorophylls are separated from each other and degradation products by high-pressure liquid chromatography and are determined by fluorescence spectroscopy (Shoaf and Lium, 1976, 1977). The difference between the ash weight and dry weight is the organic matter (biomass). The biomass/chlorophyll *a* ratio is calculated from these values.

3. Interferences

3.1 A substantial quantity of sediment may affect the chlorophyll extraction process. Inorganic matter in the sample will cause erroneously large dry and ash weights; nonliving organic matter in the sample will cause erroneously large dry (and thus organic) weights.

3.2 Exposure of the sample to heat, light, or acid can result in photochemical or chemical degradation of the chlorophylls. Large values will result from the presence of fragments of tree leaves or other plant materials that contain chlorophyll. Large populations of photosynthetic bacteria also will result in large values.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 *Analytical balance*, capable of weighing to at least 0.1 mg.

4.2 *Artificial substrates*, made of glass slides, Plexiglas or polyethylene strips, tygon tubing, styrofoam, or other materials. See figures 19 and 20 for selected types of artificial substrates.

4.3 *Auto-injector* (recommended, but not required).

4.4 *Centrifuge.*

4.5 *Centrifuge tubes*, 15 and 50 mL, conical, screwcap, graduated.

4.6 *Centrifuge tubes*, 50 mL, conical, pennyhead stopper, graduated.

4.7 *Collecting devices*, for the removal of periphyton from natural substrates. Three devices for collecting a known area of periphyton from natural or artificial substrates are shown in figure 18.

4.8 *Desiccator*, containing anhydrous calcium sulfate.

4.9 *Drying oven*, thermostatically controlled for use at 105 °C.

4.10 *Evaporation device.*

4.11 *Filters*, glass fiber, 47-mm diameter, capable of retaining particles having diameters of at least 0.45 μm.

4.12 *Filter funnel*, nonmetallic, that has vacuum or pressure apparatus.

4.13 *Fluorometer*, equipped with excitation and emission filters.

4.14 *Forceps or tongs.*

4.15 *Glass bottles*, screwcap, smallest appropriate size for the sample.

4.16 *Glass funnels.*

4.17 *Glass pan*, smallest appropriate size for scraping substrates.

4.18 *Gloves*, long-service latex.

4.19 *High-pressure liquid chromatograph* (HPLC), consisting of a solvent programmer, an isochromatic pump, an oven, and a column. (The column oven needs to be capable of maintaining a constant temperature in the 25 to 35 °C range.)

4.20 *High-vacuum pump*, capable of providing an absolute pressure of less than 1 torr.

4.21 *Muffle furnace*, for use at 500 °C.

4.22 *Pasteur pipet*, disposable.

4.23 *Porcelain crucibles.*

4.24 *Scraping device*, razor blades, stiff brushes, spatulas, or glass slides, for removing periphyton from artificial substrates. The edge of a glass microscope slide is excellent for scraping periphyton from hard, flat surfaces (Tilley, 1972).

4.25 *Separatory funnels*, 125 mL.

4.26 *Solvent-saturation pads*, 13.4×22 cm.

4.27 *Spectrometer* (spectrophotometer; fig. 57), that has a band width of 2 nm or less so absorbance can be read to ±0.001 units. Use cells that have a light path of 1 cm.

4.28 *Tissue homogenizer*, 30-mL homogenizing flasks, and blades.

4.29 *Vacuum flasks*, stoppers, glass tubing, vacuum tubing, and a sintered glass tube.

4.30 *Vacuum desiccator.*

4.31 *Vacuum oven.*

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

5.1 *Acetone*, 90 percent. Add nine volumes of acetone to one volume of distilled water.

5.2 *Chlorophyll* a *stock solution.* Transfer 1 mg chlorophyll *a* to a 100-mL volumetric flask and fill to capacity using 90-percent acetone (Note 1).

Note 1: Chlorophyll solutions undergo rapid photochemical degradation and must be stored cold (0 °C) and in the dark. Containers for solutions prepared in 5.2, 5.3, 5.4, and 5.5 are wrapped with aluminum foil as an added precaution.

5.3 *Chlorophyll* b *stock solution.* Transfer 1 mg chlorophyll *b* to a 100-mL volumetric flask and fill to capacity using 90-percent acetone.

5.4 *Chlorophyll standard solution.* Mix 25 mL chlorophyll *a* stock solution with 25 mL chlorophyll *b* stock solution in a 50-mL centrifuge tube.

5.5 *Chlorophyll working standard solutions.* Use a 5-mL pipet to prepare the following mixtures.

5.5.1 *High standard solution*, chlorophylls *a* and *b*. Add 5 mL chlorophyll standard solution to 5 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.2 *Mid-range standard solution*, chlorophylls *a* and *b*. Add 3 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.5.3 *Low standard solution*, chlorophylls *a* and *b*. Add 1 mL chlorophyll standard solution to 9 mL 90-percent acetone in a 15-mL centrifuge tube.

5.6 *Distilled or deionized water.*

5.7 *Diethyl ether*, distilled in glass, unpreserved.

5.8 *Dimethyl sulfoxide* (DMSO).

5.9 *Methyl alcohol*, 96 percent. Pour 960 mL methyl alcohol, distilled in glass, into a 1-L graduated cylinder. Add distilled water to the mark and mix.

5.10 *Nitrogen gas*, prepurified.

6. Analysis

6.1 *Sample preparation.*

6.1.1 Allow the frozen sample to thaw 2 to 3 minutes at room temperature.

6.1.2 Scrape the periphyton off the substrate into a glass pan.

6.1.3 Use 15 mL DMSO to rinse the solid material into a 30-mL homogenizing flask. Homogenize the sample until the cells have been ruptured.

CAUTION.—Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6.1.4 Transfer the sample to a 50-mL graduated centrifuge tube, and rinse the homogenizing flask and blade using 5 mL DMSO. Add the rinse to the centrifuge tube.

6.1.5 Add 20 mL diethyl ether to the centrifuge tube, screw on the cap, and shake vigorously for 10 seconds. Wait 10 seconds and shake another 10 seconds.

6.1.6 Remove the cap and slowly add, almost dropwise, 10 mL distilled water to the centrifuge tube. Secure the cap and shake gently. Vent, then shake for 10 seconds. Wait 10 seconds and shake for another 10 seconds.

6.1.7 Centrifuge at 1,000 r/min for 10 minutes.

6.1.8 Transfer the top diethyl ether layer, using a disposable pipet, to a 125-mL separatory funnel. (If the DMSO layer appears green after diethyl ether extraction, repeat 6.1.5 through 6.1.8. There are, however, some green chlorophyll derivatives not extractable using diethyl ether.)

6.1.9 Add 15 mL distilled water to the separatory funnel, and shake vigorously for 10 seconds, venting often. Allow the layers to separate. (Break emulsions by adding 1 to 2 mL acetone and swirling the funnel gently.)

6.1.10 Drain and discard the bottom layer.

6.1.11 Rinse the upper part of the separatory funnel using 2 to 3 mL acetone. Remove the bottom layer that forms in the funnel and discard.

6.1.12 Decant the diethyl ether layer through the top of the separatory funnel into a centrifuge tube. Rinse the funnel using 5 mL diethyl ether, and add the rinse to the centrifuge tube.

6.1.13 Place the centrifuge tube on the evaporation device and evaporate to 0.2 to 0.4 mL using a gentle stream of nitrogen gas.

6.1.14 Add sufficient acetone to the sample extract so the color intensity is between the color intensities of the high and low standard solutions. If the color of the sample extract is not within the specified range after the addition of 20 mL acetone, take a 1-mL aliquot of the 20 mL extract, and dilute volumetrically until the desired color intensity is obtained.

6.2 *High-pressure liquid-chromatographic analysis.*

6.2.1 Measure the absorbance of the chlorophyll stock solutions using a spectrometer. Measure the absorbance at 664 nm for chlorophyll *a* and at 647 nm for chlorophyll *b*. Record the absorbance for three replicates of chlorophylls *a* and *b*. Average the three values for chlorophyll *a* and the three values for chlorophyll *b*, separately, and record each average separately for subsequent calculations.

6.2.2 Operate the HPLC system using 96-percent methyl alcohol as the mobile phase at a flow of 1.5 mL/min until the pressure stabilizes.

6.2.3 Calibrate the instrument by injecting 10 µL of the mid-range standard solution, and record the peaks of chlorophylls *a* and *b*.

6.2.4 Verify that the response of the fluorometer is linear by injecting the high and low standard solutions.

6.2.5 Analyze the sample by injecting 10 μL of the sample extract into the HPLC. Record the peaks of chlorophylls *a* and *b*, if any.

6.3 *Dry weight and ash weight of organic matter.*

6.3.1 Bake a porcelain crucible at 500 °C for 20 minutes. Cool to room temperature in a desiccator. Silica gel is not recommended. Measure the tare weight to the nearest 0.1 mg.

6.3.2 Remove the DMSO supernatant (6.1.8) using a disposable pipet. If biomass particles are visible in the supernatant, centrifuge first and then remove the supernatant. If the supernatant is still murky, filter through a tared glass-fiber filter, burn at 500 °C, and add filter ashes to sediment in crucible.

6.3.3 Quantitatively transfer the sediment to a 30-mL porcelain crucible using a microspoon or microspatula and rinses of distilled water.

6.3.4 Place the crucible in a 105 °C oven overnight to evaporate the water.

6.3.5 Place the crucible in a desiccated (preheated to 105 °C) vacuum oven. Lower the pressure in the oven to approximately 20 torr. Leave the crucible in the oven for 2 hours. Approximately every one-half hour or hour, redraw the vacuum (without reaching atmospheric pressure in the oven) to remove the DMSO fumes from the oven.

6.3.6 Cool crucible in a vacuum desiccator to room temperature.

6.3.7 Weigh crucible to the nearest 1 mg in a desiccated balance.

6.3.8 Reheat crucible in the vacuum oven for 1 hour.

6.3.9 Cool crucible in a vacuum desiccator and weigh. If the weight is not constant, reheat until constant weight within 5 percent is obtained. This value is used to calculate the dry weight.

6.3.10 Place the crucible containing the dried residue in a muffle furnace at 500 °C for 1 hour until a constant dry weight is obtained. This value is used to calculate the ash weight (Note 2).

Note 2: The ash is wetted to reintroduce the water of hydration of the clay and other minerals that, though not evaporated at 105 °C, is lost at 500 °C. This water loss may be as much as 10 percent of the weight lost during ignition and, if not corrected, will be interpreted as organic matter (American Public Health Association and others, 1985).

7. Calculations

7.1 *Chlorophyll.*

7.1.1 Calculate the exact concentrations of the chlorophyll stock solutions from the equation:

$$C_s = \frac{A}{ab},$$

where

C_s = concentration of chlorophyll stock solution, in milligrams per liter;
A = average absorbance obtained in 6.2.1;
a = specific absorptivity [0.0877 L/mg×cm for chlorophyll *a* and 0.0514 L/mg×cm for chlorophyll *b* (Jeffrey and Humphrey, 1975)]; and
b = path length, in centimeters.

7.1.2 Verify and correct the concentrations of the chlorophyll working standard solutions in 5.5 by using the chlorophyll stock solutions determined in 7.1.1.

7.1.3 Calculate the response factor for chlorophylls *a* and *b* in the chlorophyll working standard solution:

$$RF = \frac{V \times C_m}{I_s},$$

where

RF = response factor of chlorophyll *a*, in milligrams per unit area;
V = volume of mid-range standard solution injected, in milliliters;
C_m = concentration of chlorophyll *a* or *b* in the mid-range standard solution, in milligrams per liter; and
I_s = integrated area of the component peak.

7.1.4 Use the data from 6.2.5 to calculate the concentration of chlorophyll *a* or *b* on the original substrate:

$$\text{Concentration (milligrams per square meter)} = \frac{RF \times IV_e}{A_s \times V_i \times 1{,}000},$$

where

RF = response factor of chlorophyll *a* or *b*, in milligrams per unit area;
I = integrated area of the chlorophyll *a* or *b* peak in the sample as determined in 6.2.5;
V_e = final volume of the sample extract from 6.1.14, in milliliters;
A_s = area of substrate, in square meters; and
V_i = volume of sample extract injected in 6.2.5, in microliters.

7.2 *Biomass.*

$$\text{Organic weight (milligrams per square meter)} = \frac{\text{Dry weight (milligrams)} - \text{Ash weight (milligrams)}}{\text{Area of scraped surface (square meters)}}.$$

7.3 Ratio

$$= \frac{\text{Biomass (milligrams per square meter)}}{\text{Chlorophyll } a \text{ or } b \text{ (milligrams per square meter)}}.$$

8. Reporting of results

8.1 Report concentrations of chlorophylls *a* and *b* as follows: less than 1 mg/m^2, one decimal; 1 mg/m^2 and greater, two significant figures.

8.2 Report biomass as follows: less than 1 mg/m^2, one decimal; 1 mg/m^2 and greater, two significant figures.

8.3 Report ratio to three significant figures.

9. Precision

No precision data are available.

10. Sources of information

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Adenosine triphosphate (ATP)

(B-6700-85)

Parameter and Code:
Adenosine triphosphate (μg/L): 70998

Very sensitive methods of adenosine triphosphate (ATP) analysis have been developed because of McElroy's (1947) discovery that luminescence in fireflies has an absolute requirement for ATP. ATP is determined by measuring the intensity of light produced when ATP reacts with reduced luciferin (LH_2) and oxygen (O_2) in the presence of firefly luciferase and magnesium (Mg^{+2}), producing adenosine monophosphate (AMP), inorganic pyrophosphate (PPi), oxidized luciferin (L), water (H_2O), carbon dioxide (CO_2), and light (hv). The following equation shows this reaction:

$$ATP + LH_2 + O_2 \xrightarrow[Mg^{+2}]{\text{luciferase}} AMP + PPi + L + H_2O + CO_2 + hv.$$

The bioluminescent reaction is specific for ATP. The reaction rate is proportional to the ATP concentration, and 1 photon of light is emitted for each molecule of ATP hydrolyzed. When ATP is mixed with suitably buffered enzyme and substrates, a light flash follows that decays in an exponential fashion. Either the peak height of the light flash or the integration of the area under the decay curve can be used to prepare standard curves.

The sample-collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, phytoplankton abundance may vary transversely, with depth, and with time of day. To collect a sample representative of the phytoplankton concentration at a particular depth, use a water-sampling bottle. To collect a sample representative of the entire flow of a stream, use a depth-integrated sampler (Guy and Norman, 1970; Goerlitz and Brown, 1972). For small streams, a depth-integrated sample or a point sample at a single transverse position at the centroid of flow is adequate. Study design, collection, and statistics for streams, rivers, and lakes are described in Federal Working Group on Pest Management (1974).

The analysis section (6.1 through 6.16) in the method that follows describes the extraction of ATP from the living material (algae, bacteria, or fungi) in the sample. These extraction procedures ideally should be done immediately after collection. The sample may be stored 2 to 3 hours if necessary and if the temperature and lighting conditions are maintained; for example, do not put a warm sample from a well-lighted area into a cool, dark ice chest.

1. Applications

The method is suitable for all water.

2. Summary of method

A water sample is filtered, and the ATP is extracted from the living material. The extract from the living material (containing the ATP) is injected into a suitable buffered luciferin-luciferase enzyme solution. The intensity of light produced by the subsequent reaction is measured using an ATP photometer. The reaction rate is proportional to the ATP concentration, and 1 photon of light is emitted for each molecule of ATP hydrolyzed.

3. Interferences

In general, several metals (for example, mercury) and a large concentration of salts will inhibit the reaction; therefore, washing the filter using buffered distilled water, immediately after filtration to remove most of the dissolved salts is advisable. A substantial quantity of sediment may affect the extraction process.

4. Apparatus

Most of the materials and apparatus listed in this section are available from scientific supply companies.

4.1 *Balance*, analytical.

4.2 *Constant-rate injector.*

4.3 *Cuvettes*, 6×49 mm, quartz, 1-cm light-path length.

4.4 *Cuvette caps.*

4.5 *Cuvette holder.*

4.6 *Distillation apparatus*, glass.

4.7 *Filter assemblies*, 13-mm diameter, 0.45-μm mean pore size, self-supported filters (Note 1).

Note 1: These filters are resistant to the extracting agent, dimethyl sulfoxide.

4.8 *Glass storage bottles*, approximately 150-mL capacity, and autoclavable screwcaps.

4.9 *Glass vials*, approximately 15-mL capacity, and screwcaps, 22×85 mm.

4.10 *Gloves*, long-service latex.

4.11 *Photometer*, Chem-Glow photometer and integrator, ATP photometer, or luminescence biometer.

4.12 *Pipet*, 0.1, 0.2, and 1 mL, that has disposable tips.

4.13 *Sterilizer*, horizontal steam autoclave or vertical steam autoclave.

CAUTION.—If vertical autoclaves or pressure cookers are used, they need to be equipped with an accurate pressure gauge, a thermometer with the bulb 2.5 cm above the water

level, automatic thermostatic control, metal air-release tubing for quick exhaust of air in the sterilizer, metal-to-metal-seal eliminating gaskets, automatic pressure-release valve, and clamping locks preventing removal of lid while pressure exists. These features are necessary in maintaining sterilization conditions and decreasing safety hazards.

To obtain adequate sterilization, do not overload sterilizer. Use a sterilization indicator to ensure that the correct combination of time, temperature, and saturated steam has been obtained.

4.14 *Syringe*, 50 μL, blunt-tipped (nonbeveled).

4.15 *Tubes*, graduated 12- or 15-mL centrifuge.

4.16 *Vacuum-filter stand.*

4.17 *Vacuum pump*, to provide at least 250 mm of mercury.

4.18 *Volumetric flasks*, 100-mL and 1-L sizes.

5. Reagents

Most of the reagents listed in this section are available from chemical supply companies.

All reagents are prepared using only freshly distilled water, which has an ATP value not greater than 0.1 μg/L.

5.1 *Adenosine-5-triphosphate solutions*, 1, 2.5, 10, 25, and 100 μg ATP per liter. Do the following steps rapidly because ATP is an unstable biochemical: Dissolve 119.3 mg $Na_2ATP \cdot 3H_2O$ (equivalent to 100 mg ATP) in 100 mL ATP diluent. Make two serial dilutions of 1:100 using the ATP diluent. Mix well between dilutions. The result is a 100-μg/L solution of ATP. Make 1:4, 1:10, 1:40, and 1:100 dilutions of the 100-μg/L solution using the ATP diluent to make ATP solutions of 25, 10, 2.5, and 1 μg/L concentrations. Pour small aliquots (approximately 100 μL) of the 1-, 2.5-, 10-, 25-, and 100-μg/L solutions into the cuvettes and cap using the cuvette caps. Quickfreeze the cuvettes immediately by immersing in a bath of acetone and dry ice; store at −20 °C or less.

5.2 *ATP diluent.* Dissolve 1.045 g morpholinopropane sulfonic acid (MOPS); 0.372 g ethylenediaminetetraacetic acid, disodium salt, dihydrate ($Na_2EDTA \cdot 2H_2O$); and 1.2 g magnesium sulfate ($MgSO_4$) in approximately 900 mL distilled water. Adjust the pH to 7.7 using sodium hydroxide and increase the final volume to 1 L using distilled water. If not used immediately, the solution should be autoclaved to prevent growth of micro-organisms and, thus, the production of ATP.

5.3 *Distilled water.*

5.4 *Hydrochloric acid solution*, 0.2*N*. Dilute 16.7 mL concentrated hydrochloric acid (HCl) to 1 L using distilled water.

5.5 *Luciferin-luciferase buffer solution*. The kit must be stored frozen at −20 °C or less. For daily use, dissolve one buffer-salt (MOPS and $MgSO_4$ at pH 7.4) tablet in 3 mL distilled water. Add the vial containing the lyophilized enzyme-substrate (luciferin-luciferase) powder to the buffer solution. Mix gently but completely. Do not allow the formation of bubbles because this may result in enzyme (luciferase) denaturation. Wait at least 15 minutes before using. Fresh solution must be prepared before each use but may be left at room temperature (20–24 °C) during the day. One tablet of buffer salt and one vial of enzyme-substrate powder provide enough solution for approximately 30 cuvettes.

5.6 *Morpholinopropane sulfonic acid (MOPS) solution*, 0.01*M*. Dissolve 2.09 g MOPS in approximately 900 mL distilled water. Adjust pH to 7.4 using sodium hydroxide. Increase final volume to 1 L using distilled water. Pour approximately 100 mL each into 150-mL glass bottles, cap loosely, and autoclave. After cooling, cap tightly and store at room temperature.

5.7 *Dimethyl sulfoxide (DMSO) solution*. Add nine volumes of DMSO to one volume 0.01*M* MOPS solution that was prepared in step 5.6. Mix well. Prepare fresh before each use.

CAUTION.—Latex gloves are worn to prevent the possible transport of toxic material across skin by DMSO.

6. Analysis

6.1 Shake water sample and remove 25 mL. If sample obviously contains abundant living material (for example, algae, bacteria, or fungi), this aliquot may be decreased to a volume as small as 10 mL. Record the final volume.

6.2 Pour the sample aliquot into the filter assembly containing the membrane filter, which has a graduated centrifuge tube in place and a vacuum pump attached.

6.3 Apply a vacuum no greater than 250 mm mercury.

6.4 Release vacuum immediately when filtration is almost complete so sample does not dry.

6.5 Quickly add 5 mL distilled water and filter again, this time to dryness. Release vacuum immediately.

6.6 Replace graduated centrifuge tube with a clean and dry centrifuge tube.

6.7 Pipet 0.2 mL DMSO onto sample in filter assembly and distribute evenly by rotation of filter assembly. If the 0.2 mL does not cover the sample, it may be doubled; if so, the 1 mL volume in 6.10 also should be doubled to 2 mL. Record the change so that corrections for dilutions can be made.

6.8 Wait at least 20 seconds (not more than 30).

6.9 Apply vacuum until surface is dry.

6.10 Add 1 mL of MOPS solution.

6.11 Wait 10 seconds.

6.12 Apply vacuum until surface is dry.

6.13 Repeat 6.10 through 6.12.

6.14 Record final volume; this value should be 2.2 mL.

6.15 Mix contents of centrifuge tube.

6.16 Pour contents of the centrifuge tube into small screwcap vial (approximately 15-mL volume), and quickfreeze by immersing the bottom part in an acetone and dry-ice bath. The sample must be frozen until analyzed. Storage should not exceed 30 days.

6.17 Pipet 100 μL luciferin-luciferase solution into the cuvettes.

6.18 Rinse the syringe three times using 0.2*N* hydrochloric acid by drawing acid into the entire 50-μL length of the syringe; rinse three times using MOPS solution to neutralize any remaining acid; rinse three times using distilled water.

6.19 Thaw the ATP solutions at room temperature and mix well.

6.20 Test the photometer for response to the luciferin-luciferase solution (background luminescence) and 10 μL of the five ATP solutions. Follow specific instructions for the photometer used. This procedure prepares a standard curve and is linear for this analysis.

6.21 Rinse syringe as in 6.18.

6.22 Place cuvette in photometer.

6.23 Thaw sample prepared in 6.1 through 6.16 at room temperature for analysis. Mix well.

6.24 Rinse syringe three times using the sample.

6.25 Inject 10 μL sample into the cuvette, and record response. Analyze in duplicate.

6.26 If response is too great for photometer, the sample may be diluted. Dilutions using distilled water are linear.

7. Calculations

7.1 Prepare a standard curve from the five ATP solutions. The standard curve is linear and has a slope of 1. Compute the concentration of ATP in the injected sample in micrograms ATP per liter of sample.

7.2 This ATP value is corrected for the concentration step onsite using the following equation:

$$\text{Original sample (micrograms ATP per liter)} = \frac{\text{Micrograms ATP measured per liter}}{\text{Volume of sample filtered (liters)}} \times \frac{\text{Dilution}}{\text{Volume recovered after extraction (liters)}}.$$

If undiluted, the value for dilution equals 1; the volume recovered after extraction commonly is 2.2×10^{-3} L.

8. Reporting of results

Report ATP to the nearest 0.1 μg/L.

9. Precision

Reproducibility of analysis is approximately ±2 percent (single analyst).

10. Sources of information

Federal Working Group on Pest Management, 1974, Guidelines on sampling and statistical methodologies for ambient pesticide monitoring: Washington, D.C., 59 p.

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