10200 PLANKTON*

10200 A. Introduction

Plankton are microscopic aquatic life forms with little or no ability to resist current movement and, thus, live free-floating (suspended) in natural waters. This section covers both phytoplankton and zooplankton. *Phytoplankton* are microscopic algae that occur in unicellular, colonial, or filamentous forms; most are photosynthetic and eaten by zooplankton or other filter-feeding aquatic organisms. Freshwater *zooplankton* principally consist of protozoans, rotifers, cladocerans, and copepods; marine water zooplankton are more diverse. Other planktonic microscopic aquatic organisms are dealt with elsewhere: zoosporic fungi in Section 9610F; aquatic hyphomycetes in Section 9610G; and bacteria in Part 9000.

1. Significance

Plankton, particularly phytoplankton, has long been used as a water quality indicator, both in terms of standing crop and species composition.[1–4] They strongly influence certain nonbiological aspects of water quality (e.g., pH, color, taste, oxygen concentration, and odor). Some species flourish in highly eutrophic or acidic waters, while others are sensitive to (i.e., negatively affected by) organic and/or chemical wastes. Due to narrow environmental tolerances, certain species are extremely useful in determining historical water quality and thus future management direction.[5] In addition, the composition of a phytoplankton community indicates food quality for zooplankton, with implications for fisheries.

So, the compositions of phytoplankton and zooplankton communities are critical components of water quality assessments. However, because of their transient nature and often patchy distribution, the utility of plankters as water quality indicators is limited.[6–12] Information on plankton as water quality indicators is best interpreted in the context of concurrently collected physicochemical and other biological data. Plankton also may be used to indicate the relative efficiencies of water treatment plants and the probability that groundwater sources are directly influenced by surface water.[13–18]

Some species of plankton develop noxious blooms that can decrease clarity, hurt recreational and aquacultural industries, and create offensive tastes and odors in drinking water.[19] Algal blooms may even create anoxic conditions or produce toxins that poison both aquatic and terrestrial organisms, resulting in animal or human illness or death.[20–29] So, algal blooms in both marine and freshwater environments raise ecologic, economic, and public health concerns. Both marine and freshwater species of cyanobacteria (commonly referred to as *blue-green algae*), dinoflagellates, and diatoms produce toxins. Most marine incidents are associated with dinoflagellates and diatoms, while most freshwater incidents are caused by cyanobacteria.[26,30] Several of these toxins have been found in seafood and drinking water, and have produced illness and fatalities in humans.[31,32] Sampling and analytical guidance for algal blooms and associated toxins currently are not included in *Standard Methods* (taste-and-odor compounds are discussed in Section 6040); however, general guidance is available.[33–37]

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2. References

1. Palmer, C.M. 1969. A composite rating of algae tolerating organic pollution. *J. Phycol.* 5:78.
2. Palmer, C.M. 1963. The effect of pollution on river algae. *Bull. N.Y. Acad. Sci.* 108:389.
3. Rawson, D.S. 1956. Algal indicators of trophic lake types. *Limnol. Oceanogr.* 1:18.
4. Stoermer, E.F. & J.J. Yang. 1969. Plankton Diatom Assemblages in Lake Michigan, Spec. Rep. No. 47. Great Lakes Research Div., Univ. Michigan, Ann Arbor.
5. Smol, J. 2008. Pollution of Lakes and Rivers: A Paleoenvironmental Perspective. Wiley-Blackwell, New York, N.Y.
6. Gannon, J.E. & R.S. Stemberger. 1978. Zooplankton (especially crustaceans and rotifers) as indicators of water quality. *Trans. Amer. Microsc. Soc.* 97:16.
7. Tomas, C.R., ed. 1997. Identifying Marine Phytoplankton. Academic Press, Harcourt Brace & Co., San Diego, Calif.
8. Platt, T. & W.K.W. Li, eds. 1986. Photosynthetic Picoplankton, Canadian Bull. Fish. Aquatic Sci. No. 214. Dept. Fisheries and Oceans, Ottawa, Ont.
9. Stevenson, R.J. & K.D. White. 1995. A comparison of natural and human determinants of phytoplankton communities in the Kentucky River Basin, U.S.A. *Hydrobiologia* 297:201.
10. Lange-Bertalot, H. 1979. Pollution tolerance of diatoms as criterion for water quality estimation. *Nova Hedwigia* 64:285.
11. Dixit, S.S. & J.P. Smol. 1994. Diatoms as indicators in environmental monitoring and assessment program-surface waters (EMAP-SW). *Environ. Monitor. Assess.* 31:275.
12. Stoermer, E.F. & J.P. Smol, eds. 1999. The Diatoms: Applications for the Environmental and Earth Sciences. Cambridge University Press, Cambridge, U.K.
13. Porter, S.D., T.F. Cuffney, M.E. Gurtz & M.R. Meador. 1993. Methods for Collecting Algae as Part of the National Water-Quality Assessment Program, USGS Open-file Rept. 93-409. U.S. Geological Survey, Raleigh, N.C.
14. Clark, S.C., M.L. Price, J. Flugum & R. Roberson. 1993. Groundwater under the direct influence of surface water: it is not always black or white. *In* Proc. Water Quality Technology Conf., Nov. 7–11, 1993, Miami, Fla., p. 703. American Water Works Assoc., Denver, Colo.
15. Clancy, J.L. 1992. Interpretation of microscopic particulate analysis data—A water quality approach. *In* Proc. Water Quality Technology Conference, June 25–28, 1992, Toronto, Canada, p. 1831. American Water Works Assoc., Denver, Colo.
16. U.S. Environmental Protection Agency. 1996. Microscopic Particulate Analysis (MPA) for Filtration Plant Optimization, EPA 910/R-96-001. U.S. Environmental Protection Agency, Seattle, Wash.

17. U.S. ENVIRONMENTAL PROTECTION AGENCY. 1992. Consensus Method for Determining Groundwaters Under the Direct Influence of Surface Water Using Microscopic Particulate Analysis (MPA), EPA 910/9-92-029. U.S. Environmental Protection Agency, Port Orchard, Wash.
18. HANCOCK, C.M., J.V. WARD, K.W. HANCOCK, P.T. KLONICKI & G.D. STURBAUM. 1996. Assessing plant performance using MPA. *J. Amer. Water Works Assoc.* 88:24.
19. TAYLOR, W.D., R.F. LOSEE, M. TOROBIN, G. IZAGUIRRE, D. SASS, D. KHIARI & K. ATASI. 2005. Early Warning and Management of Surface Water Taste-and-Odor Events. AWWA Research Foundation, Denver, Colo.
20. CARMICHAEL, W., ed. 1981. The Water Environment, Algal Toxins and Health. Plenum Press, New York, N.Y.
21. HUDNELL, K., ed. 2008. Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. Springer, New York, NY.
22. HUISMAN, J., H.C.P. MATTHIJS & P.M. VISSER, eds. 2005. Harmful Cyanobacteria. Springer, Dordrecht, The Netherlands.
23. HALLEGRAEFF, G.M. 1991. Aquaculturists' Guide to Harmful Australian Microalgae. Fishing Industry Training Board of Tasmania, Hobart, Tasmania, Australia.
24. WATANABE, M.F., K. HARADA, W.W. CARMICHAEL & H. FUJIKI, eds. 1996. Toxic Microcystis. CRC Press, Boca Raton, Fla.
25. HALLEGRAEFF, G.M., D.M. ANDERSON & A.D. CEMBELLA, eds. 2003. Manual of Harmful Marine Microalgae. United Nations Educational, Scientific & Cultural Org., Paris.
26. HALLEGRAEFF, G.M. 1993. A review of harmful algal blooms and their apparent global increase. *Phycologia* 32(32):79.
27. CHORUS, I. ed. 2001. Cyanotoxins: Occurrence, Causes and Consequences. Springer, Berlin.
28. DODDS, W.K., W.W. BOUSKA, J.L. EITZMANN, T.J. PILGER, K.L. PITTS, A.J. RILEY, J.T. SCHLOESSER & D.J. THORNBRUGH. 2009. Eutrophication of U.S. freshwaters: analysis of potential economic damages. *Environ. Sci. Technol.* 43(1):12.
29. HUDNELL, K., ed. 2008. Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs. Springer, New York, NY.
30. CARMICHAEL, W.W. 1997. The cyanotoxins. *Adv. Botanical Res.* 27:211.
31. FALCONER, I.R., ed. 1993. Algal Toxins in Seafood and Drinking Water. Academic Press, Harcourt Brace & Co., San Diego, Calif.
32. CHORUS, I. & J. BARTRAM. 1999. Toxic Cyanobacteria in Water. E & FN Spon, New York, N.Y.
33. WESTRICK, J.A. 2003. Everything a manager should know about algal toxins but was afraid to ask. *J. Amer. Water Works Assoc.* 95(9):26.
34. FALCONER, I.R. 2005. Cyanobacterial Toxins of Drinking Water Supplies: Cylindrospermopsins and Microcystins. CRC Press, Boca Raton, Fla.
35. GRAHAM, J.L., K.A. LOFTIN, A.C. ZIEGLER & M.T. MEYER. 2008. Cyanobacteria in lakes and reservoirs—toxin and taste-and-odor sampling guidelines. *In* U.S. Geological Survey. Techniques of Water-Resources Investigations, Book 9, Chap. A7, Sec. 7.5, (Ver. 1.0) http://water.usgs.gov/owq/FieldManual/Chapter7/7.5.html. Accessed September 2011.
36. ERDNER, D.L., J. DYBLE, L.E. BRAND, M. PARKER, R.C. STEVENS, S.K. MOORE, K. LEFEBVRE, P. BIENFANG, D.M. ANDERSON, R.R. BIDIGARE, P. MOELLER, M.L. WRABEL & K.A. HUBBARD. 2008. Centers for Oceans and Human Health: A unified approach to the challenge of harmful algal blooms. *Environ. Health* 7(Suppl 2):S2.
37. BIENFANG, P.K., M.L. PARSONS, R.R. BIDIGARE, E.A. LAWS & P.D.R. MOELLER. 2008. Ciguatera fish poisoning: A synopsis from ecology to toxicity. *In* P.J. Walsh, S.L. Smith, L.E. Fleming, H. Solo-Gabriele & W.H. Gerwick, eds. Oceans and Human Health: Risks and Remedies from the Seas, p. 257. Elsevier, New York, N.Y.

10200 B. Sample Collection

1. General Considerations

The sampling approach and site selection will depend on the study's objectives. Sampling frequency, site location, the time of day samples are collected, the type of samples collected, and how they are collected need to be based on study objectives.[1,2] Put sampling stations as close to chemical and bacteriological sampling stations as possible to ensure maximum correlation of findings. Establish enough stations in as many locations as needed to adequately define the types and quantities of plankton present in the waters studied. The water's physical nature (standing, flowing, or tidal) will greatly influence the choice of sampling stations. Using sampling sites selected by previous investigators usually ensures that historical data are available, which will lead to a better understanding of current results and provide continuity in the study of an area.

In stream and river work, put sampling stations upstream and downstream of suspected pollution sources and major tributaries, as well as at appropriate intervals throughout the reach under investigation. If possible, put stations on both sides of the river because river water may not mix laterally for long distances downstream. Similarly, investigate tributaries suspected of being polluted but interpret data from a small stream carefully because much of the plankton may be periphytic, resulting from the flowing water's scouring of natural substrates. Plankton contributions from adjacent lakes, reservoirs, and backwater areas, as well as soil organisms carried into the stream by runoff, also can influence data interpretation. In addition, the depth at which water is discharged from upstream, stratified reservoirs can affect plankton.

Because river and stream water usually is well mixed vertically, subsurface sampling (i.e., the upper meter or a composite of two or more strata) often is adequate when collecting a representative sample. There may be problems caused by stratification due to thermal discharges, mixing of warmer or colder waters from tributaries and reservoirs, salt intrusion due to tidal influences, or other circumstances that encourage well-defined pycnoclines.[3] Sample in the main channel of a river and avoid sloughs, inlets, or backwater areas unless one of the investigation's goals is to characterize such areas. Samples collected in a river's main channel are most representative of general conditions, while sloughs, inlets, and backwater areas are more representative of localized habitats. In rivers that are mixed vertically and horizontally, measure plankton populations by examining periodic samples collected at midstream 0.5 to 1 m below the surface. When setting up a sampling program, remember that data from separate samples can always be composited, but data from composites cannot be extrapolated into discrete samples.

If plankton distribution is uniform, use a random sampling scheme to accommodate statistical testing. Include a random selection of sampling sites and transects, as well as a random collection of samples from each site. On the other hand, if plankton distribution is variable or patchy, include more sampling sites, collect composite samples, and increase sample replication. Use appropriate statistical tests to determine population variability.

When sampling a lake or reservoir, use a grid network or transect lines in combination with random procedures. Take enough samples to make the data meaningful. While there is no unequivocal standard procedure, consider sampling a circular lake basin at strategic points along at least two perpendicular transects extending from shore to shore, and include the deepest point in the basin. Consider sampling a long, narrow basin at several points along at least three regularly spaced parallel transects that are perpendicular to the long axis of the basin, with the first near the inlet and the last near the outlet. Similarly, consider sampling a large bay along several parallel transects that are perpendicular to the long axis of the bay. Because many samples are required to appraise the plankton assemblage completely, it may be necessary to restrict sampling to strategic points (e.g., near water intakes and discharges, constrictions in the water body, and major bays that may influence the main basin). If only one sampling site can be established, an open water pelagic location near the deepest point in a lake or reservoir is generally considered the most representative.

In lakes, reservoirs, and estuaries where plankton populations can vary with depth, collect samples from all major depth zones or water masses, concentrating on the euphotic zone for phytoplankton and the complete water column for zooplankton. Sampling depths will be determined based on water depth at the station, the depth of a thermocline or pycnocline, or other factors. In shallow areas (2 to 3 m deep), subsurface samples collected at 0.5 to 1 m may be adequate. In deeper areas, collect samples at regular depth intervals. In estuaries, sample above and below the pycnocline. Sampling depth intervals vary for estuaries of different sizes and depths, but use depths representative of the vertical range. Composite sampling above and below the pycnocline is often used. In marine sampling, the extent of sample collection will depend on the study's intent and scope.

Special circumstances may arise over the continental shelf, where it is important to sample the entire vertical range. Take samples at stations approximately equidistant from the shore seaward. At each station, take a vertical series of samples from the water surface to nearly the bottom, gradually adding more stations across the shelf. Benthic grab samples may be taken to collect dormant resting cells or cysts. Beyond the shelf (in pelagic waters), sample in the photic zone from the surface to the thermocline (for phytoplankton) or deeper (for zooplankton). Sampling depths vary, but often are at 10- to 25-m intervals above the thermocline, at 100- to 200-m intervals from the thermocline to 1000 m deep, and then at 500- to 1000-m intervals at deeper levels.

Samples usually are referred to as "surface" or "depth" (subsurface) samples. Depth samples are taken at some stated depth, while surface samples are collected as near the water surface as possible. A "skimmed" sample of surface film plankton (neuston) can be revealing; however, ordinarily do not include a disproportionate quantity of surface film in a surface sample because neustonic plankton[4] often are trapped in the surface film with pollen, dust, and other detritus. Special methods may be needed to sample surface organisms.[5]

Sampling frequency depends on the study's intent, the range of seasonal fluctuations, meteorological conditions, the equipment's adequacy, and personnel's availability. Select a sampling frequency at some interval shorter than the plankton community's turnover rate. This requires consideration of life-cycle length, competition, predation, flushing, and current displacement. Frequent plankton sampling is desirable because of the plankton community's normal temporal variability and migratory character, but is not always practical. Daily vertical migrations occur in response to sunlight, nutrient concentrations, or predators. Random horizontal migrations or drifts are produced by winds, shifting currents, and tides. Both types of migrations will affect plankton data. Ideal characterization may require daily or more frequent sampling at multiple depths. When this is impossible, weekly, biweekly, monthly, or even quarterly sampling may still be useful for determining major population changes.

In river, stream, and estuarine regions subject to tidal influence, expect fluctuations in plankton composition over a tidal cycle. A typical sampling pattern at an estuary station includes a vertical series of samples taken from the surface, across the pycnocline, to near bottom, collected at 3-h intervals over at least two complete tidal cycles. Once a characteristic pattern is recognized, the sampling routine may be modified. If the sampling's focus does not require complete characterization but does require limiting influences, some standardization to match tidal cycles with each sample set may be adequate.

A useful series of references on freshwater and oceanographic methodology has been published.[6–12] Also, numerous taxonomic references for freshwater, estuarine, and marine phytoplankton are available in print.[13–35] In addition, several excellent Internet resources are available for verifying current taxonomy and taxonomic authority.[36–37]

2. Sampling and Storage Procedures

Once sampling locations, depths, and frequency have been determined, prepare for field sampling. Use opaque sample containers because even brief light exposure during storage will alter chlorophyll values. Sample-storage bottles should be made of polyethylene or glass to avoid metallic ion contamination, which can lead to significant errors when making algal assays or productivity measurements. Similarly, in multi-analyte sampling programs, store algal pigments in bottles without acid residues. For example, do not use bottles containing acidic preservatives for nutrients or Lugol's solution when microscopically enumerating phytoplankton: acidic preservatives preclude analyses for chlorophyll and other pigments.

To avoid confusion or error, label each container with the sampling date, cruise number, sampling station, study area (e.g., river, lake, reservoir), type of sample, and depth. Use waterproof labels and waterproof ink. When possible, enclose collection vessels in a protective container to avoid breakage. Do not add preservative to containers before sampling to avoid potentially overfilled sample bottles, inaccurate preservative additions, and contamination with potentially hazardous preservatives. Sample

size depends on the type and number of determinations to be made; the number of replicates depends on the statistical design of the study and statistical analyses selected for data interpretation. Always design a study around an objective with a predefined statistical approach rather than fit statistical analyses to data already collected.

In a field record book, note sample location, depth, type, time, meteorological conditions, turbidity, water temperature, salinity, other significant observations, and if possible, photodocument and record sample coordinates using a hand-held global positioning system (GPS). Field notebooks with waterproof paper are very suitable. Field data are invaluable when analytical results are interpreted and often help explain unusual changes due to the variable character of the aquatic environment. Collect coincident samples for chemical analyses to help define environmental variations that could affect plankton.

a. Phytoplankton: If phytoplankton densities are expected to be low (e.g., in oligotrophic waters), collect a sample larger than 1.0 L. In richer, eutrophic waters, collect a 0.1- to 1.0-L sample. Sample size may affect results: be consistent and apply experience with the waterbody or analysis techniques to obtain the correct amount of sample for meaningful analysis. Collect additional samples (0.5 to 1.0 L) if samples need to be acid cleaned for diatoms/Chrysophyte scales or need to be shipped for verification.

Nets are unsuitable for most quantitative phytoplankton sampling. Theoretically, nets capture algae larger than the mesh size, while smaller forms pass through, so nanoplankton (>2.0 to 20 μm) and picoplankton (0.2 to 2.0 um) may be completely missed. In practice, however, nets often capture larger colonies and filaments, while smaller taxa flow through. Also, the passage of larger forms is well-known, though rarely quantified. Net losses are influenced by community composition, mesh quality and size, sampling speed, volume sampled, and net clogging.[22–25]

Even when organisms are captured, differential capture, cell or colony damage, and inefficient net cleaning introduce errors that result in an unreliable, non-quantitative characterization of most phytoplankton communities. However, nets remain a powerful qualitative collection tool, especially in teaching applications.

For qualitative and quantitative evaluations, collect whole (unfiltered and unstrained) water samples with a collection bottle consisting of a cylindrical tube with stoppers at each end and a closing device. Lower the open sampler to the desired depth and trip the closing mechanism (this may involve a messenger or tugging the line). If possible, obtain composite samples from several depths or pool repetitive samples from one depth. The most commonly used samplers that operate on this principle are the Alpha, Kemmerer,[26] Niskin/Nansen, and Van Dorn[27] (Figure 10200:1) samplers.

These samplers collect all sizes of phytoplankton, which can be subsequently segregated by filtering these whole water samples through netting with various mesh sizes. (NOTE: Larger particulates may pass through smaller mesh sizes than their long axis would indicate. Select appropriate mesh sizes to carefully concentrate the various sizes of phytoplankton typical of the aquatic system being studied, and be prepared for overlap.[28,38])

Van Dorn usually is the preferred sampler for standing crop, primary productivity, and other quantitative determinations because it does not inhibit the free flow of water through the

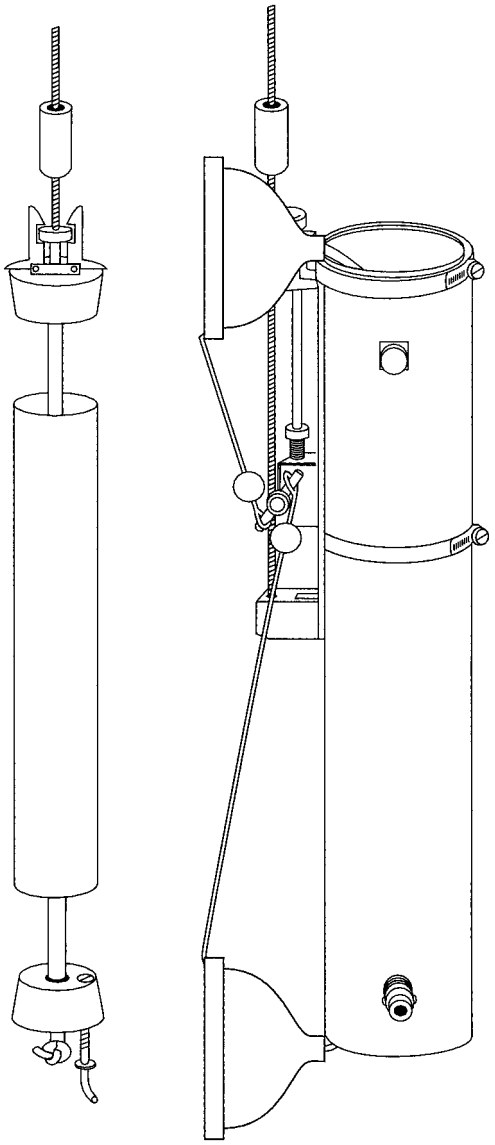


Figure 10200:1. Structural features of common water samplers, Kemmerer (left) and Van Dorn (right).

cylinder. In deep-water and marine situations, the Niskin/Nansen bottle is preferred. The Niskin/Nansen sampler has the same design as the Van Dorn sampler except it can be cast in a series on one line to sample multiple depths simultaneously with the use of auxiliary messengers. The triggering devices of these samplers are sensitive, so avoid rough handling. Always lower the sampler into the water; do not drop. Kemmerer and Van Dorn samplers have capacities of 0.5 L or more. Polyethylene or polyvinyl chloride sampling devices are preferred to metal samplers because the latter liberate metallic ions that may contaminate the sample.

In shallow waters, use a Jenkins surface mud sampler,[39] a bottle sampler modified so it is held horizontally,[40] or an appropriate bacteriological sampler.[41]

For greater collection speed, and to obtain large, accurately measured quantities of organisms, use a pump. Diaphragm and

peristaltic pumps are less damaging to organisms than centrifugal pumps.[42] Centrifugal pump impellers can damage organisms, as can passage through the hose.[43] Lower a weighted hose, attached to a suction pump, to the desired depth and pump water to the surface. Pumps can supply a homogeneous sample from a given depth or an integrated sample from the surface to a particular depth. If a centrifugal pump is used, draw samples from the line before they reach the impeller. For samples to be analyzed for organochlorine compounds, use tetrafluoroethylene (TFE) tubing.

To examine live samples, partially fill containers and store them in a refrigerator or ice chest in the dark (if not using opaque bottles); examine specimens promptly after collection.

If living material cannot be examined or if phytoplankton will be counted later, preserve the sample. There are multiple phytoplankton preservatives. Lugol's solution and glutaraldehyde are the most commonly used; others include formalin, merthiolate, and "M3" fixative. Also, adding a few crystals of copper sulfate to any preservative stock solution helps maintain the algae's color. **CAUTION: All preservatives are a hazardous chemical risk; consult the appropriate material safety data sheets (MSDSs) before working with any preservative. Glutaraldehyde and formalin, in particular, must be used in a well-ventilated area or positive flow hood.**

Lugol's solution: Lugol's solution, which can be used for most forms (e.g., naked flagellates), stains organisms that store starch (especially chlorophytes and cryptophytes) and tends to cause most cyanobacteria to settle. Unfortunately, acidic Lugol's solution dissolves the coccoliths of Coccolithophores (which are common in estuarine and marine waters), tends to cause freshwater chrysophytes and some cyanobacterial colonies (especially *Microcystis* and *Aphanizomenon*) to disintegrate, and must be spiked every 6 to 12 months because of its volatility. Lugol's solution (and, to a lesser extent, formalin) also squelches autofluorescence.

To preserve samples with Lugol's solution, add 0.3 mL Lugol's solution to 100 mL sample and store in the dark. For long-term storage, add 0.7 mL Lugol's solution per 100 mL sample. The sample should look like weak tea. If Lugol's solution cannot be re-added every 6 to 12 months, add buffered formalin to a minimum of 2.5% final concentration after 1 h (however, formalin tends to distort many cells). Alternatively, glutaraldeyhyde can be added to a final concentration of 0.25 to 0.5%, which results in less cell distortion.

Prepare Lugol's solution by dissolving 20 g potassium iodide (KI) and 10 g iodine crystals in 200 mL distilled water containing 20 mL glacial acetic acid.[44] Utermohl's[45] modification of Lugol's solution results in a neutral or slightly alkaline solution. Prepare modified Lugol's solution by dissolving 10 g KI and 5 g iodine crystals in 20 mL distilled water, then adding 50 mL distilled water in which 5 g anhydrous sodium acetate has been dissolved. This preserves Coccolithophores (which are most common in marine waters) but would be less effective for other flagellates.

Glutaraldehyde: Glutaraldehyde is in the same chemical class as formalin but used at a much lower concentration (0.25 to 0.5% versus 3 to 4% final concentration). Glutaraldehyde also causes minimal distortion, tends to preserve colony structure in most algal groups, lasts in samples for years without degradation (formalin can form crystals after 15+ years) and preserves autofluorescence. As long as the preservative percentage does not exceed 2%, no compensation calculation is needed. As a result, glutaraldehyde has gained prominence in freshwater phytoplankton and periphyton work.

Preserve samples by adding neutralized glutaraldehyde to yield a final concentration of 0.25 to 0.5%. If the sample is exceptionally dense, use a maximum concentration of 1% glutaraldehyde. Nalgene or glass bottles are suitable, and amber or opaque bottles are preferred so autofluorescence can be used in analysis. When a preserved sample has been shaken after an appropriate reaction time (about 1 h), the sample should develop temporary foam on the surface. Once preserved with glutaraldehyde, refrigeration is unnecessary and light sensitivity is much reduced; however, keep samples out of direct sunlight. **CAUTION: Keep 25% gluteraldehyde in the fume hood and work with it in a well-ventilated area.**

Formalin: To preserve samples with formalin, add 40 mL buffered formalin [20 g sodium borate ($Na_2B_2O_4$) + 1 L 37% formaldehyde] to 1 L of sample immediately after collection. **CAUTION: As with glutaraldehyde, keep concentrated fomaldehyde in the chemical fume hood and work in a well-ventilated area.** In estuarine and marine collections, adjust pH to at least 7.5 with sodium borate for samples containing Coccolithophores.

Merthiolate: To preserve samples with merthiolate, add 36 mL merthiolate solution to 1 L of sample and store in the dark. Prepare merthiolate solution by dissolving 1.0 g merthiolate, 1.5 g sodium borate, and 1.0 mL Lugol's solution in 1 L distilled water. Merthiolate-preserved samples are not sterile, but can be kept effectively for 1 year, after which time formalin or glutaraldehyde must be added.[46]

"M3" fixative: Prepare by dissolving 5 g KI, 10 g iodine, 50 mL glacial acetic acid, and 250 mL formalin in 1 L distilled water (dissolve the iodide in a small quantity of water to aid in solution of iodine). Add 20 mL fixative to 1 L sample and store in the dark.

Most preservatives distort and disrupt certain cells,[47,48] especially those with delicate forms (e.g., *Euglena, Cryptomonas, Synura, Chromulina,* and *Mallamonas*). Glutaraldehyde solution usually is least damaging for such phytoflagellates, although all preservatives create some level of preservation artifact. To become familiar with live specimens and preservation-caused distortions, use reference collection material from biological supply houses, work extensively with live material, and consult experienced co-workers. Taxonomic consistency over long-term projects is critical. Document the basis for identification carefully, making sure that morphological variation is described clearly and identification references are permanently associated with the data.

b. Zooplankton: The choice of sampler depends on the type and size distribution of zooplankton, the kind of study (distribution, productivity, etc.), and the body of water being investigated. Zooplankton populations invariably are distributed in a patchy way, making both sampling and data interpretation difficult.

To collect microzooplankton (20 to 200 μm), such as protozoa, rotifers, and immature microcrustacea, use the bottle samplers described for phytoplankton. Small zooplankters usually are sufficiently abundant to yield adequate samples in 5- to 10-L bottles; however, composite samples over depth and time are

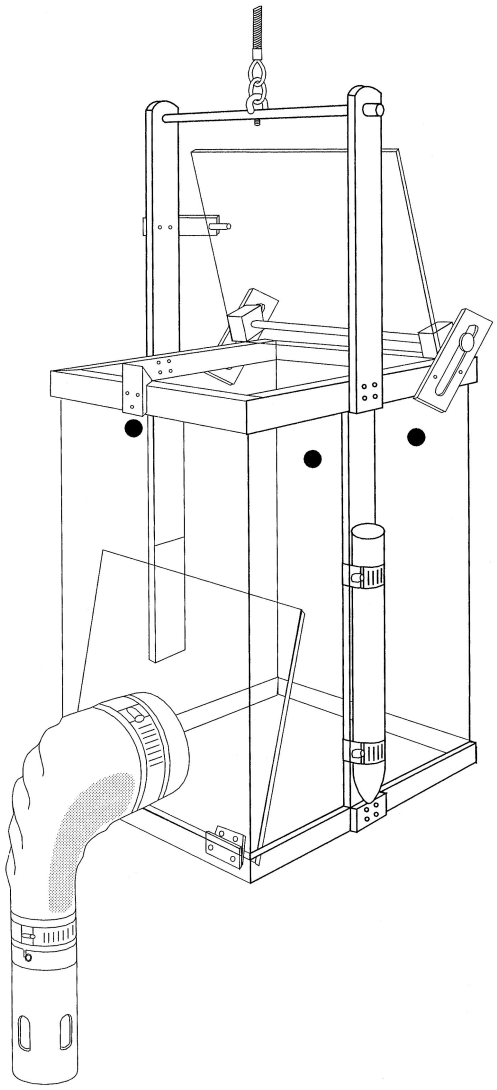


Figure 10200:2. The Schindler-Patalas plankton trap.

recommended. Water bottle samplers are suitable especially for discrete-depth samples. If depth-integrated samples are desired, use pumps or nets. Larger and more robust microzooplankters (e.g., loricate forms and crustacea) may be concentrated by passing the whole water through a 20-μm mesh net. If quantitative estimates of other nonloricate, delicate forms are required, do not screen. Fix 0.5 to 5 L of whole water to enumerate these forms.

Bottle samplers usually are unsuitable for collecting larger zooplankton (e.g., mature microcrustacea) that, unlike smaller forms, are much less numerous and are sufficiently agile to avoid capture. Although a pump can be used to sample comparatively large water volumes, and consequently adequate numbers of microcrustacea, avoidance by larger, more agile zooplankters at the pump head can cause sampling error. Consequently, larger trap samplers or nets are the preferred collection methods.

The Juday trap[49] operates on the same principle as water bottle samplers but is generally larger (10 L) and so more suitable for collecting zooplankters, especially larger copepods. However, it is awkward to use and its 10-L capacity is inadequate for oligotrophic lakes or other water bodies with few zooplankters. Also, it is constructed of metal and so is unsuitable if heavy metals analyses are required.

TABLE 10200:I. CHARACTERISTICS OF COMMONLY USED PLANKTON NETS

Silk No.	Size of Aperture μm	Approximate Open Area %	Classification
000	1024	58	Largest zooplankton and ichthyoplankton
00	752	54	Larger zooplankton and ichthyoplankton
0	569	50	Large zooplankton and ichthyoplankton
2	366	46	Large microcrustacea
6	239	44	Microcrustacea
10	158	45	Microcrustacea and most rotifers
20	76	45	Net phyto- and zooplankton
25	64	33	Nanoplankton

The Schindler-Patalas trap[50] (Figure 10200:2) usually is preferred to the Juday trap because it is constructed of clear acrylic plastic (i.e., is transparent). It can be lowered into the water with minimal disturbance and is suitable for collecting larger zooplankters. Models of 10- to 12-L capacity are available, but the 30-L size is preferred. It has no mechanical closing mechanism, so it is convenient for cold-weather sampling, when mechanical devices tend to malfunction. Like the Juday trap, it can be fitted with nets of various mesh sizes. [NOTE: Mesh sizes less than 125 μm (No. 120 and larger) may rapidly clog when large colonial or filamentous phytoplankton are abundant or zooplankters with sheaths (e.g., *Holopedium*) are present.]

Plankton nets are preferred to sampling bottles and traps in areas where plankters are few, are vertically distributed, or only qualitative data or a large biomass is needed for analysis. Because they originally were designed for qualitative sampling, modifications are required for quantitative work, and nets remain a poor choice for quantitative phytoplankton work.

The mesh size, type of material, orifice size, length, hauling method, type of tow, and volume sampled will depend on the study.[51,52] The type of netting and mesh size determine filtration efficiency, clogging tendencies, velocity, drag, and sample condition after collection. Silk, which used to be the common mesh material in plankton nets, is not recommended because its mesh openings shrink and rot with age. Nylon monofilament mesh is preferred because of its mesh size accuracy and durability. Nylon-net mesh sizes still are labeled by the silk rating system. The characteristics of commonly used nylon plankton nets are listed in Table 10200:I. Finer mesh sizes clog more readily than coarser mesh; when sizing mesh, a compromise must be made between mesh small enough to retain desired organisms effectively and large enough to preclude a serious clogging problem. If clogging occurs, there are several options, depending on whether it is phytoplankton- or zooplankton-related: decrease tow length, increase mesh size, preserve a larger volume of water, or collect whole water samples.

The maximum volume (V_M) of water that can be filtered through a net during a vertical tow can be estimated as follows:

$$V_M = \pi r^2 d$$

where:

r = radius of net orifice and
d = depth to which net is lowered.

Nets have maximum filtration volumes because mesh clogging by phytoplankton and other particles and, for fine netting, even the netting itself can cause some water to be diverted from the net's path.[53,54] Keep net towing distance as short as practical to alleviate clogging. If the net has a pronounced green or brown color after towing, it is probably clogged.

To estimate sampling volume (V_A), mount a calibrated flow meter midway between the net rims and mouth center (the meter is mounted off-center to avoid the flow reduction associated with the towing bridle).[55] Equip meter with lock mechanisms to keep it from turning in reverse or while in air. Record flow-meter readings before and after collecting sample. Calculate filtration efficiency (E) as follows:

$$E = V_A/V_M$$

If E is less than about 0.8, substantial clogging has occurred. Take steps to increase efficiency. Clogging not only decreases the volume filtered, but also leads to biased samples due to non-uniform filtration efficiency during the tow.[52] Net efficiency on a per-species basis can be determined for each system, tow depth, and sampling interval by using a vertical sampler to composite samples from tow depth to the surface.

Various types of plankton nets are shown in Figure 10200:3. Simple conical nets have been used for many years with little modification in design or improvement in accuracy. Their major source of error is that conical nets' filtration characteristics usually are unknown. Filtration efficiency in No. 20 mesh cone nets ranges from 40 to 77%. To improve efficiency, place a porous cylinder collar or nonporous truncated cone in front of the conical portion of the net. The Juday net is a commonly used net with a truncated cone. For good filtration characteristics, the ratio of the net's filtering area to orifice area should be at least 3:1. Bridles attaching the net to the towing line also adversely influence filtration efficiency and increase turbulence in front of the net, thereby increasing the potential for net avoidance by larger zooplankters. The tandem, Bongo net design (Figure 10200:3C) reduces these influences and permits duplicate samples to be collected simultaneously.

Three types of tows are used: vertical, horizontal, and oblique. Vertical tows are preferred to obtain an integrated water column sample. To make a vertical tow, lower a weighted net to a given depth, then raise vertically at an even speed of 0.5 m/s.

In small water bodies, haul the net hand over hand with a steady, unhurried motion approximating the speed of 0.5 m/s. In large bodies where long net hauls and vessel drifting are expected, use a davit, meter wheel, angle indicator, and winch. Attach a 3- to 5-kg weight to hold the net down. Determine the net's depth by multiplying the length of the extended wire by the cosine of the wire's angle with the vertical direction. Maintain wire angle as close to vertical as possible by controlling the boat's speed null against the wind drift, or wherever feasible, do vertical hauls from an anchored boat.

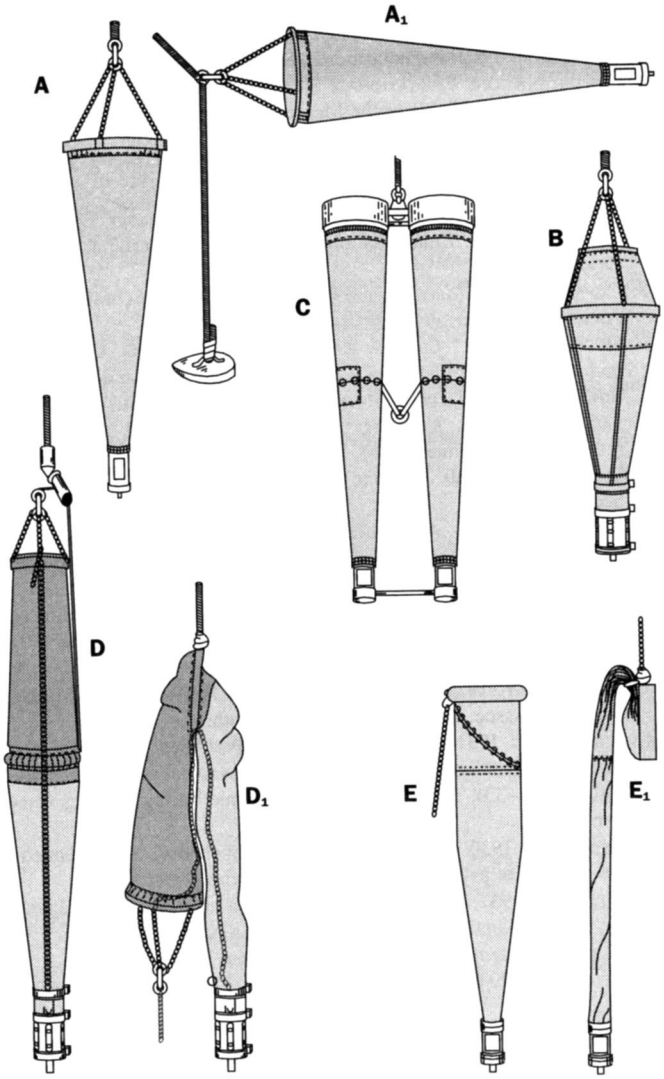


Figure 10200:3. Examples of commonly used plankton sampling nets. (A) Simple conical tow-net; A—rigged for vertical tows; A_1—for oblique or horizontal tows; (B) Wisconsin (Birge) tow-net with truncated cone to improve filtration efficiency; (C) Bongo net, can be fitted with flow meters and opening/closing mechanisms; (D) Wisconsin net fitted with messenger-activated closing mechanism, D—open, D_1—closed; (E) Free-fall net, E—open, E_1—closed.

Vertical and oblique tows collect a composite sample, and horizontal tows collect a sample at a discrete depth. Oblique tows usually are preferred over vertical tows in shallow water or wherever a longer net tow is required. For oblique tows, lower the net or sampler to some predetermined depth and then raise it at a constant rate as the boat moves forward. Oblique tows do not necessarily sample a true angle from the bottom to the surface. Under best conditions, the pattern is somewhat sigmoid due to boat acceleration and slack in the tow line.

Horizontal tows usually are used to obtain depth distribution information on zooplankton. Although a variety of horizontal

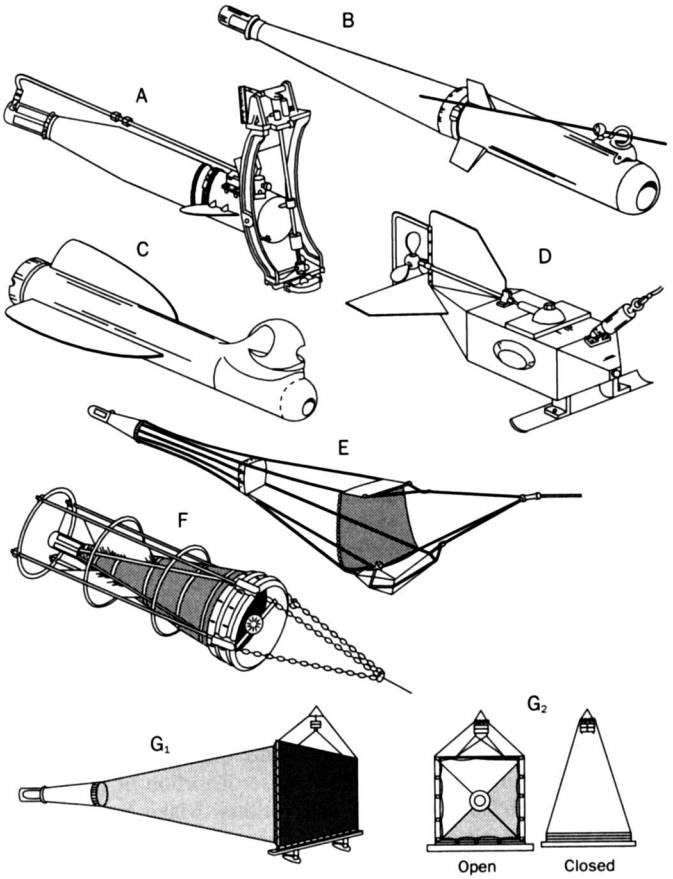


Figure 10200:4. Examples of commonly used high-speed zooplankton samplers. (A) Clarke-Bumpus sampler; (B) Miller sampler; (C) Hardy plankton indicator; (D) Hardy continuous plankton recorder; (E) Issacs-Kidd mid-water trawl; (F) Gulf V sampler; (G) Tucker trawl, G_1-sideview, G_2-front view open and closed.

samplers is available (see Figure 10200:4), use the Clarke-Bumpus sampler [56] for quantitative collection of zooplankton because of its built-in flowmeter and opening–closing device. For horizontal tows, use a boat equipped as above and determine sampler depth as above. Lower sampler to preselected depth, open, tow at that depth for 5 to 10 min, then close and raise it.

A variety of zooplankton sampling methods can be used in flowing water. The choice depends largely on flow velocity. Properly weighted bottles, traps and pump hoses, and nets can be used in medium- to slow-flowing waters. In turbulent, well-mixed waters, collect surface water via bucket and filter it through the appropriately sized mesh. Select sample size based on zooplankter concentration.

Give plankton nets proper care and maintenance. Do not let particulate dry on the net because it can significantly reduce size of mesh apertures and increase frequency of clogging. Wash net thoroughly with water after each use, and let it dry completely before storage. Periodically clean with a warm soap solution. Because nylon net is susceptible to deterioration from abrasion and sunlight, guard against unnecessary wear and store in the dark.

Traps and nets do not work well in shallow areas with aquatic vegetation, so use a length of lightweight rubber or polyethylene tubing with netting stretched over one end and rope tied to the other.[57] Use tubing that is 5- to 10-cm diameter and long enough to reach from the surface to the bottom. Affix the netting with tape or rubber bands, so it will stay in place in water but can be removed easily after sampling. Lower the open end of the tubing (the end with the rope attached) into the water until it almost touches the bottom and then use the rope to pull it up again, while keeping the covered end above the water surface. When the open end emerges from the water, let the covered end fall in. Pull the tubing into the boat, open end first, and let the water in the tube drain through the netting. When the zooplankton has been concentrated in a small volume, just above the netting, remove the netting over a container and catch the concentrated sample. Wash netting and end of tubing into the container to ensure that all the zooplankton is collected. This method is not limited to areas with aquatic vegetation. It is an excellent method of obtaining an integrated sample from any shallow area. In standing waters, collect tow samples by filtering 1 to 5 m^3 of water.

Zooplankton samples most often are preserved with 70% ethanol[58] or 5% buffered formalin; glutaraldehyde or Lugol's solution will work, but not as well. Ethanol preservative is preferred for materials to be stained in permanent mounts or stored. Formalin may be used for the first 48 h of preservation with subsequent transfer to 70% ethanol. Formalin may distort pleomorphic forms, such as protozoans and rotifers. Make formalin in sucrose-saturated water to minimize carapace distortion and loss of eggs in crustaceans, especially cladocerans.[59] Bouin's fixative—picric acid saturated in calcium carbonate-buffered formaldehyde containing 5% (v/v) acetic acid—produces reasonable results for soft-bodied microzooplankton.[60] Dilute Bouin's fixative 1:19 with the sample. Because rapid fixation is necessary, pour the sample onto the fixative or inject fixative rapidly into the sample.

Use a narcotizing agent (e.g., carbonated water, menthol-saturated water, or neosynephrine) to prevent or reduce the contraction or distortion of organisms, especially rotifers, cladocerans, and many marine invertebrates.[61,62] Adding a few drops of detergent prevents preserved organisms from clumping. Preserve samples as soon as most animal movement has ceased—usually within a half hour of narcotization. To prevent evaporation, add 5% glycerin to the concentrated sample. In turbid samples, differentiate animal and detrital material by adding 0.04% rose bengal stain, which intensely stains the carapace (shell) of zooplankters and is a good general cytoplasmic stain. Taxonomic consistency over long-term projects is critical. Document the basis for identification carefully, making sure that morphological variation is described clearly and identification references are permanently associated with the data.

3. References

1. U.S. Environmental Protection Agency. 1982. Handbook for Sampling and Sample Preservation of Water and Wastewater, EPA-600/4-82-029. U.S. Environmental Protection Agency, Washington, D.C.
2. Graham, J.L., K.A. Loftin, A.C. Ziegler & M.T. Meyer. 2008. Cyanobacteria in lakes and reservoirs—toxin and taste-and-odor

sampling guidelines. *In* U.S. Geological Survey, Techniques of Water-Resources Investigations, Book 9, Chap. A7, Sec. 7.5, (Ver. 1.0) http://water.usgs.gov/owq/FieldManual/Chapter7/7.5.html. Accessed September 2011.
3. Parker, B.C. & R.F. Hatcher. 1974. Enrichment of surface freshwater microlayers with algae. *J. Phycol.* 10:185.
4. Marshall, H.G. & L. Burchardt. 2005. Neuston: Its definition with a historical review regarding its concept and community structure. *Arch. Hydrobiol.* 164(4):429.
5. Marshall, H.G. & M.I. Gladyshev. 2009. Neuston in aquatic ecosystems. *In* G.E. Likens, ed. Encyclopedia of Inland Waters, Vol.1. Elsevier, Oxford, U.K.
6. Wetzel, R.G. 1983. Limnology. Saunders College Publishing, Philadelphia, Pa.
7. Wetzel, R.G. & G.E. Likens. 1991. Limnological Analyses. Springer-Verlag, New York, N.Y.
8. Taguchi, S. & K. Nakajima. 1971. Plankton and seston in the sea surface of three inlets of Japan. *Bull. Plankton Soc. Japan* 18:20.
9. United Nations Educational, Scientific and Cultural Organization. 1966. Determination of Photosynthetic Pigments in Sea-water, Monogr. Oceanogr. Methodol. No. 1. United Nations Educational, Scientific & Cultural Org., Paris.
10. United Nations Educational, Scientific and Cultural Organization. 1968. Zooplankton Sampling, Monogr. Oceanogr. Methodol. No. 2. United Nations Educational, Scientific & Cultural Org., Paris.
11. United Nations Educational, Scientific and Cultural Organization. 1973. A Guide to the Measurement of Marine Primary Production under Some Special Conditions, Monogr. Oceanogr. Methodol. No. 3. United Nations Educational, Scientific & Cultural Org., Paris.
12. Sournia, A., ed. 1978. Phytoplankton Manual, Monogr. Oceanogr. Methodol. No. 6. United Nations Educational, Scientific & Cultural Org., Paris.
13. Cupp, E.E. 1943. Marine plankton diatoms of the west coast of North America. *Bull. Scripps Inst. Oceanogr*. 5:1.
14. Hustedt, F. 1927–66. Die Kieselalgen Deutschlands, Österreichs und der Schweiz mit Berucksichtigung der Übrigen Lander Europas Sowie der Angrenzenden Meeresgebiete. *In* L. Rabenhorst. Kryptogamen-Flora, Vol. 7: Teil 1 (1927–30); Teil 2 (1931–59); Teil 3 (1961–66). Akademie Verlag, Leipzig, Germany.
15. Lebour, M.V. 1930. The Planktonic Diatoms of Northern Seas. Ray Soc., London.
16. Hendey, N.I. 1964. An introductory account of the smaller algae of British coastal waters, V. bacillariophyceae (Diatoms). *Fish. Invest. Min. Agr. Fish. Food (G.B.),* Ser. IV:1.
17. Dodge, J.D. 1975. The prorocentrales (Dinophyceae); II. Revision of the taxonomy within the genus *Prorocentrum. Bot. Limnol. Soc*. 71:103.
18. Lebour, M.V. 1925. The Dinoflagellates of Northern Seas. Marine Biological Assoc. Plymouth, U.K.
19. Schiller, J. 1931–37. Dinoflagellatae (Peridineae) in monographischer Behandlung. *In* L. Rabenhorst. Kryptogamen-Flora, Vol. 10; Teil 1 (1931–33); Teil 2 (1935–37). Akademie Verlag, Leipzig, Germany.
20. Schiller, J. 1930. Coccolithineae. *In* L. Rabenhorst. Kryptogamen-Flora, Vol. 10, p. 89. Akademie Verlag, Leipzig, Germany.
21. Geitler, L. 1932. Cyanophyceae von Europa unter Berucksichtigung der anderen Kontinente. *In* L. Rabenhorst. Kryptogamen-Flora, Vol. 14, p. 1. Akademie Verlag, Leipzig, Germany.
22. Allen, E.J. 1919. A contribution to the quantitative study of plankton. *J. Marine Biol. Assoc. U.K.* 12(1):1.
23. Graham, J.L. & J.R. Jones. 2007. Microcystin distribution in physical size class separations of natural plankton communities. *Lake Reservoir Mgmt.* 23(2):161.
24. Hardy, A.C. 1956. The Open Sea; Its Natural History: The World of Plankton. Houghton Mifflin Company, Boston, Mass.
25. Johnstone, J., A. Scott & H.C. Chadwick. 1924. The Marine Plankton. The University Press of Liverpool Limited, Liverpool, U.K.
26. Welch, P.S. 1948. Limnological Methods. Blakiston Co., Philadelphia, Pa.
27. Strickland, J.D.H. & T.R. Parsons. 1968. A Practical Manual of Sea Water Analysis, Fish. Res. Board Can. Bull. No. 167. Queen's Printer, Ottawa, Ont.
28. Dussart, B.M. 1965. Les différentes catégories de plancton. *Hydrobiologia* 26:72.
29. Komárek, J. & K. Anagnostidis. 2001. Cyanoprokaryota, Teil 1; Chroococcales. *In* H. Ettl, G. Gärtner, H. Heynig & D. Mollenhauer, eds. Süsswasserflora von Mitteleuropa, Band 19(1). Elsevier, Heidelberg.
30. Komárek, J. & K. Anagnostidis. 2005. Cyanoprokaryota, Teil II; Oscillatoriales. *In* B. Büdel, G. Gärtner, L. Krienitz & M. Schagerl, eds. Süsswasserflora von Mitteleuropa, Band 19(2). Elsevier, Heidelberg.
31. Krammer, K & H. Lange-Bertalot. 2000. Bacillariophyceae, Teil 3; Centrales, Fragilariaceae, Eunotiaceae. Süsswasserflora von Mitteleuropa, Band 2(3). Spektrum Akademischer Verlag, Heidelberg.
32. Prescott, G.W., C.E.M. Bicudo & W.C. Vinyard. 1982. A Synopsis of North American Desmids; Part II. Desmidiaceae: Placodermae, Sec. 4. University of Nebraska Press, Lincoln.
33. Wehr, J.D. & R.G. Sheath. 2003. Freshwater algae of North America. Academic Press, Amsterdam.
34. John, D.M., B.A. Whitton & A.J. Brook. 2002. The Freshwater Algal Flora of the British Isles. University of Cambridge Press, Cambridge, UK.
35. Tomas, C.R. 1997. Identifying Marine Phytoplankton. Academic Press, Harcourt Brace & Co., San Diego.
36. Brands, S.J. (comp.) 1989–present. The Taxonomicon. Universal Taxonomic Services, Zwaag, The Netherlands. http://www.taxonomicon.net. Accessed September 2011.
37. Integrated Taxonomic Information System (ITIS). 2010. http://www.itis.gov. Accessed September 2011.
38. Sieburth, J.McN., V. Smetacek & J. Lenz. 1978. Pelagic ecosystem structure: Heterotrophic compartments of plankton and their relationship to plankton size fractions. *Limnol. Oceanogr*. 23:1256.
39. Mortimer, C.H. 1942. The exchange of dissolved substances between mud and water in lakes. *J. Ecol.* 30:147.
40. Vollenweider, R.A. 1969. A Manual on Methods for Measuring Primary Production in Aquatic Environments, IBP Handbook No. 12. Blackwell Scientific Publ., Oxford, England.
41. Geldreich, E.E., H.D. Nash, D.F. Spino & D.J. Reasoner. 1980. Bacterial dynamics in a water supply reservoir: a case study. *J. Amer. Water Works Assoc.* 72:31.
42. Beers, J.R. 1978. Pump sampling. *In* A. Sournia, ed. Phytoplankton Manual. United Nations Educational, Scientific & Cultural Org., Paris.
43. Exton, R.J., W.M. Houghton, W. Esaias, L.W. Haas & D. Hayward. 1983. Spectral differences and temporal stability of phycoerythrin fluorescence in estuaries and coastal waters due to the domination of labile cryptophytes and stable cyanobacteria. *Limnol. Oceanog*. 28:1225.
44. Edmondson, W.T., ed. 1959. Freshwater Biology, 2nd ed. John Wiley & Sons, New York, N.Y.
45. Utermohl, H. 1958. Zur Vervollkommung der quantitativen phytoplankton Methodik. *Mitt. Int. Ver. Limnol.* No. 9.
46. Weber, C.I. 1968. The preservation of phytoplankton grab samples. *Trans. Amer. Microsc. Soc.* 87:70.

47. PAERL, H.W. 1984. An evaluation of freeze fixation as a phytoplankton preservation method for microautoradiography. *Limnol. Oceanog.* 29:417.
48. SILVER, M.W. & P.J. DAVOLL. 1978. Loss of ^{14}C activity after chemical fixation of phytoplankton: Error source for autoradiography and other productivity measurements. *Limnol. Oceanog.* 23:362.
49. JUDAY, C. 1916. Limnological apparatus. *Trans. Wis. Acad. Sci*. 18:566.
50. SCHINDLER, D.W. 1969. Two useful devices for vertical plankton and water sampling. *J. Fish. Res. Board Can.* 26:1948.
51. SCHWOERBEL, J. 1970. Methods of Hydrobiology. Pergamon Press, Toronto, Ont.
52. TRANTER, D.J., ed. 1980. Reviews on Zooplankton Sampling Methods. United Nations Educational, Scientific & Cultural Org., Switzerland.
53. GANNON, J.E. 1980. Towards improving the use of zooplankton in water quality surveillance of the St. Lawrence Great Lakes. *Can. Tech. Rep. Fish. Aquat. Sci.* 976:87.
54. ROBERTSON, A. 1968. Abundance, Distribution, and Biology of Plankton in Lake Michigan with the Addition of a Research Ships of Opportunity Project, Spec. Rep. No. 35. Great Lakes Research Div., Univ. Michigan, Ann Arbor.
55. EVANS, M.S. & D.W. SELL. 1985. Mesh size and collection characteristics of 50-cm diameter conical plankton nets. *Hydrobiologia* 122:97.
56. CLARKE, G.L. & D.F. BUMPUS. 1940. The Plankton Sampler: An Instrument for Quantitative Plankton Investigations, Spec. Publ. No. 5. Limnological Soc. America.
57. PENNAK, R.W. 1962. Quantitative zooplankton sampling in littoral vegetation areas. *Limnol. Oceanog*. 7:487.
58. BLACK, A.R. & S.I. DODSON. 2003. Ethanol: a better preservation technique for Daphnia. *Limnol. Oceanog.* (Methods) 1:45.
59. HANEY, J.F. & D.J. HALL. 1973. Sugar-coated Daphnia; A preservation technique for Cladocera. *Limnol. Oceanog*. 18:331.
60. COATS, D.W. & J.F. HEINBOKEL. 1982. A study of reproduction and other life cycle phenomena in plankton protists using an acridine orange fluorescence technique. *Mar. Biol*. 67:71.
61. GANNON, J.E. & S.A. GANNON. 1975. Observations on the narcotization of crustacean zooplankton. *Crustaceana* 28(2):220.
62. STEEDMAN, H.F. 1976. Narcotizing agents and methods. *In* H.F. Steedman, ed. Zooplankton Fixation and Preservation, Monogr. Oceanogr. Methodol. No. 4. United Nations Educational, Scientific & Cultural Org., Paris.

10200 C. Concentration Techniques

The organisms in water samples often must be concentrated in the laboratory before analysis. Ultimately, when calculating a concentration from an actual count, the multiplication factor should be less than 25 (i.e., every organism or cell counted should not represent more than about 25 organisms or cells in the natural sample). The multiplication factor is a function of the concentrated sample's concentration and volume. Three techniques for concentrating phytoplankton are sedimentation, membrane filtration, and centrifugation. A special technique for zooplankton also is described below.

1. Sedimentation/Settling

Sedimentation is the preferred concentration method because it is nonselective (unlike filtration) and nondestructive (unlike filtration or centrifugation), although many picoplankton, smaller nanoplankton, and actively swimming flagellates (in unpreserved samples) may not settle completely. Also, this approach may be too slow if results are needed quickly. The volume concentrated varies inversely with the abundance of organisms and is related to sample turbidity.

Allow 1 h settling per millimeter of column depth. For a sample preserved with Lugol's solution (2 to 4 ml/L), allow about 0.5 h settling/mm depth.[1] The sample may be concentrated in a series of steps by quantitatively transferring concentrate from the initial container to sequentially smaller ones. Use cylindrical settling chambers with thin, clear glass bottoms. Apply a height-to-diameter ratio no larger than 5:1 to avoid excessive chamber wall influence and currents in the chamber. Fill settling chambers without forming a vortex, keep them vibration-free, and move them carefully to avoid non-random distribution of settled matter. When siphoning supernatant to obtain the desired concentrate (usually 2 to 3 mL; 5 mL for diatom mounts), do it slowly, do not agitate the water, and hold the end of the siphon or pipet directly below the water's surface. Store concentrated sample in a closed, labeled container (remember that samples preserved with Lugol's solution will need to be re-spiked every 6 to 12 months).

2. Membrane Filtration

The filtration method permits the use of high magnification to enumerate small plankters (e.g., flagellates and cyanobacteria); it essentially concentrates the sample while providing a countable preparation. This section emphasizes preparation for microscopy, although glass-fiber filters (GF/F) (and membrane filters) are also used to isolate phytoplankton for pigment analysis (see 10200H). However, delicate forms (e.g., "naked" flagellates) can be distorted by even gentle filtration. When populations are dense and the detritus content is high, the filter clogs quickly and silt may crush organisms or obscure them from view. However, settling under high particulate circumstances also yields a difficult sample. Filtration offers the opportunity to make permanent mounts, allows for fast sample preparation when rapid results are needed to support management decisions (as in water treatment), and enhances the use of autofluorescence (thin preparation).

Pour a measured volume of well-mixed sample into a funnel equipped with a membrane filter (25-mm filter diameter; 0.45-μm pore size). Apply a vacuum of less than 50 kPa ($<$25 mm Hg) to the filter until about 0.5 cm of sample remains. Break vacuum, then apply low vacuum (about 12 kPa, 2 to 3 mm Hg) to remove remaining water. Do not dry filter.

For samples with a low phytoplankton and silt content, this method increases the probability of observing less abundant forms.[2] Samples also may be concentrated on a filter, inverted onto a microscope slide, and quick-frozen so plankton can be

transferred from the filter to the slide. Alternatively, oil can be added to make the filter slightly translucent.[3,4]

Both autotrophic and heterotrophic picoplankton may be collected and counted via filtration and subsequent epifluorescence microscopy.[5–7] Wet a backing 0.45-μm Nuclepore filter with distilled water and place filter on the stem. Place a black 0.2-μm Nuclepore filter over the other filter. Based on cell concentrations, filter generally 1 to 2 mL of water sample through the filter apparatus, using a hand pump exerting a vacuum of 10 mm mercury. Filter sample until the meniscus disappears from the top filter. Remove the 0.2-μm filter and mount in oil on a slide (see 10200D.2*a*).

3. Centrifugation

Plankton can be concentrated via batch or continuous centrifugation. Centrifuge batch samples at 1000 g for 20 min. The Foerst continuous centrifuge is no longer recommended as a quantitative device, but existing programs may continue using it to ensure continuity with previously collected data. Although centrifugation accelerates sedimentation, it often damages fragile organisms, and is not preferable for quantitative analysis.

4. Sand Filter/Backwash

This method uses sand to filter out phytoplankters and then uses a backwash step to remove algae from the filter media. Although this method destroys many species, especially fragile or large forms, and yields differential recovery of species, it is still in use in some water utilities with limited laboratory resources. It is the least preferable concentration method.

5. Zooplankton Concentration

Zooplankton samples often need to be concentrated in the field, especially when large water bottles or pump sampling methods are used. Moreover, samples obtained via nets or other methods sometimes need to be further concentrated for storage or preparation for examination. When only small volume reductions are needed, pour sample back into the bucket of traps or nets. When processing large volumes of water (as in pump sampling), use larger plankton buckets or funnels with more water-volume retention and filtration surface area. Construct a filter funnel similar to that shown in Figure 10200:5 of clear acrylic plastic or other suitable material.[8] The apparatus' volume and mesh size depend on the volume of water to be filtered and the size of organisms to be retained. The filter funnel's mesh size normally is the same as that of the net or other field sampling device.

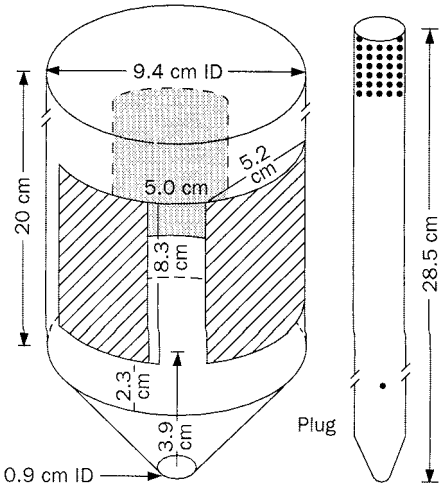


Figure 10200:5. Filter funnel for concentrating zooplankton. This device, originally designed for rotifers, can be modified for other zooplankters by changing the dimensions and mesh size. (After Likens and Gilbert.[8]).

6. References

1. Furet, J.E. & K. Benson-Evans. 1982. An evaluation of the time required to obtain sedimentation of fixed algal particles prior to enumeration. *Brit. Phycol. J.* 17:253.
2. McNabb, C.D. 1960. Enumeration of freshwater phytoplankton concentrated on the membrane filter. *Limnol. Oceanogr.* 5:57.
3. Hewes, C.D. & O. Holm-Hansen. 1983. A method for recovering nanoplankton from filters for identification with the microscope: The filter-transfer-freeze (FTF) technique. *Limnol. Oceanogr.* 28:389.
4. Hewes, C.D., F.M.H. Reid & O. Holm-Hansen. 1984. The quantitative analysis of nanoplankton: A study of methods. *J. Plankton Res.* 6:601.
5. MacIsaac, E.A. & J.G. Stockner. 1993. Enumeration of phototrophic picoplankton by autofluorescence microscopy. *In* P.F. Kemp, B.F. Sherr, E.B. Sherr & J.J. Cole, eds. Handbook of Methods in Aquatic Microbial Ecology. Lewis Publ., Boca Raton, Fla.
6. Caron, D.A. 1983. Technique for enumeration of heterotrophic and phototrophic nanoplankton using epifluorescence microscopy and comparison with other procedures. *Appl. Environ. Microbiol.* 46: 491.
7. Marshall, H.G. 2002. Autotrophic picoplankton: Their presence and significance in marine and freshwater ecosystems. *Virginia J. Sci.* 53:13.
8. Likens, G.E. & J.J. Gilbert. 1970. Notes on quantitative sampling of natural populations of planktonic rotifers. *Limnol. Oceanogr.* 15:816.

10200 D. Preparing Slide Mounts

Counting methods may use temporary counting cells, semi-permanent slide mounts, or permanent slide mounts. Chamber or mount choice for phytoplankton and zooplankton will depend on available resources, analysis time line, organism size range, and microscope specifications. A method is also included for acid cleaning and mounting diatoms for species identifications and valve counts. Generally, use multiple chambers or mounts (often three per sample), regardless of processing procedure, to help account for subsample variability.[1]

1. Phytoplankton Temporary and Semi-Permanent Wet Slide Mounts

Agitate settled sample concentrate long enough to ensure thorough mixing (50 to 100 times), and withdraw a subsample with an accurately calibrated pipet. Clean pipet thoroughly between samples. To prepare wet slide mounts, transfer 0.1 mL to a glass slide, place a cover slip over the sample, and ring the cover slip with an adhesive (e.g., clear nail polish) to prevent evaporation. For procedures on preparing temporary counting chambers (Sedgwick-Rafter, Palmer-Maloney, nanoplankton and hemacytometer chambers/cells), see 10200F. For semi-permanent mounts, add a few drops of glycerin to the slide. As the sample ages, the water evaporates, leaving the organisms embedded in the glycerin. If the cover slip is ringed with adhesive, the slide can be retained for a few years if stored in the dark.

2. Phytoplankton Permanent Slide Mounts

a. Membrane filter mounts, oil-cleared: Suggested membrane filters include mixed ester/cellulose ester filters.* Method does not work well with non-organic filters (glass fiber filters or polycarbonate filters). Pre-concentration is not required with this method, but a test mount should be completed first to ensure that algal density on the filter is 10 to 20 natural units/field at the counting magnification. If sample volume is less than 0.5 mL, suspend in 10 mL filtered distilled water before mounting to encourage a random distribution on the filter. Place two drops of immersion oil on a labeled slide. Agitate the sample long enough to ensure thorough mixing (50 to 100 times), and withdraw a subsample with an accurately calibrated pipet. Clean pipet thoroughly between samples.

Immediately after filtering (see 10200C.2), place filter on top of oil with a pair of forceps and add two drops of oil to top of filter. The oil impregnates the filter, making it transparent. Impregnation typically occurs in 24 to 48 h but can be completed in 1 to 2 h by applying heat (70°C). Once the filter has cleared, place a few more drops of oil on it and cover with a cover slip. The mounted filter is now ready for microscopic examination.

Oil-cleared filters will cloud after several months. Alternatively, mount membrane filters in mounting medium.† Immerse filters in 1-propanol to displace residual water and transfer to xylol for several minutes to clear filters. Place a section of filter or entire filter on a microscope slide with the mounting medium, cover with a cover glass, and dry at low temperature.[1]

b. Membrane filter mounts, permanent HPMA: Suggested membrane filters are mixed cellulose ester filters (see *a* above). Method does not work with non-organic filters (glass fiber filters or polycarbonate filters). The hexamethylphosphoramide (HPMA) method for producing algal sample slides provides an optically clear background while permanently infiltrating and preserving the sample for archival purposes.[2,3] Mounting distortion is minimal, and magnifications of 100 to 1000× can be used on the same specimen. If samples are preserved in glutaraldehyde (final concentration of 0.25 to 0.50%), epifluorescence can be used on the sample while counting.

Agitate sample long enough to ensure thorough mixing (50 to 100 times), and withdraw a subsample with an accurately calibrated pipet. Filter subsample as described in 10200C.2.

Immediately after filtering, carefully place the filter face down on a 25-mm (#1) coverslip with a pair of forceps and add 2 to 3 drops of pre-polymerized HPMA‡ on the back of the filter. The resin clears the filter and impregnates algal cells. Place filters with HPMA in a drying oven at 60°C for 24 hours. Once filter has cleared and polymerized, place a few more drops of HPMA on it and adhere it to a 25 mm × 75 mm slide. Place back in the drying oven for 24 to 72 hours. The preparation is permanent when the coverslip's outer rim is completely polymerized. The internal part of the preparation may be liquid, but as long as the outer rim is solid, the slide is permanent (HPMA only polymerizes in the presence of oxygen). The mounted filter can be stored indefinitely at room temperature and is now ready for microscopic examination.

c. *Sedimented slide mounts:* There is a technique available for making permanent resin mounts of natural phytoplankton deposited via sedimentation on a microscope slide or cover glass and dehydrated via ethanol vapor substitution.[4,5] This method takes several days to complete; follow settling-duration guidelines and do not exceed a 5:1 height-to-depth ratio for the settling tower (see 10200C.1).

3. Diatom Mounts

Samples concentrated for diatom analysis via settling or centrifugation may contain dissolved materials (e.g., marine salts, preservative, and detergents) that will leave interfering residues. Wash well with distilled water before preparing slide(s). Transfer several drops of washed concentrate via a large-bore disposable pipet or large-bore dropper to a cover glass on a hot plate warmed enough to increase the evaporation rate but not cause boiling (use a large-bore pipet or dropper to prevent accidental exclusion of larger forms or those forming colonies or chains). If the uncleaned material is very concentrated, improve diatom distribution by adding the drops to a cover glass already flooded with distilled water. Redistribute on cover slip, if necessary (using a pipet), to produce a homogenous distribution of frustules. Evaporate to dryness. Repeat addition and evaporation

* Millipore HA, Pall GN, or equivalent.
† Permount, Fisher Scientific Co., Millipore HA, Pall GN, or equivalent.
‡ HPMA, SPI Supplies Division of Structure Probe, Inc., P.O. Box 656, West Chester, PA 19381-0656.

until enough sample has been transferred to the cover glass without producing a residue so dense that organisms cannot be recognized. If in doubt about the density, examine under a compound microscope. After evaporation, incinerate the residue on the cover glass on a 300 to 500°C hot plate; alternatively, use a muffle furnace. This usually requires 20 to 45 min. Mount as described below.

Clean samples chemically for diatom analysis as described elsewhere.[6–8] Mix equal volumes of concentrated nitric acid (HNO_3), sulfuric acid (H_2SO_4), or 50% H_2O_2 and sample. **CAUTION: When working with concentrated acids or caustics, wear safety goggles and an acid-resistant apron and gloves, and work in a chemical fume hood. This reaction will be highly exothermic!** Add a few grains of potassium dichromate ($K_2Cr_2O_7$)[5] to facilitate digestion of the filter and cellular organic matter. Add more dichromate if solution color changes from yellow to green. Place sample on a hot plate and boil down to about one-third the original volume. This process destroys organic matter, leaving only diatom shells (frustules). Alternatively, dichromate may be omitted (or not), and the treated sample may be left to stand overnight. Omitting the boiling and dichromate probably will leave cells intact, which is helpful when identifying heterovalver or heteropolar diatoms. Cool, wash with distilled water, and mount as described below. Transfer cleaned frustules to a cover glass and dry as described below.

Place a drop of mounting medium in the center of a labeled slide. Use 25- × 75-mm slides with frosted ends. Using a suitable high-refractive-index (1.6+) microscopic mounting medium§ ensures permanent, easily handled mounts for examination under oil immersion. Heat slide to near 90°C for 1 to 2 min before applying the heated cover slip with its sample residue to hasten evaporation of solvent in the mounting medium. Remove slide to a cool surface and, during cooling (5 to 10 s), apply firm but gentle pressure to cover glass with a broad, flat instrument. To prevent resin crystallization, ring cover slip with clear fingernail polish.

4. Zooplankton Mounts

For zooplankton analyses, withdraw a 1- to 5-mL subsample from the concentrate and dilute or concentrate further as necessary. Transfer sample to a counting cell or chamber (see 10200G) for analysis as a wet mount. Use polyvinyl lactyl phenol‖ to prepare semi-permanent zooplankton mounts. The mounts are good for about a year, and then the clearing agent causes organisms to deteriorate. For long-term storage, ring cover slip with clear lacquer (fingernail polish) to retard mountant crystallization. For permanent mounting, other mountants are available, and some stains not only highlight features but also partially clear the animals (e.g., Lignin Pink Double stain, Bio-Quip).#

For the protozoan portion of the microzooplankton, a protargol staining procedure[9] not only provides a permanent mount but also reveals the cytological details often necessary for identification.

This procedure is qualitative and especially important in taxonomic studies of ciliated protozoa.

5. References

1. VOLLENWEIDER, R.A. 1969. A Manual on Methods for Measuring Primary Production in Aquatic Environments, IBP Handbook #12. Blackwell Scientific, Oxford, U.K.
2. MILLIPORE FILTER CORPORATION. 1966. Biological examination of water, sludge and bottom materials. Millipore Techniques, Water Microbiology, p. 25.
3. CRUMPTON, W.G. 1987. A simple and reliable method for making permanent mounts of phytoplankton for light and fluorescence microscopy. *Limnol. Oceanogr.* 32:1154.
4. ST. AMAND, A. & S.R. CARPENTER. 1993. Plankton vertical structure. *In* S.R. Carpenter & J.F. Kitchell, eds. The Trophic Cascade in Lakes. Cambridge University Press, Cambridge, U.K.
5. SANFORD, G.R., A. SANDS & C.R. GOLDMAN. 1962. A settle-freeze method for concentrating phytoplankton in quantitative studies. *Limnol. Oceanogr*. 14:790.
6. CRUMPTON, W.G. & R.G. WETZEL. 1981. A method for preparing permanent mounts of phytoplankton for critical microscopy and cell counting. *Limnol. Oceanogr.* 26:976.
7. PATRICK, R. & C.W. REIMER. 1966. The Diatoms of the United States, Vol. 1, Monogr. 13. Philadelphia Acad. Natur. Sci., Philadelphia, Pa.
8. BARBOUR, M.T., J. GERRITSEN, B.D. SNYDER & J.B. STRIBLING. 1999. Rapid Bioassessment Protocols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish, 2nd ed., EPA 841-B-99-002. Off. Water, U.S. Environmental Protection Agency, Washington, D.C.
9. HOHN, M.H. & J. HELLERMAN. 1963. The taxonomy and structure of diatom populations for three eastern North American rivers using three sampling methods. *Trans. Amer. Microsc. Soc.* 62:250.
10. SMALL, E.B. & D.H. LYNN. 1985. Phylum Ciliophora Doflein, 1901. *In* J.J. Lee, S.H. Hunter & E.C. Bovee, eds. An Illustrated Guide to the Protozoa. Soc. Protozoology, Lawrence, Kansas.

§ Naphrax, Brunel Microscopes, Unit 2, Vincients Road, Bumpers Farm Industrial Estate, Chippenham, Wiltshire SN14 6NQ U.K., or equivalent.
‖ Biomedical Specialists, Box 1687, Santa Monica, CA.

CMC-10, Master's Chemical Co., P.O. Box 2382, Des Plaines, IL.; Hydramount, Biomedical Specialists, Box 1687, Santa Monica, CA.; or equivalent.

10200 E. Microscopes and Calibrations

1. Compound Microscope

Use either a standard or an inverted compound microscope to identify and enumerate algae. Equip either with a mechanical stage that can move all parts of a counting cell past the objective lens. Standard equipment is a set of 10, 12.5, or 15× oculars and 10, 20, 40, and 100× objectives. Use objectives to provide adequate working distance for the counting chamber. Magnification requirements vary based on the plankton fraction being investigated, the type of microscope, counting chamber used,

and optics. With standard objectives, the Sedgwick-Rafter chamber limits magnification to about 200× and the Palmer-Maloney cell limits magnification to about 500×. The *useful upper limit of magnification* for any objective is 1000 times the numerical aperture (NA). Above this magnification, no greater detail can be resolved. Use combinations of oculars, intermediate magnifiers, and objectives to obtain the greatest magnification without exceeding the useful limit of magnification. Generally, the upper magnification limit of the standard or inverted compound microscope is 1250×. When the limit is exceeded, empty magnification results. *Empty magnification* is when the image is larger but not any clearer. Optics that enhance contrast [e.g., phase contrast, differential interference contrast (Nomarski), or interference reflection contrast (IRC)[1]] are useful and may be essential to identify phytoplankton accurately.

2. Stereoscopic Microscope

A *stereoscopic microscope* is essentially two complete microscopes assembled into a binocular instrument to give a stereoscopic view and an erect rather than an inverted image. Use this microscope to study and count large plankters (e.g., mature microcrustacea). Combine 10 to 15× paired oculars with 1 to 8× objectives; this bridges the gap between the hand lens and compound microscope, providing magnification from 10 to 120×. Alternatively, use a good zoom-type instrument with comparable magnification.

3. Inverted Compound Microscope

Many laboratories routinely use an inverted compound microscope for plankton counting.[2–5] In this instrument, the objectives are below a movable stage and the illumination comes from above, so analysts can view organisms that have settled to the bottom of a chamber. Place samples in a cylindrical settling chamber with a thin, clear glass bottom. Chambers of various capacities are available; the appropriate size depends on organism density. After a suitable settling period (see 10200C.1), count organisms in the settling chamber.

The major advantage of the inverted microscope is that by simply rotating the nosepiece, a specimen can be examined (or counted) directly in the settling chamber at any desired magnification. When used with an oil that is viscous enough not to run down the objective, oil-immersion objectives are useful and have excellent resolution. No preparation or manipulation other than settling is required. Generally, examine a preserved sample when performing counts. Techniques are available for samples with an abundance of floating organisms.[6]

4. Epifluorescence Microscope

An epifluorescence microscope may be either standard or inverted. It uses incident light to excite electrons in intracellular compounds (e.g., pigments or absorbed stains), and the energy emitted during electron return-to-the-ground state is measured as fluorescent light. The technique has been applied to microscopic identification of chlorophyll *a*, phycoerythrin, phycocyanin-containing cells (autotrophs), and nonpigmented heterotrophic plankton. Fluorescent stains (e.g., primulin or proflavin) also have been used to differentiate nanoplanktonic primary and secondary producers.[7–9] Excitation and emission wavelengths are unique for each pigment and stain, and require distinct light filter combinations and light sources. Select the filter combinations appropriate for the particular application. Concentrate samples via membrane filtration (see 10200C and 10200D, depending on application).

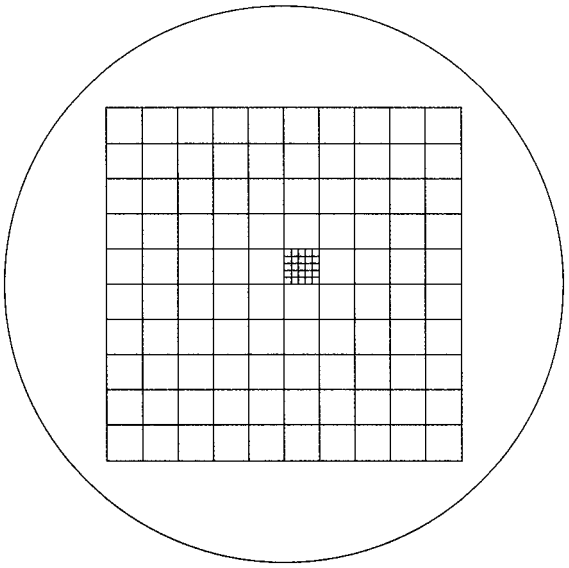


Figure 10200:6. Ocular micrometer ruling. A Whipple micrometer reticule is illustrated.

Epifluorescence microscopy is particularly useful for enumerating the picoplankton and heterotrophic flagellate populations common to most aquatic systems, or differentiating morphologically similar algal divisions—especially in high particulate samples—because differential pigment composition creates different fluorescent patterns. Use epifluorescence microscopy as a complementary procedure to standard light microscope counting techniques.

5. Microscope Calibration

Microscope calibration is essential. The usual calibration equipment is an ocular micrometer (Whipple grid, reticle, or reticule) placed in the microscope's eyepiece and a stage micrometer with a standardized, accurately ruled scale on a glass slide. There are several designs available for both phytoplankton and zooplankton. The Whipple disk (Figure 10200:6) has an accurately ruled grid subdivided into 100 squares. One square near the center is subdivided further into 25 smaller squares. The grid's outer dimensions are such that, with a 10× objective and a 10× ocular, it delimits an area of about 1 mm^2 on the microscope stage. Because this area may differ from one microscope to another, carefully calibrate the ocular micrometer for each microscope.

With the ocular and stage micrometers parallel and in part superimposed, match the line at the left edge of the Whipple grid with the zero mark on the stage micrometer scale (Figure 10200:7). Determine the width of the Whipple grid image to the nearest 0.01 mm from the stage micrometer scale. If the width is exactly 1 mm (1000 μm), the larger squares will be 1/10 mm (100 μm)

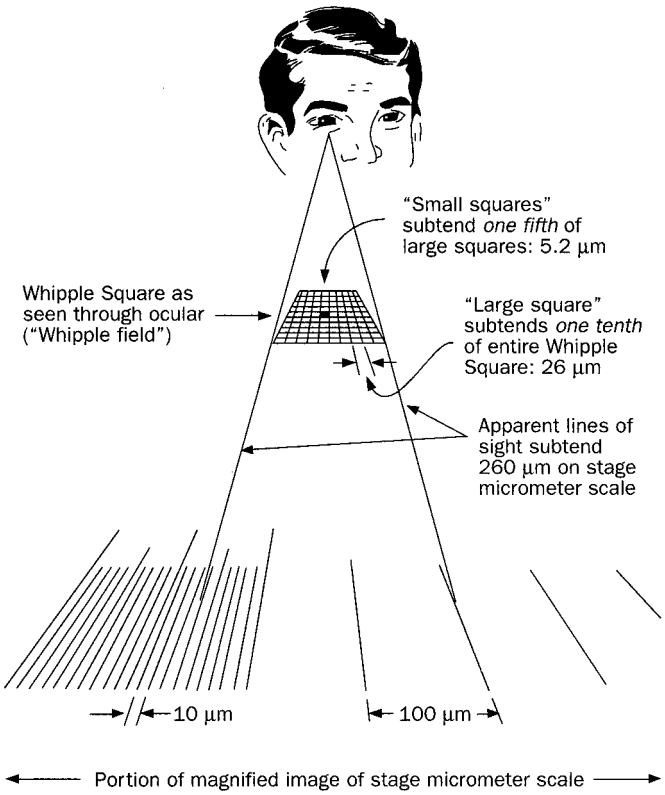


Figure 10200:7. Calibration of Whipple Square, as seen with 10× ocular and 43× objective (approximately 430× total magnification).

on a side and each of the smaller squares will be 1/50 mm (20 μm).

When the microscope is calibrated at higher magnifications, the entire scale on the stage micrometer will not be seen; make measurements to the nearest 0.001 mm. Additional calibration details are available.[10]

6. References

1. SIVER, P.A. & J. HINSCH. 2001. The use of interference reflection contrast in the examination of diatom valves. *J. Phycol.* 36(3):616.
2. WETZEL, R.G. & G.E. LIKENS. 1991. Limnological Analyses, 2nd ed. Springer-Verlag, New York, N.Y.
3. LUND, J.W.G., C. KIPLING & E.D. LECREN. 1958. The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. *Hydrobiologia* 11:143.
4. SICKO-GOAD, L. & E.F. STOERMER. 1984. The need for uniform terminology concerning phytoplankton cell size fractions and examples of picoplankton from the Laurentian Great Lakes. *J. Great Lakes Res*. 10:90.
5. HASLE, G. 1978. The inverted microscope method. *In* A. Sournia, ed. Phytoplankton Manual, Monograph. Oceanogr. Methods No. 6. United Nations Educational, Scientific & Cultural Org., Paris.
6. REYNOLDS, C.S. & G.H.M. JAWORSKI. 1978. Enumeration of natural *Microcystis* populations. *Brit. Phycol. J.* 13:269.
7. DAVIS, P.G. & J. MCN. SIEBURTH. 1982. Differentiation of phototrophic and heterotrophic nanoplankton populations in marine waters by epifluorescence microscopy. *Ann. Inst. Oceanogr.* 58:249.
8. CARON, D.A. 1983. Techniques for enumeration of heterotrophic and phototrophic nanoplankton, using epifluorescence microscopy, and comparison with other procedures. *Appl. Environ. Microbiol*. 46:491.
9. SHERR, E.B. & B.F. SHERR. 1983. Double-staining epifluorescence techniques to assess frequency of dividing cells and bacteriovory in natural populations of heterotrophic microprotozoa. *Appl. Environ. Microbiol.* 46:1388.
10. JACKSON, H.W. & L.G. WILLIAMS. 1962. Calibration and use of certain plankton counting equipment. *Trans. Amer. Microsc. Soc.* 81:96.

10200 F. Phytoplankton Counting Techniques

1. Counting Units

Some phytoplankton are unicellular, while others are multicellular (colonial or filamentous). Listed below are suggestions for reporting concentration or density:

Enumeration Method	Counting Unit	Reporting Unit
Total cell count	One cell	Cells/mL
Natural unit count (clump count)	One organism (any unicellular organism, natural colony, or filament)	Natural Units/mL
Areal standard unit count*	400 μm^2	Units/mL

* Areal standard unit = area of four small squares in a Whipple grid at a magnification of 200×, is microscope-specific, and is the least preferred reporting unit.

The variety of configurations poses a problem in enumeration. For example, should a four-celled colony of *Scenedesmus* (see Section 10900, Plates 32, 34) be reported as one colony or four individual cells? Generally, both cells and natural units should be enumerated. A *natural unit* is the unit that appears in the environment and that aquatic organisms encounter. Making a total cell count can be time-consuming and tedious, especially when colonies consist of thousands of individual cells; however, cells per colony/filament can be estimated closely, if done carefully. The natural unit or clump is the most easily used system; however, it is not necessarily the most accurate quantitatively because handling and preserving samples may dislodge cells from the colony periphery (especially in *Microcystis* and other cyanobacteria with dilute sheaths; this can be a large problem in Lugol's preserved samples). The cell/natural unit method also does not reflect

the abundance of biomass or biovolume without additional measurement and calculation (see 10200I).

For most applications, biomass data are preferred, and cell dimensions and abundance are needed to convert from cell counts to biomass, assuming a specific gravity of 1.0. Measure enough cells (generally 10 to 30) to get a reliable average or range (corresponding to size categories applied to each species). If the focus is on biologically meaningful units (e.g., particle sizes), then natural units with an average or range of size dimensions are most appropriate. The most useful counts will provide natural units, average natural unit size [greatest axial linear dimension (GALD)], average cells per unit, and average size per cell, allowing a series of calculations pertinent to various applications of the data.

Never mix and match units among different taxa within a count. For example, do not report *Merismopedia* as "units" of 4 cells, *Aphanizomenon* as filaments, and *Microcystis* as cells. This will make data interpretation difficult and comparisons of long-term data sets impossible because idiosyncrasies among counters over the years tend to get lost. Whatever method is chosen, identify it clearly in reporting results and understand the implications for data analysis.[1]

If the distribution of organisms is random and the population fits a Poisson distribution, the counting error may be estimated.[2] For example, the approximate 95% confidence limit, as a percentage of the number of natural units counted (N), equals:

$$\frac{2}{\sqrt{N}}(100\%)$$

So if 100 units are counted, the 95% confidence limit approximates $\pm 20\%$. For a count of 400 units, the limit is about 10%. Natural units, not cells, are used because natural units are statically encountered during counting, not cells. Most counts are conducted to 300 to 400 natural units, spread among multiple slides or counting chambers.

2. Counting Procedures

To enumerate plankton, use a counting cell or chamber that limits the volume and area for ready calculation of population densities. Counting live material with motile taxa is not recommended because they often will avoid heat and light (or be attracted to light, depending on the species), and move in and out of the field of view.

When counting with a Whipple grid, establish a convention for tallying organisms lying on an outer boundary line. For example, when counting a "field" (entire Whipple square), designate the top and left boundaries as "no-count" sides, and the bottom and right boundaries as "count" sides. Thus, tally every plankter touching a "count" side from the inside or outside but ignore any touching a "no-count" side. If significant numbers of filamentous or other large forms cross two or more boundaries of the grid, count them separately at a lower magnification and include their number in the total count.

To identify organisms, use standard bench references (see 10200B.1 and Section 10900) and check current literature. New taxonomic resources are constantly being published.

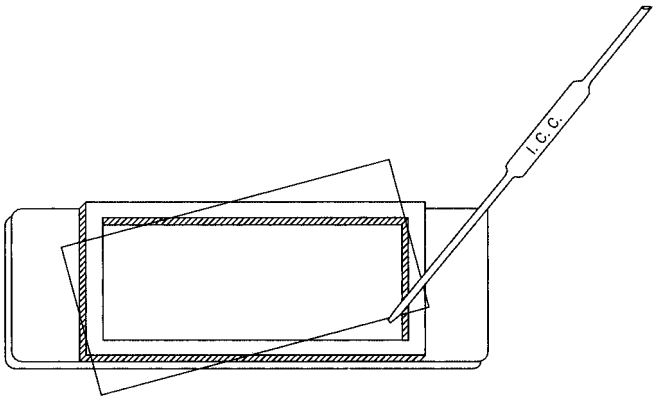


Figure 10200:8. Counting cell (Sedgwick-Rafter), showing method of filling. SOURCE: WHIPPLE, G.C., G.M. FAIR & M.C. WHIPPLE. 1927. The Microscopy of Drinking Water. John Wiley & Sons, New York, N.Y

For aquatic habitats subject to ongoing monitoring, it is often helpful to develop a habitat-specific pictorial key or voucher collection from accumulated data and images. Do not count dead cells or broken diatom frustules. Tally empty centric and pennate diatoms separately as "dead centric diatoms" or "dead pennate diatoms" for use in converting the diatom species proportional count to a count per milliliter, if that methodology applies to your study. Determining which cells were dead or live at collection time is often subjective; results depend on such variables as preservative used, age of collections, and purpose of the study. This becomes especially difficult with many of the tiny diatoms. Generally, if the cell wall and at least one plastid are intact, the natural unit is counted as live.

Magnification is important in phytoplankton identification and enumeration. Although magnifications of 100 to 200× are useful for counting large organisms or colonies, much higher magnifications often are required. It is useful to categorize phytoplankton-counting techniques according to magnification because this will affect density calculations.

a. Low-magnification (up to 200×) methods: A Sedgwick-Rafter (S-R) cell is commonly used for counting plankton because it is easily manipulated and provides reasonably reproducible data when used with a calibrated microscope equipped with an eyepiece measuring device (e.g., the Whipple grid). The S-R cell is about 50 mm long × 20 mm wide × 1 mm deep. Its total bottom area is about 1000 mm^2 and total volume is about 1000 mm^3 (1 mL). Carefully check the exact length and depth of the cell with a micrometer and calipers before use.

The cell's greatest disadvantage is that high-magnification objectives cannot be used. As a result, the S-R cell is not appropriate for examining nanoplankton.

1) Filling the cell—First, place the cover glass diagonally across the cell and transfer sample with a large-bore pipet (Figure 10200:8). Placing the cover slip diagonally across the cell helps prevent air bubbles from forming in cell corners. It often will rotate slowly and cover the inner portion of the S-R cell during filling. Do not overfill because a sample depth greater than 1 mm would produce an invalid count. During lengthy examinations, do not permit large air spaces (caused by evaporation) to develop in the chamber. To prevent such air spaces

from forming, occasionally place a small drop of distilled water on edge of cover glass.

Before counting, let the S-R cell stand for at least 15 min to settle plankton. Count plankton on the bottom of the S-R cell. Some phytoplankton—notably some blue-green algae or motile flagellates in unpreserved samples—may not settle but rise to the underside of the cover slip. When this occurs, count these organisms separately by refocusing, and add to total of those counted on the cell bottom to derive total number of organisms. Count algae in strips or fields.

2) Strip counting—In this context, a *strip* the length of the S-R cell is a volume about 50 mm long, 1 mm deep, and the width of the total Whipple grid.

The number of strips to be counted is a function of the precision desired and the number of units (cells, colonies, or filaments) per strip. Derive number of plankton in the S-R cell as follows:

$$\text{No./mL} = \frac{C \times 1000\ \text{mm}^3}{L \times D \times W \times S}$$

where:

C = number of organisms counted,
L = length of each strip (S-R cell length), mm,
D = depth of a strip (S-R cell depth), mm,
W = width of a strip (Whipple grid image width), mm, and
S = number of strips counted.

Multiply or divide number of cells per milliliter by a correction factor to adjust for sample dilution or concentration. Ultimately, the factor by which counted cells are multiplied should not be much higher than 25 for reliable results.

3) Field counting—On samples containing many plankton (10 or more plankters per field), make field counts rather than strip counts. Count plankters in random fields, each consisting of one Whipple grid. The number of fields counted will depend on plankton density and statistical accuracy desired (see 10200F.1). Calculate the number of plankton per milliliter as follows:

$$\text{No./mL} = \frac{C \times 1000\ \text{mm}^3}{A \times D \times F}$$

where:

C = number of organisms counted,
A = area of a field (Whipple grid image area), mm^2,
D = depth of a field (S-R cell depth), mm, and
F = number of fields counted.

Multiply or divide the number of cells per milliliter by a correction factor to adjust for sample dilution or concentration. Again, for reliable results, the factor by which counted cells are multiplied should not be much higher than 25.

b. Intermediate magnification (low to 500×) methods: The Palmer-Maloney (P-M) nanoplankton cell[3] is designed specifically for nanoplankton enumeration. It has a circular chamber that is 17.9-mm diam and 0.4-mm deep, and holds 0.1 mL. The shallow depth permits use of 40 to 45× objectives with sufficient working distance. The principal disadvantage of the P-M cell is that these magnifications (400 to 450×) often are insufficient for nanoplankton identification and enumeration.

Because a relatively small sample portion is examined in the P-M cell, do not use it unless the sample contains a dense population (10 or more plankters per field). Such a small sample portion from a less dense population leads to serious underestimation of density. This can be overcome by counting more area over more chambers; again, use the guide of a maximum multiplication factor of 25.

With the cover slip in place, pipet sample into one of the 2- × 5-mm channels on the side of the chamber. After a 10-min settling period, count the plankters in random fields (the number of fields depend on plankton density and variety, and statistical accuracy desired). Strips may be counted in this or any other circular cell by measuring the effective diameter and counting two perpendicular strips that cross at the center.

Calculate the number per milliliter as follows:

$$\text{No./mL} = \frac{C \times 1000\ \text{mm}^3}{A \times D \times F}$$

where:

C = number of organisms counted,
A = area of a field (Whipple grid image), mm^2,
D = depth of a field (P-M cell depth), mm, and
F = number of fields counted.

Multiply or divide the number of cells per milliliter by a correction factor to adjust for sample dilution or concentration.

Other, similar chambers are now available that function much like P-M chambers. For example, the standard medical hemacytometer (used to enumerate blood cells) has a ruled grid machined into a counting plate and is fitted with a ground-glass cover slip. The grid is divided into 1-mm^2 divisions, and the chamber is 0.1 mm deep. Pipet sample into chamber and view under 450× magnification. Count all cells within the grid. The chamber manufacturer provides a detailed instruction sheet on calculations and proper usage. One disadvantage of the hemacytometer is that the sample must have a very high plankton density to yield statistically reliable data, or else analysts must view much more area over multiple chambers.

c. High-magnification methods: Examining phytoplankton at high magnification requires the use of oil-immersion objectives. Suitable procedures include inverted microscope chambers, membrane filter mounts, sedimented slide mounts, the Lackey drop method, and diatom mounts.

1) Inverted microscope counts—Prepare a sample for examination by filling the settling chamber. After the desired settling time (see 10200C.1), transfer the chamber to the microscope stage. Count perpendicular strips across the center of the bottom cover glass. Strip counts may be made via a Whipple grid or special counting oculars with a pair of adjustable parallel hairs and one cross hair. Determine the width of the strip with a stage micrometer, and tally organisms as they pass the cross hair, which functions as a reference point. Hold strip width constant for any series of samples. Alternatively, examine random nonoverlapping fields until at least 100 units of the dominant species are counted. To be most accurate, particularly because algae distribution may be non-uniform, count the entire chamber floor.

Alternatively, make a random field-minimum count to attain a precision of at least 85%.[4] This can be overcome by counting more area over more chambers; again, use the guide of a maximum multiplication factor of 25.

$$\text{Strip count (No./mL)} = \frac{C \times A_t}{L \times W \times S \times V}$$

where:

C = number of organisms counted,
A_t = total area of bottom of settling chamber, mm^2,
L = length of a strip, mm,
W = width of a strip (Whipple-grid image width), mm,
S = number of strips counted, and
V = volume of sample settled, mL.

$$\text{Field count (No./mL)} = \frac{C \times A_t}{A_f \times F \times V}$$

where:

A_f = area of a field (Whipple grid image area), mm^2,
F = number of fields counted, and
other terms are as defined above.

2) Membrane filter mounts—Concentrate sample as directed in 10200C.2 or prepare membrane filter as directed in 10200D.2*a* and 2*b*.

Examine samples, which are concentrated on unlined membrane filters and mounted in oil or HPMA, as described above. Count enough random fields to ensure desired level of statistical accuracy (see 10200F.1). Select magnification level and size of microscope field (quadrat) so the most abundant species appear in at least 70% but not more than 90% of microscopic fields examined (80% is optimum). Adjust microscope field size by using entire field of view, or part or all of the Whipple grid/ocular micrometer. Examine at least 30 random microscope fields and record number of fields in which each species occurred, depending on density. Report results as organisms per milliliter, calculated as follows:

$$\text{(No./mL)} = \frac{N \times Q}{V \times D}$$

where:

N = density (organisms/field) from Table 10200:II,
Q = number of fields per filter,
V = milliliters filtered, and
D = dilution factor (0.96 for 4% formalin preservative) (Dilution factor is not necessary for preservative at a concentration ≤2%).

3) Sedimented slide mounts—Examine mounts that were prepared as directed in 10200D.2*c*.

4) Lackey drop method—The Lackey drop (microtransect) method[5] is a simple method of obtaining counts of considerable accuracy with samples containing a dense plankton population. It is similar to the S-R strip count.

Table 10200:II. Conversion Table for Membrane Filter Technique (Based on 30 Scored Fields)

Total Occurrence	F* %	N†
1	3.3	0.03
2	6.7	0.07
3	10.0	0.10
4	13.3	0.14
5	16.7	0.18
6	20.0	0.22
7	23.3	0.26
8	26.7	0.31
9	30.0	0.35
10	33.3	0.40
11	36.7	0.45
12	40.0	0.51
13	43.3	0.57
14	46.7	0.63
15	50.0	0.69
16	53.3	0.76
17	56.7	0.83
18	60.0	0.91
19	63.3	1.00
20	66.7	1.10
21	70.0	1.20
22	73.3	1.32
23	76.7	1.47
24	80.0	1.61
25	83.3	1.79
26	86.7	2.02
27	90.0	2.30
28	93.3	2.71
29	96.7	3.42
30	100.0	?

* $F = \dfrac{\text{total number of species occurrences} \times 100}{\text{Total number of fields examined}}$
† N = number of organisms per field.

Prepare slides as directed in 10200D.1. Oil immersion objectives can be used with the semipermanent slides. Count organisms in enough strips to ensure desired level of statistical accuracy (see 10200F.1). Calculate number of organisms per milliliter as follows:

$$\text{(No./mL)} = \frac{C \times A_t}{A_s \times S \times V}$$

where:

C = number of organisms counted,
A_t = area of cover slip, mm^2,
A_s = area of one strip, mm^2,
S = number of strips counted, and
V = volume of sample under the cover slip, mL.

5) Diatom mounts—Prepare samples as directed in 10200D.3.

For diatom species proportional count, examine diatom samples under oil immersion at a magnification of at least 900×. Scan lateral strips the width of the Whipple grid/field until at least 500 valves are counted (2 valves per cell). Available time

and required accuracy dictate the number of valves to be counted. Determine percentage abundance of each species from tallied counts, and calculate counts per milliliter of each species by multiplying percent abundance by total live and dead diatom count obtained from the plankton counting chamber. For more accuracy (if clearly indicated by material and study objectives), distinguish between living and dead diatoms at the species level.

6) Phytoplankton staining technique—Staining algae enables analysts to differentiate between "live" and "dead" diatoms[6] so total phytoplankton can be enumerated in a single sample without sacrificing detailed diatom taxonomy. It also results in permanent reference slides. This procedure is most useful when diatoms are major components of phytoplankton and distinguishing between living and dead diatoms is important. Other stains can be used to highlight features that facilitate separation of major taxonomic groups. Alternatively, if the sample was preserved in glutaraldehyde, epifluorescence can be used.

For diatom work, preferably preserve samples in Lugol's solution, glutaraldehyde, or formalin (see 10200B.2*a*). For analysis, thoroughly mix the sample and filter a portion through a 47- or 25-mm-diam membrane filter (pore diam 0.45 or 0.65 μm). Use a vacuum of 16 to 20 kPa (<25 mm Hg), and never let sample dry. Add 2 to 5 mL aqueous acid fuchsin solution to the filter and let stand for 20 min. (Create aqueous acid fuchsin solution by dissolving 1 g acid fuchsin in 100 mL distilled water to which 2 mL glacial acetic acid has been added.) After staining, filter sample, wash briefly with distilled water, and filter again. Administer successive rinses of 50, 90, and 100% propanol to the sample while filtering. Soak for 2 min in a second 100% propanol wash, filter, and add xylene. At least two washes are required; let the final one soak 10 min before filtering. Trim the xylene-soaked filter and place on a microscope slide on which there are several drops of mounting medium.* Apply several more drops of medium to top of filter and install a cover glass. Carefully squeeze out excess mounting medium. Make the final mount permanent by lacquering the edges of the cover glass.

Count organisms using the most appropriate magnification. "Live" diatoms typically are red while "dead" ones are unstained. Oil immersion is necessary when identifying diatoms to species and many other algae. Count either strips or random fields, and calculate plankton densities per milliliter:

$$\text{No./mL} = \frac{C \times A_t}{A_c \times V}$$

where:

C = number of organisms counted,
A_t = total area of effective filter before trimming and mounting,
A_c = area counted (strips or fields), and
V = volume of sample filtered, mL.

* Permount, Fisher Scientific Co., or equivalent.

3. References

1. Ingram, W.M. & C.M. Palmer. 1952. Simplified procedures for collecting, examining, and recording plankton in water. *J. Amer. Water Works Assoc.* 44:617.
2. Venrick, E.L. 1978. How many cells to count. *In* A. Sournia, ed. Phytoplankton Manual, Monograph. Oceanogr. Methods No. 6, United Nations Educational, Scientific & Cultural Org., Paris.
3. Palmer, C.M. & T.E. Maloney. 1954. A New Counting Slide for Nanoplankton, Spec. Publ. No. 21. American Soc. Limnology & Oceanography.
4. Sournia, A., ed. 1978. Phytoplankton Manual, Monogr. Oceanogr. Methodol. No. 6. United Nations Educational, Scientific & Cultural Org., Paris.
5. Lackey, J.B. 1938. The manipulation and counting of river plankton and changes in some organisms due to formalin preservation. *Pub. Health Rep.* 53:2080.
6. Owen, B.B., Jr., M. Afzal & W.R. Cody. 1978. Staining preparations for phytoplankton and periphyton. *Brit. Phycol. J.* 13:155.

10200 G. Zooplankton Counting Techniques

1. Subsampling

Count entire samples with low zooplankton numbers (<200 zooplankters) without subsampling. Most zooplankton samples will contain more organisms than can be enumerated practically, however, so use a subsampling procedure. Before subsampling, remove and enumerate all large uncommon organisms (e.g., fish larvae in fresh water or coelenterates, decapods, fish larvae, etc., in salt water). Subsample by the pipet or splitting method.

In the pipet method, adjust sample to a convenient volume in a graduated cylinder or Imhoff cone. Concentrating plankton via a rubber bulb and clear acrylic plastic tube with fine mesh netting fitted on the end is convenient and accurate (Figure 10200:9). For smaller microzooplankton, use the sedimentation techniques described for concentrating phytoplankton. Transfer sample to a beaker or other wide-mouth vessel for subsampling via a Hensen-Stempel or similar wide-bore pipet. Gently stir sample completely and randomly with the pipet and quickly withdraw 1 to 5 mL. Transfer to a suitable counting chamber.

Alternatively, subsample by splitting via any of a number of devices of which the Folsom plankton splitter[1] is best known (Figure 10200:10). Level splitter before using. Place sample in the splitter and divide into subsplits. Rinse splitter into subsamples. Repeat until a workable number (200 to 500 individuals) is obtained in a subsample. Exercise care to provide unbiased splits. Even when using the Folsom splitter, unbiased subsamples cannot be unquestioningly assumed;[2] therefore, count animals in several subsamples from the same sample to verify that the splitter is unbiased and to determine the sampling error introduced by using it.

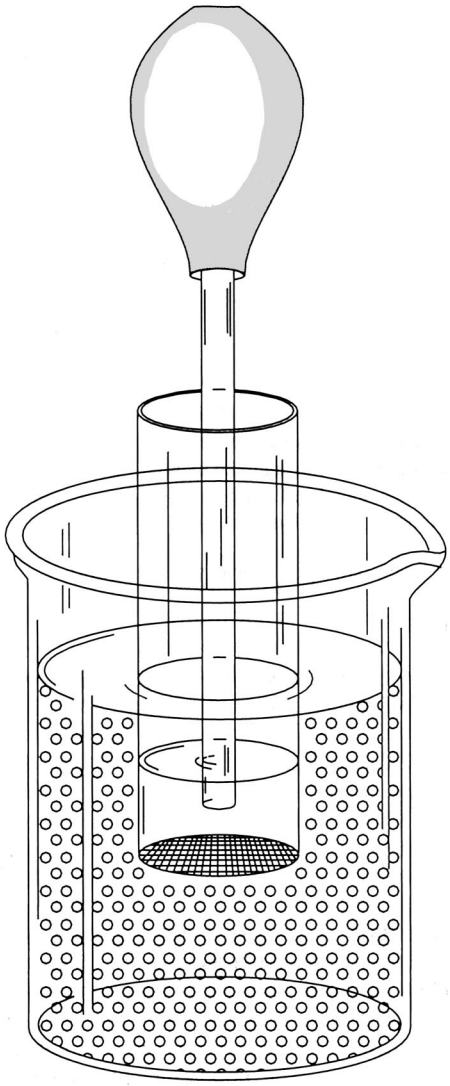


Figure 10200:9. A simple, efficient device for concentrating plankton. The tube is lowered into the beaker containing the sample. Water filtering into the tube is removed via rubber bulb. The filter is nylon monofilament screen cloth that is glued to the bottom of the tube. The mesh size should be small enough to prevent zooplankton from entering filtrate (after Dodson and Thomas[5]).

Another method permits abundance estimates of more equivalent levels of precision among taxa than obtained with either the Hensen-Stempel pipet or the Folsom splitter.[3] Normal counting procedures tally organisms based on their abundance in a sample. Therefore, in a sample with a dominant organism making up 50% of total numbers, the tally of the dominant taxon will be large and have a small error. However, errors related to subdominants will increase as the tally of each taxon decreases. By accepting one level of precision, the technique[3] has been developed to obtain the same error about dominants and subdominants, permitting quantitative comparisons between taxa over successive times or between stations.

2. Enumeration

Using a compound microscope and a magnification of 100×, enumerate small zooplankton (protozoa, rotifers, and nauplii) in a 1- to 5-mL clear acrylic plastic counting cell fitted with a glass cover slip. For larger, mature microcrustacea, use a counting chamber holding 5 to 10 mL. A Sedgwick-Rafter cell is unsuitable because of its size. An open counting chamber 80 × 50 × 2 mm deep is desirable; however, an open chamber is difficult to move without jarring and disrupting the count. Place a mild detergent solution in the chamber before counting to reduce organism movements, or use special counting trays with parallel or circular grooves or partitions.[4,5] Count microcrustacea with a binocular dissecting microscope at 20 to 40× magnification. If identification is questionable, remove organisms with a microbiological transfer loop and examine at a higher magnification under a compound microscope.

Report zooplankton as number per liter or number per cubic meter, depending on the system and study objectives:

$$\text{No./m.}^3 = \frac{C \times V'}{V'' \times V'''}$$

where:

C = number of organisms counted,
V' = volume of the concentrated sample, mL,
V'' = volume counted, mL, and
V''' = volume of the grab sample, m^3.

To obtain organisms per liter, divide by 1000.

3. References

1. Longhurst, A.R. & D.L.R. Seibert. 1967. Skill in the use of Folsom's plankton sample splitter. *Limnol. Oceanogr.* 12:334.
2. McEwen, G.F., M.W. Johnson & T.R. Folsom. 1954. A statistical analysis of the Folsom sample splitter based upon test observations. *Arch. Meteorol. Geophys. Bioklimatol., Ser.* A, 6:502.
3. Alden, R.W., III, R.C. Dahiya & R.J. Young, Jr. 1982. A method for the enumeration of zooplankton samples. *J. Exp. Mar. Biol. Ecol.* 59:185.
4. Gannon, J.E. 1971. Two counting cells for the enumeration of zooplankton micro-crustacea. *Trans. Amer. Microsc. Soc.* 90:486.
5. Dodson, A.N. & W.H. Thomas. 1964. Concentrating plankton in gentle fashion. *Limnol. Oceanogr.* 9:455.

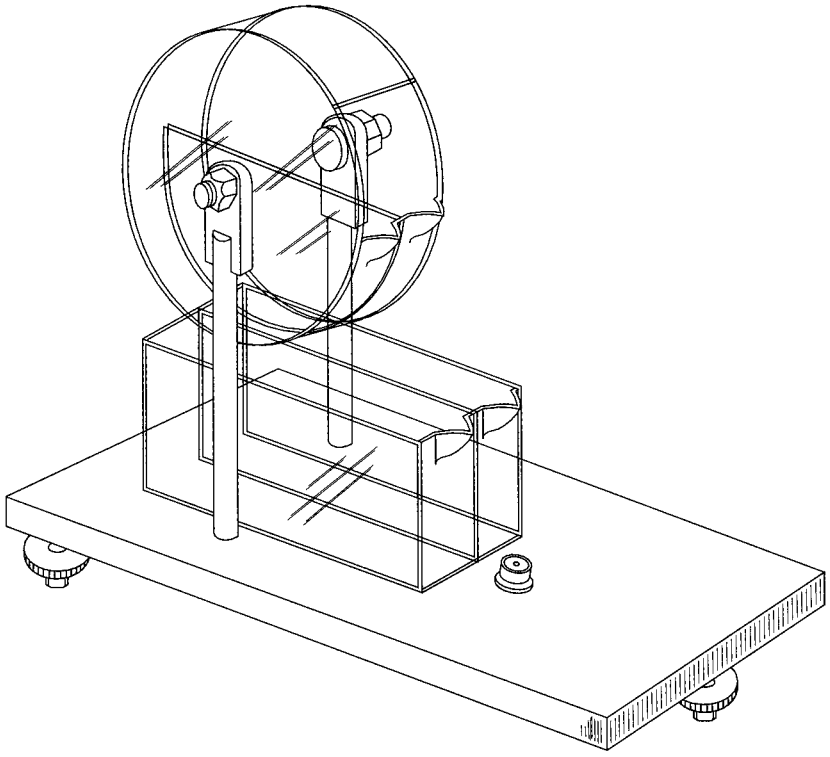


Figure 10200:10. The Folsom plankton splitter.

10200 H. Chlorophyll

Photosynthetic pigment concentrations are used extensively to estimate phytoplankton biomass.[1,2] All green plants contain chlorophyll *a,* which constitutes about 1 to 2% of the dry weight of planktonic algae. Other pigments in phytoplankton include chlorophylls *b* and c, xanthophylls, phycobilins, and carotenes. Important chlorophyll degradation products found in the aquatic environment are chlorophyllides, pheophorbides, and pheophytins. The presence or absence of various photosynthetic pigments is used, among other features, to identify major algal groups.

Three methods to determine chlorophyll *a* in phytoplankton are the spectrophotometric,[3–5] fluorometric,[6–8] and high-performance liquid chromatographic (HPLC) techniques.[9] Fluorometry is more sensitive than spectrophotometry, requires less sample, and can be used for in-vivo measurements.[10] These optical methods can significantly under- or overestimate chlorophyll *a* concentrations,[11–18] in part because the absorption and fluorescence bands of co-occurring accessory pigments and chlorophyll degradation products overlap.

Pheophorbide *a* and pheophytin *a,* two common degradation products of chlorophyll *a,* can interfere with the determination of chlorophyll *a* because they absorb light and fluoresce in the same region of the spectrum as chlorophyll *a* does. If these pheopigments are present, significant errors in chlorophyll *a* values will result. Pheopigments can be measured either via spectrophotometry or fluorometry, but in marine and freshwater environments, the fluorometric method is unreliable when chlorophyll *b* co-occurs, unless a non-acidification method is used (EPA Method 445.0). On acidifying chlorophyll *b*, the resulting fluorescence emission of pheophytin *b* coincides with that of pheophytin *a,* causing underestimation and overestimation of chlorophyll *a* and pheopigments, respectively. The non-acidification method has the following advantages: more accurate chlorophyll *a* data, less labor intensive, fewer resources needed, and very sensitive.

HPLC is a useful method for quantifying photosynthetic pigments,[9,13,15,16,19–21] including chlorophyll *a*, accessory pigments (e.g., chlorophylls *b* and *c*), and chlorophyll degradation products (chlorophyllides, pheophorbides, and pheophytins). Pigment distribution is useful for quantitative assessment of phytoplankton community composition and zooplankton grazing activity.[22]

1. Pigment Extraction

Glass fiber filters are preferred for removing algae from water. The glass fibers help break the cells during grinding, larger volumes of water can be filtered, and no precipitate forms after acidification. Inert membrane filters, such as polyester filters, may be used when these factors are irrelevant. Filters taken from water with pH above 6 may be placed in airtight plastic bags and stored frozen for 28 days. Process samples from naturally acidic water with pH less than 6 promptly after filtration to prevent possible chlorophyll degradation from residual acidic water on the filter. (*Naturally acidic water* has a pH below 6 due to humic acid or the contents of senescent cells, not preservatives.)

Chlorophyll can be extracted from cells, typically collected on a filter, with several solvents (e.g., acetone, ethanol, and methanol). The procedure described here uses acetone. Conduct this procedure with chlorophyll extracts in subdued light to avoid degradation. Use opaque containers or wrap with aluminum foil. The pigments are extracted from plankton concentrate with aqueous acetone and the extract's absorbance (optical density) is determined via a spectrophotometer. The ease with which chlorophylls are removed from cells varies considerably with different algae. To completely extract pigments consistently, disrupt the cells mechanically with a tissue grinder.

a. Equipment and reagents:

1) *Tissue grinder:** Successfully macerating glass fiber filters in tissue grinders with grinding tube and pestle of conical design may be difficult. Preferably use round-bottom grinding tubes with a matching pestle that has grooves in the TFE tip. Alternatively, sonication may be used.

2) *Clinical or benchtop centrifuge* (5000 to 7500 rpm) [$g = (0.00001118r)(rpm)$, where r = centrifuge's radius].

3) *Centrifuge tubes,* 15-mL graduated, screw-cap.

4) *Filtration equipment,* filters, glass fiber† or membrane (0.45-μm porosity, 47-mm diameter); vacuum pump (or lab vacuum); solvent-resistant filter assembly (e.g., an all-glass filter-support apparatus that can be cleaned with water and organic solvents), 1.0-μm pore size;‡ 10-mL solvent-resistant syringe.

5) *Saturated magnesium carbonate solution:* Add 1.0 g finely powdered $MgCO_3$ to 100 mL distilled water.

6) *Aqueous acetone solution:* Mix 90 parts acetone (reagent-grade BP 56°C) with 10 parts saturated magnesium carbonate solution. For HPLC pigment analysis, mix 90 parts HPLC-grade acetone with 10 parts distilled water.

b. Extraction procedure:

1) Concentrate sample by centrifuging or filtering as soon as possible after collection. If processing must be delayed, hold samples on ice or at 4°C and protect from exposure to light. Use opaque bottles because even brief exposure to light during storage will alter chlorophyll values. Rinse sample storage container with about 20 mL organic-free lab water (which is also passed through the same sample filter to make sure all cells are collected). Use glassware and cuvettes that are clean and acid-free. Add approximately 2 mL of $MgCO_3$ solution to sample just before filtering process is completed. $MgCO_3$ solution acts as a pH buffer to keep chlorophyll from degrading.

2) Place sample in a tissue grinder, cover with 2 to 3 mL 90% aqueous acetone solution, and macerate at 500 rpm for 1 min. Use TFE/glass grinder for a glass-fiber filter and glass/glass grinder for a membrane filter.

3) Transfer sample to a screw-cap centrifuge tube, rinse grinder with a few milliliters 90% aqueous acetone, and add the rinse to the extraction slurry. Adjust total volume to 10 mL with 90% aqueous acetone (additional techniques are used for HPLC analyses; see 10200H.4). Use solvent sparingly and avoid excessive dilution of pigments. Steep samples at least 2 h at 4°C in

* Kontes Glass Co., Vineland,NJ 08360: Glass/glass grinder, Model No. 88855, which requires adaptor 78800 and a stirrer motor 790040 operated at 500 rpm.: Glass/TEE grinder, Model 886000; or equivalent.

† Whatman GF/F (0.7 μm), GFB (1.0 μm), Gelman AE (1 μm),[23] or equivalent.

‡ Gelman Acrodisc, or equivalent.

the dark. Glass fiber filters of 25- and 47-mm diam§ have dry displacement volumes of 0.03 and 0.10 mL, respectively, and introduce errors of about 0.3 and 1.0% if a 10-mL extraction volume is used.

4) Clarify by filtering through a solvent-resistant disposable filter [e.g., a 0.45 μm PTFE 13 mm syringe filter (to minimize retention of extract in filter and filter holder, force 1 to 2 mL air through filter after extract)] or by centrifuging in closed tubes for 20 min at 500 *g* or 3000 rpm [$g = (0.00001118r)(rpm)$, where r = centrifuge's radius]. Decant clarified extract into a clean, calibrated, 15-mL, screw-cap centrifuge tube and measure total volume. Proceed as in 10200H.2, 3, 4, or 5.

2. Spectrophotometric Determination of Chlorophyll

a. Equipment and reagents:

1) *Spectrophotometer,* with a narrow band (pass) width (0.5 to 2.0 nm) because the chlorophyll absorption peak is relatively narrow. At a spectral band width of 20 nm, the chlorophyll *a* concentration may be underestimated by as much as 40%.

2) *Cuvettes,* with 1-, 4-, and 10-cm path lengths.

3) *Pipets,* 0.1- and 5.0-mL.

4) *Hydrochloric acid,* HCl, 0.1*N*.

b. Determination of chlorophyll a *in the presence of pheophytin* a: Chlorophyll *a* may be overestimated by including pheopigments that absorb near the same wavelength as chlorophyll *a.* Adding acid to chlorophyll *a* results in loss of the magnesium atom, converting it to pheophytin *a.* Acidify carefully to a final molarity of not more than $3 \times 10^{-3}M$ to prevent certain accessory pigments from changing to absorb at the same wavelength as pheophytin *a.*[13] When a solution of pure chlorophyll *a* is converted to pheophytin *a* via acidification, the absorption-peak ratio (absorbance 664/absorbance 665) of 1.70 is used to correct the apparent chlorophyll *a* concentration for pheophytin *a.*

Samples with an absorbance 664 before:absorbance 665 after acidification ratio (664_b:665_a) of 1.70 are considered to contain no pheophytin *a* and to be in excellent physiological condition. Solutions of pure pheophytin show no reduction in absorbance 665 upon acidification and have a 664_b:665_a ratio of 1.0. Thus, mixtures of chlorophyll *a* and pheophytin *a* have absorption peak ratios ranging between 1.0 and 1.7. These ratios are based on the use of 90% acetone as solvent. Using 100% acetone as solvent results in a chlorophyll *a* before-to-after acidification ratio of about 2.0.[3]

Spectrophotometric procedure—Transfer 3 mL clarified extract to a 1-cm cuvette and read absorbance at 750 and 664 nm. Acidify extract in the cuvette with 0.1 mL 0.1*N* HCl. Gently agitate the acidified extract and, 90 s after acidification, read absorbance at 750 and 665 nm. The volumes of extract and acid, and the time after acidification are critical for accurate, consistent results.

The absorbance 664 before acidification should be between 0.1 and 1.0. For very dilute extracts, use cuvettes with a longer path. If a larger cell is used, add a proportionately larger volume of acid. Correct absorbance obtained with larger cuvettes to 1 cm before making calculations.

§ Whatman GF/F, or equivalent.

Subtract the 750-nm absorbance value from the readings before (absorbance 664 nm) and after acidification (absorbance 665 nm).

Using the corrected values, calculate chlorophyll *a* and pheophytin *a* per cubic meter as follows:

$$\text{Chlorophyll } a, \text{ mg/m}^3 = \frac{26.7\,(664_b - 665_a) \times V_1}{V_2 \times L}$$

$$\text{Pheophytin } a, \text{ mg/m}^3 = \frac{26.7\,[1.7(665_a) - 664_b] \times V_1}{V_2 \times L}$$

where:

V_1 = volume of extract, L,
V_2 = volume of sample, m^3,
L = light path length or width of cuvette, cm, and
664_b, 665_a = absorbance of 90% acetone extract before and after acidification, respectively.

The value 26.7 is the absorbance correction and equals

$$A \times K$$

where:

A = absorbance coefficient for chlorophyll *a* at 664 nm = 11.0, and
K = ratio expressing correction for acidification.

$$= \frac{\left(\frac{664_b}{665_a}\right) \textit{pure chlorophyll a}}{\left(\frac{664_b}{665_a}\right) \textit{pure chlorophyll a} - \left(\frac{664_b}{665_a}\right) \textit{pure pheophytin a}}$$

$$= \frac{1.7}{1.7-1.0} = 2.43$$

c. Determination of chlorophyll a, b, *and* c *(trichromatic method):* Spectrophotometric procedure—Transfer extract to a 1-cm cuvette and measure absorbance at 750, 664, 647, and 630 nm. Choose a cell path length or dilution to give absorbance 664 between 0.1 and 1.0.

Use the absorbance readings at 664, 647, and 630 nm to determine chlorophyll *a, b,* and *c*, respectively. The absorbance reading at 750 nm is a correction for turbidity. Subtract this reading from each of the pigment absorbance values of the other wavelengths before using them in the equations below. Because the extract's absorbance at 750 nm is sensitive to changes in the acetone-to-water proportions, adhere closely to the 90 parts acetone:10 parts water (v/v) formula for pigment extraction. Turbidity can be removed easily via filtration through a disposable, solvent-resistant filter attached to a syringe or by centrifuging for 20 min at 500 *g*.

Calculate the concentrations of chlorophyll *a, b,* and *c* in the extract by inserting the corrected optical densities in following equations:[5]

a) C_a = 11.85(absorbance 664) – 1.54(absorbance 647) – 0.08(absorbance 630)

b) C_b = 21.03(absorbance 647) – 5.43(absorbance 664) – 2.66(absorbance 630)

c) C_c = 24.52(absorbance 630) –7.60(absorbance 647) – 1.67(absorbance 664)

where:

C_a, C_b, and C_c = concentrations of chlorophyll *a, b,* and *c,* respectively, mg/L, and

Absorbance 664, 647, and 630 = corrected optical densities (with a 1-cm light path) at the respective wavelengths.

After determining the concentration of pigment in the extract, calculate the amount of pigment per unit volume as follows:

$$\text{Chlorophyll } a, \text{ mg/m}^3 = \frac{C_a \times \text{ extract volume, L}}{\text{Volume of sample, m}^3}$$

3. Fluorometric Determination of Chlorophyll *a*

The fluorometric method for chlorophyll *a* is more sensitive than the spectrophotometric method, so smaller samples can be used. To achieve acceptable results, calibrate the fluorometer spectrophotometrically with a sample from the same source. Optimum sensitivity for chlorophyll *a* extract measurements is obtained at an excitation wavelength of 430 nm and an emission wavelength of 663 nm. A method for continuously measuring chlorophyll *a* in vivo is available but is reported to be less efficient than the in-vitro method given here (yielding about one-tenth as much fluorescence per unit weight as the same amount in solution). Pheophytin *a* also can be determined fluorometrically.[24]

a. Equipment and reagents: In addition to those listed under 10200H.1*a* and 2*a*:

Fluorometer,‖ equipped with a high-intensity F4T.5 blue lamp; photomultiplier tube R-446 (red-sensitive); sliding window orifices 1, 3, 10, and 30×; and filters for light emission (CS-2-64) and excitation (CS-5-60). A high-sensitivity door is preferable.

b. Extraction procedure: Prepare sample as directed in 10200H.1*b*.

1) Calibrate fluorometer with a known concentration of chlorophyll solution as follows. Prepare chlorophyll extract and analyze spectrophotometrically. Prepare serial dilutions of the extract to provide concentrations of approximately 2, 6, 20, and 60 μg chlorophyll *a*/L. Make fluorometric readings for each solution at each sensitivity setting (sliding window orifice): 1, 3, 10, and 30×. Using the values obtained, derive calibration factors to convert fluorometric readings in each sensitivity level to concentrations of chlorophyll *a,* as follows:

$$F_s = \frac{C'_a}{R_s}$$

where:

F_s = calibration factor for sensitivity setting *S,*
R_s = fluorometer reading for sensitivity setting *S,* and,
C'_a = concentration of chlorophyll *a* determined spectrophotometrically, μg/L.

2) Measure sample fluorescence at sensitivity settings that will provide a midscale reading. (Avoid using the 1× window because of quenching effects.) Convert fluorescence readings to concentrations of chlorophyll *a* by multiplying the readings by the appropriate calibration factor.

c. Determination of chlorophyll a *in the presence of pheophytin* a: This method normally is not applicable to freshwater samples. See discussions under 10200H and 2*b*.

1) Equipment and reagents—In addition to those listed under 10200H.1*a* and 2*a*, pure chlorophyll *a*# (or a plankton chlorophyll extract with a spectrophotometric before-and-after acidification ratio of 1.70 containing no chlorophyll *b*).

2) Fluorometric procedure—Calibrate fluorometer as directed in ¶ *b*1) above. Determine extract fluorescence at each sensitivity setting before and after acifidication. Calculate calibration factors *(F_s)* and before-and-after acidification fluorescence ratio by dividing the fluorescence reading obtained before acidification by the one obtained after acidification. Avoid readings on the 1× scale and those outside the range of 20 to 80 fluorometric units.

3) Calculations—Determine the "corrected" chlorophyll *a* and pheophytin *a* in sample extracts via the following equations:[8,24]

$$\text{Chlorophyll } a, \text{ mg/m}^3 = F_s \frac{r}{r-l}(R_b - R_a)\frac{V_e}{V_e}$$

$$\text{Pheophytin } a, \text{ mg/m}^3 = F_s \frac{r}{r-l}(rR_b - R_a)\frac{V_e}{V_e}$$

where:

F_s = conversion factor for sensitivity setting *S* (see 10200H.2*b*),
R_b = fluorescence of extract before acidification,
R_a = fluorescence of extract after acidification,
r = R_b/R_a, as determined with pure chlorophyll *a* for the instrument (Redetermine r and F_S if filters or light source are changed),
V_e = volume of extract, and
V_s = volume of sample.

d. Extraction of whole water, nonfiltered samples: Alternatively, to prevent cell lysis during filtration, extract whole water sample.

1) Equipment and reagents—Fluorometer equipped with a high-sensitivity R928 phototube** with output impedance of 36 ma/W at 675 nm and a high-sensitivity door. Place neutral density filter (40 to 60N) in the rear light path,†† selected to permit reagent blanking on the highest sensitivity scale.

2) Extraction procedure—Decant 1.5 mL sample into screw-cap test tube and add 8.5 mL 100% acetone. Mix with vortex mixer and hold in the dark for 6 h at room temperature. Filter

‖ Model 10-005, Turner Designs, Sunnyvale, CA, or equivalent.

Purified chlorophyll *a,* Sigma Chemical Company, St. Louis, MO., or equivalent.
** Hammamatsu Corp., Middlesex, NJ, or equivalent.
†† If using Model 10-005, Turner Designs, or equivalent.

through glass fiber filter‡‡ or centrifuge. Measure fluorescence as described in 10200H.3 and estimate concentrations as in ¶ *c* above. Humic substances interfere, so if they are present, filter a sample portion (see 10200H.1*b*) and process filtrate with sample. Subtract filtrate (blank) fluorescence from that of sample.

4. High-Performance Liquid Chromatographic Determination of Algal Chlorophylls and Their Degradation Products

a. Equipment and reagents: In addition to those listed for pigment extraction, 10200H.1*a*:

1) *High-pressure liquid chromatograph* capable of a flow rate of 2.0 mL/m.

2) *High-pressure injector valve* equipped with a 100-μL sample loop.

3) *Guard column* (4.0 × 0.5 cm, C_{18} packing material, 3-μm particle size, or equivalent protection system) to extend life of primary column.

4) *Reverse-phase HPLC column.*§§

5) *Fluorescence detector* capable of excitation at 430 ± 30 nm and measuring emissions at wavelengths greater than 600 nm.

6) *Data recorder device:* Strip chart recorder or, preferably, an electronic integrator.

7) *Syringe,* glass, 250-μL.

8) *HPLC eluents:* System A (80:15:5 blend of methanol, reagent water, and ion-pairing solution, respectively) and System B (80:20 blend of methanol and acetone, respectively). Use HPLC-grade solvents; measure volumes before mixing. Filter eluents through a solvent-resistant 0.4-μm filter before use and degas with helium. Prepare the ion-pairing (IP) solution from 15 g tetrabutylammonium acetate‖‖ and 77 g ammonium acetate## made up to 1 L with reagent water.[15]

9) *Calibration standards:* Individually dissolve 1 mg each pure chlorophyll *a* and *b*‖‖ in 100 mL 90% acetone. Determine the exact concentrations spectrophotometrically (ϵ_{664} for chlorophyll *a* in 90% acetone = 87.67 L/g · cm; ϵ_{647} for chlorophyll *b* in 90% acetone = 51.36 L/g · cm).[5] Prepare pheophytin $a + a'$ and $b + b'$ standards from the primary chlorophyll *a* and *b* standards via acidification with hydrochloric acid; correct respective concentrations for Mg^{2+} loss. Extract chlorophyll *c* with 90% acetone from diatoms, purify by thin-layer chromatography (TLC)[25] and calibrate spectrophotometrically (ϵ_{631} for a mixture containing equal amounts of chlorophylls c_1 and c_2 in 90% acetone containing 1% pyridine = 42.6 L/g · cm; the absence of this small amount of pyridine is presumed to cause only small differences in the absorption properties of chlorophyll *c*.[26] Alternatively, determine the chlorophyll *c* content of a 90% acetone extract made from diatoms spectrophotometrically (chlorophyll $c_1 + c_2$, μg/mL = $24.36E_{630} - 3.73E_{664}$)[5] and use as standard. Prepare chlorophyllide *a* from diatoms,[27] purify by TLC[25] and calibrate spectrophotometrically in 90% acetone (ϵ_{664} for chlorophyllide *a* = 128 L/g · cm).[28] Prepare pheophorbide *a* by acidification of chlorophyllide *a,* purify by TLC,[25] and calibrate spectrophotometrically in 90% acetone (ϵ_{665} for pheophorbide *a* = 69.8 L/g · cm).[28] Standards stored under nitrogen in the dark at –20°C are stable for about 1 month.

b. Procedure:

1) Set up and equilibrate HPLC with solvent System A at a flow rate of 2 mL/min. Adjust fluorometer sensitivity to provide full-scale reading with the most concentrated chlorophyll *a* standard.

2) Calibrate HPLC by preparing working standards from the primary standards (on day of use). Once the standards' retention times are determined for a particular system, simplify standardization by preparing serial dilutions from mixed standards. Prepare separately mixed standards for the chlorophylls and chlorophyllide *a* and for the pheophytins and pheophorbide *a.* Mix 1-mL portions of standards with 300 μL ion-pairing solutions and equilibrate for 5 min before injection (use of ion-pairing agents greatly enhances separation of dephytolated pigments, chlorophyllide *a,* chlorophyll *c,* and pheophorbide *a*).

Prepare blanks by mixing 1 mL 90% acetone with 300 μL IP solution. Rinse syringe twice with 150 μL standard and draw about 250 μL standard into syringe for injection. Place syringe in injector valve and completely fill the 100-μL sample loop. Construct calibration curves by plotting fluorescence peak areas (or heights) against standard pigment concentrations.

3) Prepare samples for injection by mixing a 1-mL portion of the 90% acetone pigment extract with 300 μL IP solution.

4) Use a two-step solvent program to optimize separation of chorophylls from their degradation products.[15] After injection, change from solvent System A to System B over 5 min and follow with System B for 15 min at a flow rate of 2 mL/min. Re-equilibrate the column with System A for 5 min before the next injection, for a total analysis time of about 25 min. Degas the solvent systems with helium during analysis. Increase lifetime of HPLC column by storing it in 100% methanol between runs. Periodically flush the HPLC with reagent water to avoid buildup of ion pairing agents.

5) Calculate individual pigment concentrations using the following formula:

$$C_i = \frac{A_s F_i V_E}{V_I V_S}$$

where:

C_i = individual pigment concentration, mg/L,
A_s = area of individual pigment peak from sample injection,
F_i = standard response factor (mg pigment/0.1 mL standard divided by corresponding peak area).
V_I = injection volume (0.1 mL),
V_E = extraction volume, mL, and
V_S = sample volume, L.

6) This method is designed only to quantify chlorophylls and their degradation products. To detect carotenoid pigments, which also are present in 90% acetone extracts but do not fluoresce, use absorbance spectroscopy (at about 440 nm).[21]

7) The elution order and approximate retention times for the major chlorophyll pigments and their degradation products are shown in Figure 10200:11. The detection limits [signal-to-noise (*s/n*) ratio = 2] vary with fluorometer configuration and flow rate; however, they range from 10 to 100 pg per injection for

‡‡ Whatman GF/F or equivalent.
§§ Microsorb C_{18} column, 10 cm long, 3-μm particle size, Rainin Co., or equivalent.
‖‖ Fluka Chemical Corp., 980 South Second Street, Ronkonkoma, NY, or equivalent.
Sigma Chemical Company, or equivalent.

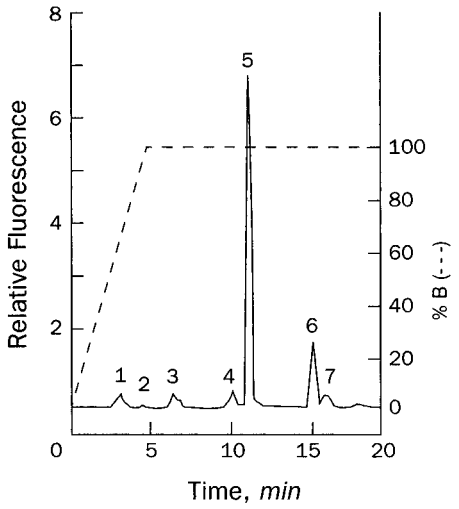


Figure 10200:11. Reverse-phase HPLC chromatogram for a fivefold dilution of EPA sample. Injection volume 100 μL; peaks detected by fluorescence spectroscopy (λ_{ex}: 400–460 nm; λ_{ex}: >600 nm). Peak identities are: 1—chlorophyllide *a;* 2—chlorophyll *c;* 3—pheophorbide *a;* 4—chlorophyll *b;* 5—chlorophyll *a;* 6—pheophytin *a;* and 7—pheophytin *a′*. The chlorophyll *b* degradation products, pheophytin *b* and pheophytin *b′*, were below detection limits. Peak identities confirmed by on-line diode array spectroscopy (350–550 nm).

most chlorophylls and their degradation products.[15,21,29] The accuracy of the HPLC method depends primarily on purity of pigment standards. Preferably measure the standards' absorption spectra (350 to 750 nm) and compare with published data. Pigment purity also can be assessed via HPLC analysis if there are no co-eluting contaminants with absorption and their fluorescence bands overlap those of the standards. HPLC and spectrophotometrically derived pigment concentrations for available EPA standards agree reasonably well (±20%) if spectrophotometric results are corrected for pheopigments and HPLC results are expressed as pigment equivalents (e.g., chlorophyll *a* equivalents = chlorophyllide *a* + chlorophyll *a* + chlorophyll *a′*, provided that proper molecular weight corrections are applied).[30] Thus, if significant amounts of chlorophyll derivatives are present, pigment concentrations determined spectrophotometrically will be overestimated. The agreement between HPLC and fluorometrically derived results depends on the presence of accessory chlorophylls *b*, *c*, and their derivatives. Triplicate injections of a fivefold dilution of an EPA sample gave coefficients of variation of 7.5% (chlorophyllide *a*), 9.1% (chlorophyll *c*), 13.4% (pheophorbide *a*), 9.6% (chlorophyll *b*), 0.5% (chlorophyll *a*), 6.2% (pheophytin *a*), and 22.9% (pheophytin *a′*), with an average value of 10% for the seven pigments analyzed.

5. High-Performance Liquid Chromatographic Determination of Algal Chlorophyll and Carotenoid Pigments

a. Equipment and reagents: In addition to those listed for pigment extraction, 10200H.1*a*:

1) *High-performance liquid chromatographic pump* capable of gradient delivery of three solvents at a flow rate of 1 mL/min.

2) *High-pressure injector valve* equipped with a 200-μL sample loop.

3) *Guard column* (50 × 4.6 mm, C_{18} packing material,*** 5-μm particle size) to extend life of primary column.

4) *Reverse-phase HPLC column* with endcapping (250 × 4.6 mm, 5-μm particle size, C_{18} column***).

5) *Variable wavelength or filter absorbance detector* with low-volume flow-through cell. Detection wavelength is 436 nm.

6) *Data recording device:* Strip chart recorder or, preferably, an electronic integrator or computer equipped with hardware and software for chromatographic data analysis.

7) *Syringe,* glass, 500-μL.

8) *HPLC eluents:* Eluent A [80:20 blend of methanol and 0.5*M* ammonium acetate (v:v), respectively; pH 7.2]; Eluent B [90:10 blend of acetonitrile and water (v:v), respectively], and Eluent C (ethyl acetate). Use HPLC-grade solvents. Measure volumes before mixing. Filter eluents through a solvent-resistant 0.4-μm filter before use and degas with helium.

9) *Calibration standards:* Chlorophylls *a* and *b*, and β,β-carotene can be purchased,††† as can zeaxanthin and lutein.‡‡‡ Other pigment standards can be purified from plant extracts via thin-layer chromatography (TLC)[25] or preparative-scale HPLC. Determine concentration of all standards using a monochromator-based spectrophotometer in the appropriate solvents before calibrating the HPLC system. The recommended extinction coefficients for the most common algal pigments found in freshwater systems are given in Table 10200:III. Measure absorbance in a 1-cm cuvette at the appropriate wavelength (usually at λ_{max}) and 750 nm (to correct for light scattering). Calculate standards concentrations as follows:

$$C_i = \frac{(A_{\lambda max} - A_{750\ nm})}{E_{lcm} \times b} \times 1000$$

where:

C_i = individual pigment concentration, mg/L,
A = absorbance at specific wavelength,
E_{1cm} = weight-specific absorption coefficient, L/g · cm,
b = pathlength of cuvette, cm, and
1000 = conversion factor, g to mg.

Standards stored under nitrogen in the dark at –20°C are stable for about 1 month.

b. Procedure:

1) Set up and equilibrate the HPLC with Eluent A at a flow rate of 1 mL/min.

2) Calibrate the HPLC using working standards (about 0 to 1000 ng/mL) prepared from primary standards on day of use. Mix 1 mL standard with 300 μL distilled water, shake, and equilibrate for 5 min before injection (diluting standards and sample extracts with water increases the pigments' affinity for the column in the loading step, resulting in better separation of more polar pigments). Rinse syringe twice with 300 μL standard and draw 500 μL standard into syringe for injection. Place

*** Spherisorb ODS-2, Phase Separations Inc., Norwalk, CT, or equivalent.
††† Sigma Chemical Co., St. Louis, MO, or equivalent.
‡‡‡ Roth Chemical Co., distributed by Atomergic Chemetals Corp., Farmingdale, NY, or equivalent.

TABLE 10200:III. EXTINCTION COEFFICIENTS AND CHROMATOGRAPHIC PROPERTIES OF PIGMENTS SEPARATED BY REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (CF. FIGURE 10200:12)

Pigment Identity	Wavelength (solvent) *nm*	E_{1cm} *L/g · cm*	Ref. No.	Retention Time *min*	% c.v. (n = 3 inj)	Absorption Maxima in Eluent* *nm*		
Chlorophyllide *a*	664 (90% acetone)	128.0	28	7.8	5.7	nd†	nd	nd
Chlorophyll c_{1+2}	631 (90% acetone)	42.6	26	8.9	0.6	444	576	630
Peridinin	466 (acetone)	134.0	33	10.0	1.2		472	
Fucoxanthin	449 (acetone)	160.0	44	11.0	0.9		446	(466)
Neoxanthin	439 (ethanol)	224.3	35	11.5	5.9	416	441	470
Violaxanthin	443 (ethanol)	255.0	35	13.2	2.6	416	440	470
Diadinoxanthin	448 (acetone)	223.0	36	14.6	6.0	422	446	476
Lutein	445 (ethanol)	255.0	35	17.5	0.7	(422)	446	476
Zeaxanthin	450 (ethanol)	254.0	35	18.0	2.2	(428)	454	478
Chlorophyll *b*	647 (90% acetone)	51.36	5	21.1	1.0	456	596	646
Chlorophyll *a*	664 (90% acetone)	87.67	5	22.3	0.8	431	618	665
β,β-carotene	453 (90% acetone)‡	262.0	35	25.4	2.0	427	462	480

* All absorption maxima are from Wright *et al.*[31] except those for chlorophyll c_{1+2} (R.R. Bidigare and M. Latasa, unpublished data).
† Not determined.
‡ Because of a potential insolubility problem of β,β-carotene in ethanol, prepare this standard in 90% acetone, not ethanol. It is assumed that the extinction coefficient of β,β-carotene in 90% acetone is the same as that in ethanol.

syringe in injector valve, overfilling the 200 μL sample loop 2.5-fold. To check for possible interferences in extraction solvent and/or filter, prepare a blank by extracting a glass fiber filter in 90% acetone; mixing 1 mL 90% acetone filter extract with 300 μL distilled water; and injecting into the HPLC. Plot absorbance peak areas (or heights) against standard pigment concentrations. Calculate response factors as the slope of the regression between the weights of injected standards (ng) and the areas of parent pigment (plus areas of structurally related isomers, if present). These isomers contribute to the standards' absorption signal; disregarding them results in overestimation of pigments in sample extracts.[32]

3) Prepare samples for injection by mixing a 1-mL portion of the 90% acetone pigment extract with 300 μL distilled water, shake, and equilibrate for 5 min before injection.

4) Following sample injection, use a gradient program to optimize separation of chlorophyll and carotenoid pigments. The system described in Table 10200:IV has been developed from the original method[31] to ensure elution of most hydrophobic pigments. Degas solvent system with helium during analysis. Periodically flush HPLC with distilled water to avoid accumulating ion-pairing reagents.

5) Routinely determine peak identities by comparing sample peaks' retention times with those of pure standards. Confirm peak identities spectrophotometrically by collecting eluting peaks from the column outlet (or directly with an on-line diode array spectrophotometer). Table 10200:III lists absorption maxima for the most common pigments found in freshwater systems.

6) Calculate individual pigment concentrations using the formula given in 10200H.4*b*5).

7) This method is designed to separate chlorophyll and carotenoid pigments (Figure 10200:12); however, it also separates major chlorophyll breakdown products.

8) Method precision was assessed by making triplicate injections of a mixture of phytoplankton and plant extracts. Coefficients of variation ranged from 0.6 to 6.0% (Table 10200:III). Using an appropriate internal standard increases precision.

Further information on these pigments and on analysis methods is available.[33–37]

6. References

1. ROTT, E. 1980. Spectrophotometric and chromatographic chlorophyll analysis: comparison of results and discussion of the trichromatic method. *Ergebn. Limnol.* (Suppl. to *Arch. Hydrobiol.*) 14:37.
2. MARKER, A.F.H., E.A. NUSCH, H. RAI & B. RIEMANN. 1980. The measurement of photosynthetic pigments in freshwaters and stan-

TABLE 10200:IV. HPLC SOLVENT SYSTEM PROGRAM

Time *min*	Flow Rate *mL/min*	Percentage of Eluent A	B	C	Conditions
Analysis protocol:					
0.0	1.0	100	0	0	Injection
2.0	1.0	0	100	0	Linear gradient
2.6	1.0	0	90	10	Linear gradient
13.6	1.0	0	65	35	Linear gradient
18.0	1.0	0	31	69	Linear gradient
23.0	1.0	0	31	69	Hold
25.0	1.0	0	100	0	Linear gradient
26.0	1.0	100	0	0	Linear gradient
34.0	1.0	100	0	0	Hold
Shutdown protocol:					
0.0	1.0	100	0	0	Analysis complete
3.0	1.0	0	100	0	Linear gradient
6.0	1.0	0	0	100	Linear gradient
16.0	1.0	0	0	100	Washing
17.0	0.0	0	0	100	Shutdown

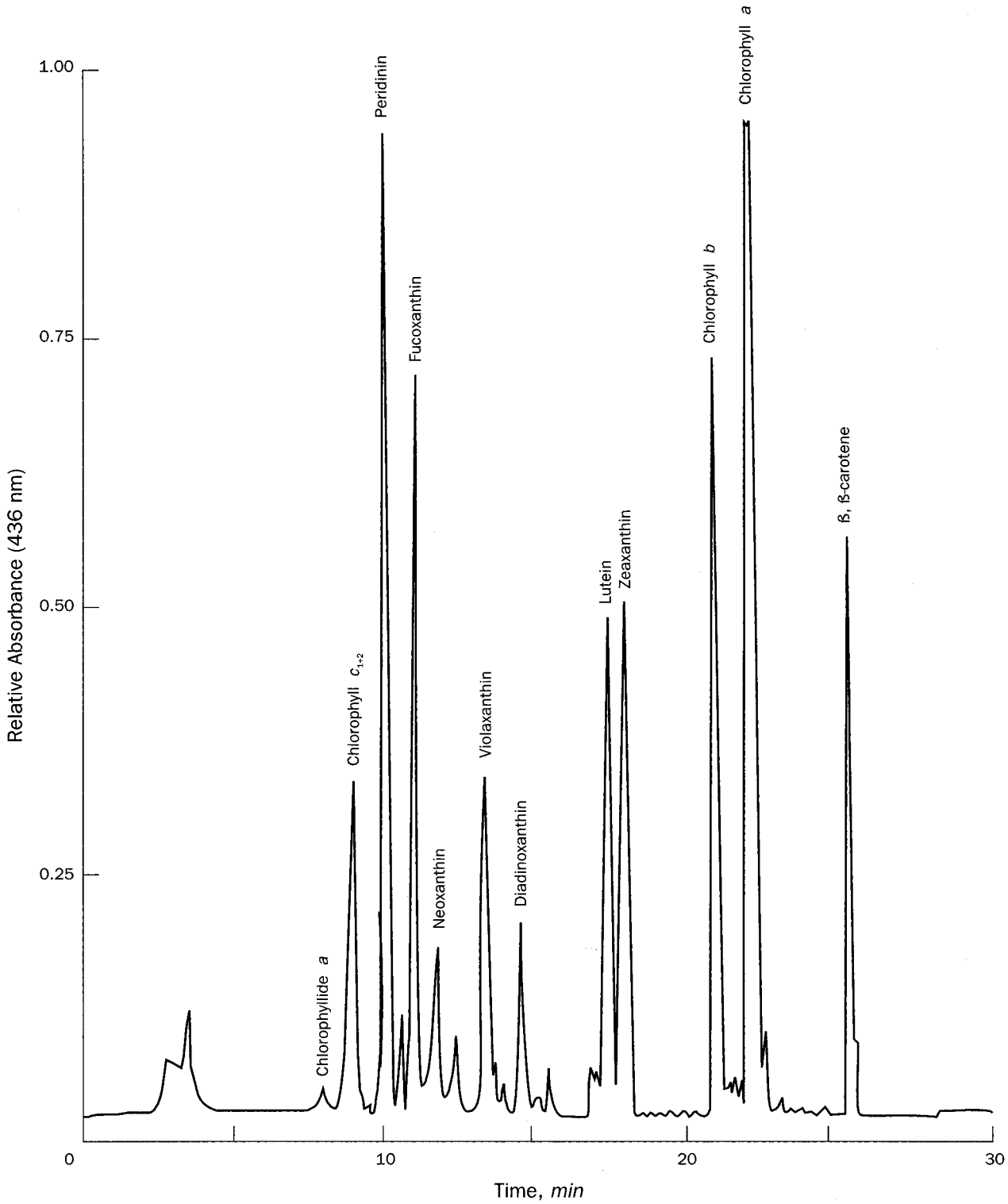


Figure 10200:12. Reverse-phase HPLC pigment chromatogram for a mixture of common algal pigments found in freshwater systems. For further data, see Table 10200:III. Sample contained a natural extract with authentic known additions. The small unlabeled peaks are pigment degradation products.

dardization of methods: Conclusions and recommendations. *Ergebn. Limnol.* (Suppl. to *Arch. Hydrobiol.*) 14:91.
3. Lorenzen, C.J. 1967. Determination of chlorophyll and pheopigments: spectrophotometric equations. *Limnol. Oceanogr.* 12:343.
4. Fitzgerald, G.P. & S.L. Faust. 1967. A spectrophotometric method for the estimation of percentage degradation of chlorophylls to pheopigments in extracts of algae. *Limnol. Oceanogr.* 12:335.
5. Jeffrey, S.W. & G.F. Humphrey. 1975. New spectrophotometric equations for determining chlorphylls *a*, *b*, and *c*, in higher plants, algae and natural phytoplankton. *Biochem. Physiol. Pflanzen* 167:191.
6. Yentsch, C.S. & D.W. Menzel. 1963. A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorescence. *Deep Sea Res.* 10:221.
7. Loftus, M.E. & J.H. Carpenter. 1971. A fluorometric method for determining chlorophylls *a, b,* and *c. J. Mar. Res.* 29:319.
8. Holm-Hansen, O., C.J. Lorenzen, R.W. Holmes & J.D.H. Strickland. 1965. Fluorometric determination of chlorophyll. *J. Cons. Cons. Perma. Int. Explor. Mer.* 30:3.
9. Bidigare, R.R., L. Van Heukelem & C.C. Trees. 2005. Analysis of algal pigments by high-performance liquid chromatography. *In* R.A. Andersen, ed. Algal Culturing Techniques. Academic Press, New York, N.Y.
10. Lorenzen, C.J. 1966. A method for the continuous measurement of *in vivo* chlorophyll concentration. *Deep Sea Res.* 13:223.
11. Jacobsen, T.R. 1978. A quantitative method for the separation of chlorophylls *a* and *b* from phytoplankton pigments by high-pressure liquid chromatography. *Mar. Sci. Comm.* 4:33.
12. Brown, L.M., B.T. Hargrave & M.D. Mackinnon. 1981. Analysis of chlorophyll *a* in sediments by high-performance liquid chromatography. *Can. J. Fish. Aquat. Sci.* 38:205.
13. Gieskes, W.W. & G.W. Kraay. 1983. Unknown chlorophyll *a* derivatives in the North Sea and the tropical Atlantic Ocean revealed by HPLC analysis. *Limnol. Oceanogr.* 28:757.
14. Gowen, R.J., P. Tett & B.J.B. Wood. 1983. Changes in the major dihydroporphyrin plankton pigments during the spring bloom of phytoplankton in two Scoottish sea-lochs. *J. Mar. Biol. Assoc. U.K.* 63:27.
15. Mantoura, R.F.C. & C.A. Llwewllyn. 1983. The rapid determination of algal chlorophyll and caroteniod pigments and their breakdown products in natural waters by reverse-phase high-performance liquid chromatography. *Anal. Chim. Acta* 151:297.
16. Gieskes, W.W.C. & G.W. Kraay. 1984. Phytoplankton, its pigments, and primary production at a central North Sea station in May, July and September 1981. *Neth. J. Sea Res.* 18:51.
17. Hallegraeff, G.M. & S.E. Jeffrey. 1985. Description of new chlorophyll *a* alteration products in marine phytoplankton. *Deep Sea Res.* 32:697.
18. Trees, C.C., M.C. Kennicutt II & J.M. Brooks. 1985. Errors associated with the standard fluorometric determination of chlorophylls and phaeopigments. *Mar. Chem.* 17:1.
19. Eskins, K., C.R. Schofield & H.J. Dutton. 1977. High-performance liquid chromatography of plant pigments. *J. Chromatogr.* 135:217.
20. Wright, S.W. & J.D. Shearer. 1984. Rapid extraction and high performance liquid chromatography of chlorophylls and carotenoids from marine phytoplankton. *J. Chromatogr.* 294:281.
21. Bidigare, R.R., M.C. Kennicutt II & J.M. Brooks. 1985. Rapid determination of chlorophylls and their degradation products by high-performance liquid chromatography. *Limnol. Oceanogr.* 30:432.
22. Jeffry, S.W. 1974. Profiles of photosynthetic pigments in the ocean using thin-layer chromatography. *Mar. Biol.* 26:101.
23. Phinney, D.A. & C.S. Yentsch. 1985. A novel phytoplankton chlorophyll technique: toward automated analysis. *J. Plankton Res.* 7:633.
24. Strickland, J.D.H. & T.R. Parsons. 1968. A Practical Manual of Sea Water Analysis, Fish. Res. Board Can. Bull. No. 167. Queen's Printer, Ottawa, Ont.
25. Jeffrey, S.W. 1981. An improved thin-layer chromatographic technique for marine phytoplankton pigments. *Limnol. Oceanogr.* 26:191.
26. Jeffrey, S.W. 1972. Preparation and some properties of crystalline chlorophyll c_1 and c_2 from marine algae. *Biochim. Biophys. Acta* 279:15.
27. Barrett, J. & S.W. Jeffrey. 1971. A note on the occurrence of chlorophyllase in marine algae. *J. Exp. Mar. Biol. Ecol.* 7:255.
28. Lorenzen, C.J. & J. Newton Downs. 1986. Specific absorption coefficients of chlorophyllide *a* and pheophorbide *a* in 90 percent acetone, and comments on the fluorometric determination of chlorophyll and pheopigments. *Limnol. Oceanogr.* 31:449.
29. Sartory, D.P. 1985. The determination of algal chlorophyllous pigments by high performance liquid chromatography and spectrophotometry. *Water Res.* 19:605.
30. Murray, A.P., C.F. Gibbs & A.R. Longmore. 1986. Determination of chlorophyll in marine waters: Intercomparison of a rapid HPLC method with full HPLC, spectrophotometric and fluorometric methods. *Mar. Chem.* 19:211.
31. Wright, S.W., S.W. Jeffrey, R.F.C. Mantoura, C.A. Llewellyn, T. Bjornland, D. Repeta & N. Welschmeyer. 1991. Improved HPLC method for the analysis of chlorophylls and carotenoids from marine phytoplankton. *Mar. Ecol. Prog. Ser.* 77:183.
32. Bidigare, R.R. 1991. Analysis of algal chlorophylls and carotenoids. *In* D.C. Hurd & D.W. Spencer, eds. Marine Particles: Analysis and Characterization. America Geophysical Union, Washington, D.C.
33. Jeffrey, S.W. & F.T. Haxo. 1968. Photosynthetic pigments of symbiotic dinoflagellates (zooxanthallae) from corals and clams. *Biol. Bull.* 135:149.
34. Jensen, A. 1978. Chlorophylls and carotenoids. *In* J.A. Helleburst & J.S. Craige, eds. Handbook of Phycological Methods: Physiological and Biochemical Methods. Cambridge University Press, Cambridge, England.
35. Davis, B.H. 1976. Carotenoids. *In* T.W. Goodwin, ed. Chemistry and Biochemistry of Plant Pigments. Academic Press, New York, N.Y.
36. Johansen, J.E., W.A. Svec & S. Liaaen-Jensen. 1974. Carotenoids of the Dinophyceae. *Phytochem.* 13:2261.
37. Arar, E.J. & G.B. Collins. 1992. Method 445.0: *In vitro* determination of chlorophyll *a* and pheophytin *a* in marine and freshwater phytoplankton by fluorescence. *In* Methods for the Determination of Chemical Substances in Marine and Estuarine Environmental Samples. U.S. Environmental Protection Agency, Cincinnati, Ohio.

10200 I. Determination of Biomass (Standing Crop)

Biomass is a quantitative estimate of the total mass of living organisms in a given area or volume. It may include the mass of a population (*species biomass*) or of a community (*community biomass*) but gives no information on community structure or function. The most accurate methods for estimating biomass are dry weight, ash-free dry weight, and volume of living organisms. Indirect methods include estimates of total carbon, caloric content, nitrogen, lipids, carbohydrates, silica (diatoms), and chlorophyll (algae). Adenosine triphosphate[1] (ATP) and deoxyribonucleic acid[2,3] (DNA) also have been used as indirect estimates.

Most biomass estimates can be affected by organic and inorganic detritus; ATP and DNA analyses include contributions from bacterial flora.[4] Biomass calculated from direct cell counts and dimensional measurements will incorporate error but avoid including extraneous matter, including sediments and detritus, that other techniques cannot segregate from their results.

1. Chlorophyll *a*

Chlorophyll *a* is used as an algal biomass indicator.[5] Assuming that chlorophyll *a* constitutes, on average, 1.5% of the ash-free dry weight of algae, estimate the algal biomass by multiplying the chlorophyll *a* content by 67 (see 10200H, for chlorophyll *a* methodology). The ratio of chlorophyll to dry weight will vary among major taxonomic groups, however, so the ratio might be adjusted based on knowledge of algal composition.

2. Biovolume (Cell Biovolume)

Plankton data derived on a volume-per-volume basis often are more useful than numbers per milliliter because they allow for analysis of individual taxa or species contributions to community structure and function on a biomass basis (e.g., 1 cell-Chlamydomonas: ~100 μm^3 vs. 1 cell-Ceratium: ~40,000 μm^3).[6] Determine cell biovolume by using the simplest geometric configuration (e.g., sphere, cone, cylinder, wedge) that best fits the shape of the cell being measured.[7–10] Measure only the biovolume of living cells, not sheaths and setae. An organism's cell size can differ substantially in different waters or in the same water at different times of the year, so for each sampling period, average the measurements of 10 to 30 individuals of each species or taxon (use a larger number of individuals for extremely variable genera like *Microcystis*). Calculate the total biovolume of any species by multiplying the average cell biovolume (in μm^3) by the number of cells per milliliter.

Compute total wet algal biovolume as:

$$V_t = \sum_{i=1}^{n} (N_i \times V_i)$$

where:

V_t = total plankton cell biovolume, mm^3/L,
N_i = number of organisms of the ith species/L, and
V_i = average biovolume of cells of ith species, μm^3.

The carbon biomass in cells of individual phytoplankton components may also be estimated directly from cell biovolume measurements.[8]

3. Volume/Surface Area

An estimation of natural-unit surface area is valuable when analyzing interactions between the natural unit and surrounding waters. Compute average surface area in μm by using similar geometric configurations and multiplying by the number per milliliter of the species being considered.[2] Take care to subtract open areas (lorica openings) or surfaces that abut each other (e.g., valve faces that touch in multiple cell diatom colonies). Natural unit volume and area estimates allow analysis of nutrient dynamics, grazing, and suspension dynamics.

4. Displacement Volume

This method[11] measures the volume of liquid that a sample displaces. Displacement volume may be determined via several methods. For simple, direct measurement, place sample in a sieve whose mesh is equal to or smaller than that of the capture net. Let sample drain and transfer to a measured volume of water in a graduated cylinder. Measure the new volume (sample plus known volume). The displacement volume equals the new volume minus the first measured volume of water. This method works well for macroalgal samples and zooplankton.

5. Gravimetric Methods

The plankton community's biomass can be estimated from gravimetric determinations, although silt and organic detritus interfere. Determine dry weight by placing 100 mg wet concentrated sample in a clean, ignited, and tared porcelain crucible and dry at 105°C for 24 h. Alternatively, filter a known volume of sample through 0.45-μm-porosity membrane or a pre-rinsed, dried, and pre-weighed glass-fiber filter. (NOTE: The small sample used in direct filtration may lead to error if not handled properly.) Cool sample in a desiccator and weigh. Obtain ash-free weight by igniting the dried sample at 500°C for 1 h in a muffle furnace. Cool, rewet ash with distilled water, and bring to constant weight at 105°C. (The ash is rewetted to restore water of hydration to clays and other minerals; this may amount to as much as 10% of weight lost during incineration.[12]) The *ash-free dry weight* is the difference between the dry weight and the weight of the ash residue after ashing. When comparing mixed assemblages, ash-free weight is preferred to dry weight because the ash content of samples varies greatly. Ash may constitute 50% or more of the dry weight of phytoplankton with inorganic structures, such as diatoms. In other samples, the ash content is only about 5% of dry weight.

6. Adenosine Triphosphate (ATP)

The only way to determine the total viable plankton biomass is to measure adenosine triphosphate (ATP) in plankton. ATP occurs in all plants and animals, but only in living cells; it is not

associated with nonliving particulate. The ratio of ATP to biomass varies from species to species, but appears to be constant enough to permit reliable estimates of biomass based on ATP measurements.[13] The method is simple and relatively inexpensive, and the instrumentation is stable and reliable. The method also has many potential applications in entrainment and bioassay work, especially plankton mortality studies.

a. Equipment and reagents:

1) *Glassware:* clean, sterile, dry borosilicate glass flasks, beakers, and pipets.

2) *Filters:* 47-mm-diam, 0.45-μm-porosity membrane filters.

3) *Filtration equipment.*

4) *Freezer* (–20°C).

5) *Boiling water bath.*

6) *Detection instruments* designed specifically for measuring ATP.*

7) *Microsyringes:* 10-, 25-, 50-, 100-, and 250-μL.

8) *Reaction cuvettes and vials.*

9) *Tris buffer* (0.02*M*, pH 7.75): Dissolve 7.5 g trishydroxymethylaminomethane in 3 L distilled water and adjust pH to 7.75 with 20% HCl. Autoclave 150-mL portions at 115°C for 15 min.

10) *Luciferin-luciferase enzyme preparation:*† Rehydrate frozen (–20°C) lyophilized extracts of firefly lanterns with Tris buffer as directed by the supplier; let stand at room temperature 2 to 3 h, then centrifuge at 300 × *g* for 1 min and decant the supernatant into a clean, dry test tube; let stand at room temperature for 1 h.

11) *Purified ATP standard:* Dissolve 12.3 mg disodium ATP in 1 L distilled water and dilute 1.0 mL to 100 mL with Tris buffer; 0.2 mL = 20 ng ATP.

b. Procedure:

1) Calibration—To determine the calibration factor, *F*, prepare a series of dilutions of purified ATP standard and record the light emission from several portions of each concentration of standard. Correct mean area of standards by subtracting peak reading or mean area of several blanks using 0.2 mL Tris buffer. Calculate calibration factor F_S:

$$F_s = \frac{C}{A_s}$$

where:

F_s = calibration factor at sensitivity *S*,
A_s = peak reading or mean area under standard ATP curve corrected for blank, and
C = concentration of ATP in standard solution, ng/mL.

2) Sample analysis—Collect a 1- to 2-L sample in a clean, sterile sampler. Pass through a 250-μm net to remove large zooplankton[13] and filter through a 47-mm, 0.45-μm-porosity filter by applying a vacuum of about 30 kPa. (**IMPORTANT: Break suction before the last film of water is pulled through the filter.**) Quickly place filter in a small beaker. Immediately cover filter with 3 mL boiling Tris buffer, using an automatic pipet. Place beaker in boiling water bath for 5 min and, with a Pasteur pipet, transfer extract to a clean, dry, calibrated test tube. Rinse filter and beaker with 2 mL boiling Tris buffer; combine extracts, record volume, bring volume up to 5 mL with Tris buffer, cover tubes with parafilm and, if samples cannot be analyzed immediately, freeze at –25°C. Extracts may be stored for many months in a freezer. Prepare at least triplicate extracts of each sample.

The analytical procedure depends on detection equipment used. If a scintillation counter is used, pipet 0.2 mL enzyme preparation (blank) into a glass vial. Measure the enzyme preparation's light emissions for 2 to 3 min at sensitivity settings near those anticipated for the sample. Add 0.2 mL sample extract to the vial, record the time, and swirl. Start recording light output 10 s after combining ATP extract and enzyme preparation; record output for 2 to 3 min, using the same time period for all samples. Determine the mean of the areas under the curves obtained and correct by subtracting the mean of the areas under the curves obtained from blanks prepared as directed in Strickland and Parsons.[14]

c. Calculations: Calculate ATP concentration:

$$ATP,\ \text{ng/L} = \frac{A_c \times V_e \times F_s}{V_s}$$

where:

A_c = mean corrected area under extract curves,
V_e = extract volume, mL,
F_s = calibration factor, and
V_s = volume of sample, L.

Assuming an ATP content of 2.4 μg ATP/mg dry weight organic matter,[15] total living plankton biomass (*B*), as dry weight organic matter, is given as:

$$B,\ \text{mg/L} = \frac{ATP}{(2.4)(1000)}$$

7. References

1. Holm-Hansen, O. & C.R. Booth. 1966. The measurement of adenosine triphosphate in the ocean and its ecological significance. *Limnol. Oceanogr.* 11:510.
2. Holm-Hansen, O., W.H. Sutcliffe, Jr. & J. Sharp. 1968. Measurement of deoxyribonucleic acid in the ocean and its ecological significance. *Limnol. Oceanogr.* 13:507.
3. Holm-Hansen, O. 1969. Determination of microbial biomass in ocean profiles. *Limnol. Oceanogr.* 14:740.
4. Paerl, H.W., M.M. Tilzer & C.R. Goldman. 1976. Chlorophyll *a* vs. ATP as algal biomass indicators in lakes. *J. Phycol.* 12:242.
5. Creitz, G.I. & F.A. Richards. 1955. The estimation and characterization of plankton populations by pigment analysis. *J. Mar. Res.* 14:211.
6. Kutkuhn, J.H. 1958. Notes on the precision of numerical and volumetric plankton estimates from small sample concentrations. *Limnol. Oceanogr.* 3:69.
7. Vollenweider, R.A. 1969. A Manual on Methods for Measuring Primary Production in Aquatic Environments, IBP Handbook No. 12. Blackwell Scientific Publ., Oxford, England.

* Beckman, JRB, Turner Designs, or equivalent.
† Dupont, Sigma Chemical, or equivalent.

8. SMAYDA, T.J. 1978. From phytoplankters to biomass. *In* A. Sournia, ed. Phytoplankton Manual, Monograph. Oceanogr. Methods, No. 6. United Nations Educational, Scientific & Cultural Org., Paris.
9. HILLENBRAND, H., C.D. DURSELEN, D. KIRSCHTEL, U. POLLINGHER & T. ZOHARY. 1999. Biovolume calculation for pelagic and benthic microalgae. *J. Phycol.* 35:403.
10. OLRIK, K., P. BLOMQVIST, P. BRETTUM, G. CRONBERG & P. ELORANTA. 1998. Methods for quantitative assessment of phytoplankton in freshwaters, part I. Naturvårdsverket, Stockholm
11. JACOBS, F. & G.C. GRANT. 1978. Guidelines for Zooplankton Sampling in Quantitative Baseline and Monitoring Programs, EPA-600/3-78-026. U.S. Environmental Protection Agency, Washington, D.C.
12. NELSON, D.J. & D.C. SCOTT. 1962. Role of detritus in the productivity of a rock-outcrop community in a Piedmont stream. *Limnol. Oceanogr.* 7:396.
13. RUDD, J.W.M. & R.D. HAMILTON. 1973. Measurement of adenosine triphosphate (ATP) in two precambrian shield lakes of northwestern Ontario. *J. Fish. Res. Board Can.* 30:1537.
14. STRICKLAND, J.D.H. & T.R. PARSONS. 1968. A Practical Manual of Sea Water Analysts, Fish. Res. Board Can. Bull. No. 167. Queen's Printer, Ottawa, Ont.
15. WEBER, C.I. 1973. Recent developments in the measurement of the response of plankton and periphyton to changes in their environment. *In* G. Glass, ed. Bioassay Techniques and Environmental Chemistry. Ann Arbor Science Publ., Ann Arbor, Mich.

10200 J. Metabolic Rate Measurements

The aquatic community's physiological condition and spectrum of biological interactions must be considered when evaluating the state of natural waters. In earlier studies, numbers, species composition, and biomass were prime considerations. Recognition of this approach's limitations led to the measurement of rates of metabolic processes, such as photosynthesis (productivity), nitrogen fixation, respiration, and electron transport. These provide a better understanding of the aquatic ecosystem's complex nature. An indication of photosynthetic efficiency can be determined via the productivity index (mg C fixed/unit chlorophyll *a*).[1]

1. Nitrogen Fixation

An organism's ability to fix nitrogen is a great competitive advantage and plays a major role in population dynamics. Two reliable methods for estimating nitrogen-fixation rates in the laboratory are the 15*N* isotope tracer method[2,3] and the acetylene reduction method.[4] Because the rate of nitrogen fixation varies greatly with different organisms and with the concentration of combined nitrogen, nitrogen-fixation rates cannot be used to estimate biomass of nitrogen-fixing organisms. However, the acetylene reduction method is useful in measuring nitrogen budgets and in algal assay work.[5]

2. Productivity, Oxygen Method

In this context, *productivity* is the rate at which inorganic carbon is converted to an organic form. Chlorophyll-bearing organisms (phytoplankton, periphyton, macrophytes) serve as primary producers in the aquatic food chain. Photosynthesis ultimately results in the formation of a wide range of organic compounds, release of oxygen, and reduction of carbon dioxide (CO_2) in surrounding waters. Primary productivity[6] can be determined by measuring the changes in oxygen and CO_2 concentrations.[7]

In poorly buffered waters, pH can be used to detect variations in the system. As CO_2 is removed during photosynthesis, pH rises. This shift can be used to estimate both photosynthesis and respiration.[8] The sea and many fresh waters are too highly buffered to make this useful, but it has been applied successfully to productivity studies in some lake waters.

Two methods for measuring the rate of carbon uptake and net photosynthesis in situ are the oxygen method[9] and the carbon-14 method.[10] In both methods, clear (light) and darkened (dark) bottles are filled with water samples and suspended at regular depth intervals for 3 to 4 hours (maximum rate) or for a 24-hour period (integrate rate), or samples are incubated under controlled conditions in environmental growth chambers in the laboratory.

The basic reactions in algal photosynthesis involve uptake of inorganic carbon and release of oxygen:

$$CO_2 + H_2O \rightarrow (CH_2O)_x + O_2$$

The chief advantages of the oxygen method are that it provides estimates of gross and net productivity and respiration, and that analyses can be performed with inexpensive laboratory equipment and common reagents. The dissolved oxygen (DO) concentration is determined at the beginning and end of the incubation period. Productivity is calculated based on the assumption that one carbon atom is assimilated for each oxygen molecule released.

a. Equipment:

1) *BOD bottles,* numbered, 300-mL, clear and opaque borosilicate glass, with ground glass stopper and flared mouth, for sample incubation. Acid-clean the bottles, rinse thoroughly with distilled water, and just before use, rinse with water being tested. Do not use detergents containing phosphorus.

If suitable opaque bottles are not available, make clear BOD bottles opaque by painting them black and wrapping them with black waterproof tape. As a further precaution, wrap entire bottle in aluminum foil or place in light-excluding container during incubation.

2) *Supporting line or rack* that does not shade suspended bottles.

3) *Nonmetallic, opaque acrylic plastic Van Dorn sampler* or equivalent, 3- to 5-L capacity.

4) *Equipment and reagents for DO determinations:* See Section 4500-O.

5) *Pyrheliometer.*

6) *Submarine photometer.*

7) *Thermometer.*

b. Procedure:

1) Using a pyrheliometer, obtain a profile of the input of solar radiation for the photoperiod.

2) Using a submarine photometer, determine depth of euphotic zone (the region that receives 1% or more of surface illumination). Select depth intervals for bottle placement. To closely approximate the photosynthesis-depth curve, place samples at intervals equal to one-tenth the depth of the euphotic zone. Estimate productivity in relatively shallow water with fewer depth intervals.

3) Measure oxygen concentration via a probe or titration and temperature and salinity to determine whether water is supersaturated with oxygen (see Table 4500-O:I). If water is supersaturated, bubble nitrogen gas through sample to lower initial oxygen concentration to less than 80% saturation.

4) Keep samples out of direct sunlight during handling. Introduce samples taken from each preselected depth into duplicate clear, darkened, and initial-analysis bottles. Insert sampler's delivery tube to bottom of sample bottle and fill so three volumes of water are allowed to overflow. Remove tube slowly and close bottle. Use water from the same grab sample to fill a "set" (one light, one dark, and one initial bottle).

5) Immediately treat (fix) samples taken to chemically determine initial DO (see Section 4500-O) with manganous sulfate ($MnSO_4$), alkaline iodide, and sulfuric acid (H_2SO_4) or check with an oxygen probe. Analyses may be delayed several hours, if necessary, if samples are fixed or iced and stored in the dark.

6) Suspend duplicate paired clear and darkened bottles at the depth from which the samples were taken and incubate for at least 2 h, but never longer than it takes for oxygen-gas bubbles to form in clear bottles or DO to be depleted in dark bottles.

7) At the end of the exposure period, immediately determine DO as described above.

c. Calculations: The increase in oxygen concentration in the light bottle during incubation is a measure of net production, which (because oxygen is used concurrently in respiration) is somewhat less than total (gross) production. The loss of oxygen in the dark bottle is used as an estimate of total plankton respiration. Thus:

$$\text{Net photosynthesis} = \text{light bottle DO} - \text{initial DO}$$

$$\text{Respiration} = \text{initial DO} - \text{dark bottle DO}$$

$$\text{Gross photosynthesis} = \text{light bottle DO} - \text{dark bottle DO}$$

Average results from duplicates.

1) Calculate the gross or net production for each incubation depth and plot:

$$\text{mg fixed carbon/m}^3 = \text{mg oxygen released/L} \times 12/32 \times 1000\ \text{L/m}^3 \div K$$

where K is the photosynthetic quotient (PQ), which ranges from 1 to 2 depending on nitrogen supply.[11,12]

Use the factor 12/32 to convert oxygen to carbon; under ideal conditions, 1 mole of O_2 (32 g) is released for each mole of fixed carbon (12 g).

2) In this context, *productivity* is the rate of production; it generally is reported in grams carbon fixed per square meter per day. Determine the productivity of a vertical column of water 1 m square by plotting productivity for each exposure depth and graphically integrating the area under the curve.

3) Using the solar radiation profile and photosynthesis rate during incubation, adjust the data to represent phytoplankton productivity for the entire photoperiod. Because photosynthetic rates vary widely during the daily cycle,[13,14] do not attempt to convert data to other test circumstances.

3. Productivity, Carbon-14 Method

Add solution of radioactive carbonate ($^{14}CO_3^{2-}$) to light and dark bottles that have been filled with sample, as described for the oxygen method. After incubation in situ, collect the plankton on a membrane filter, treat with hydrochloric acid (HCl) fumes to remove inorganic carbon-14, and assay for radioactivity. The quantity of fixed carbon is proportional to the fraction of assimilated radioactive carbon. This procedure differs from the oxygen method in that it directly measures carbon uptake and measures only net photosynthesis.[15] It is basically more sensitive than the oxygen method but fails to account for organic materials that leach from cells[16,17] during incubation.

a. Equipment and reagents:

1) *Pyrheliometer.*

2) *Submarine photometer.*

3) *BOD bottles and supporting apparatus:* See 10200J.2*a*1) and 2).

4) *Membrane-filtering device and 25-mm filters* with porosities of 0.22, 0.30, 0.45, 0.80, and 1.2 μm.

5) *Counting equipment for measuring radioactivity:* Scaler with end-window tube, gas flow meter, or liquid scintillation counter (see Section 7030B.3). The thin-window tube is the least expensive detector and, when used with a small scaler, provides acceptable data at modest cost.

6) *Fuming chamber:* Use a glass desiccator with conc HCl about 1.4 cm deep in desiccant chamber. The fuming chamber is recommended for filter decontamination.[18,19]

7) *Syringe or pipet,* nonmetallic.

8) *Chemical reagents:* See Sections 4500-CO_2 (Carbon Dioxide) and 2320 (Alkalinity).

9) *Radioactive carbonate solutions:*

a) *Sodium chloride dilution solution*, 5% NaCl (w/v): Add 0.3 g sodium carbonate (Na_2CO_3) and one pellet sodium hydroxide (NaOH) per liter. Use for marine studies only.

b) *Carrier-free radioactive carbonate solution,* commercially available in sealed vials containing about 5 μCi ^{14}C/mL. Confirm absence of suspended and dissolved toxic metals[20] or filter and pass through an ion-exchange column.*

c) *Working solutions* with activities of 1, 5, and 25 μCi ^{14}C/2 mL. For fresh-water studies, use carrier-free radioactive carbonate. For marine-water studies, prepare by diluting carrier-free radioactive carbonate solution with NaCl dilution solution.

d) *Stock ampules:* Prepare ampules containing 2 mL of required working solution. Fill ampules and autoclave sealed ampules at 121°C for 20 min.[21]

* Chelex 100 or equivalent.

b. Procedure:

1) Using a pyrheliometer, obtain a record of incident solar radiation for the photoperiod.

2) Determine depth intervals for sampling and incubation as described above.

3) Use duplicate light and dark bottles at each depth. Also, use dark bottles or bottles harvested at time zero. Fill bottles with sample, add 2 mL radioactive carbonate solution (using a nonmetallic pipet) to the bottom of each bottle, and mix thoroughly by repeated inversion. The concentration of carbon-14 should be about 10 μCi/L in relatively productive waters, to 100 μCi/L or more in oligotrophic (open ocean) waters. To obtain statistical significance, have at least 1000 counts per minute (cpm) in the filtered sample. Take duplicate samples at each depth to determine initial concentration of inorganic carbon (CO_2, HCO_3^-, and CO_3^{2-}) available for photosynthesis (see Section 4500-CO_2). For estuarine and marine samples, estimate total inorganic carbon concentrations with a simple titration procedure[22] and make initial temperature, salinity, and pH measurements.

4) Incubate samples for up to 4 h. If measurements are required for the entire photoperiod, overlap 4-h periods from dawn until dusk. A 4-h incubation period may be sufficient if energy input is used as the basis for extrapolating incubation period to entire photoperiod. For incubation procedure, see 10200J.2*b*6).

5) After incubation, remove sample bottles and immediately place in the dark. Filter unpreserved samples promptly. Avoid sample preservation to avoid lysing cells or inadvertently including extracellular products.

6) Filter two portions of each sample through a membrane filter whose pore size is consistent with quantitative retention of plankton. Although the 0.45-μm pore filter usually is adequate, determine the sample-retention efficiency of a wide range of pore sizes immediately before analysis.[23,24] Apply about 30 kPa of vacuum during filtration. Excess vacuum may cause extensive cell rupture and loss of radioactivity through the membrane.[25] Use maximum sample volume consistent with rapid filtration (1 to 2 min), but do not clog filter.

7) Place membranes in HCl fumes for 20 min. Count filters as soon as possible, although extended storage in a desiccator is acceptable.

8) Determine radioactivity by counting with an end-window tube, windowless gas flow detector, or liquid scintillation counter.

9) Determine counting geometry of thin-window and windowless gas flow detectors.[26] Using three ampules of carbon-14, prepare a series of barium carbonate ($BaCO_3$) precipitates on tared 0.45-μm membrane filters, as directed below. The precipitates will contain the same amount of carbon-14 activity, but their thicknesses will range from 0.5 to 6.0 mg/cm^2. Dilute each ampule to 500 mL with a solution of 1.36 g Na_2CO_3/L CO_2-free distilled water. Pipet 0.5-mL portions into each of seven conical flasks containing 0, 0.5, 1.5, 2.5, 3.5, 4.5, and 5.5 mL, respectively, of a solution of 1.36 g Na_2CO_3/L CO_2-free distilled water. Add, respectively, 0.3, 0.6, 1.2, 1.8, 2.4, 3.0, and 3.6 mL 1.04% barium chloride ($BaCl_2$) solution. Let $BaCO_3$ precipitate stand 2 h with gentle swirling every half hour. Collect each precipitate on a filter (using an apparatus with a filtration area comparable to that of the samples). With suction, dry filters without washing; place in a desiccator for 24 h, weigh, and count. The counting rate increases exponentially as precipitate thickness decreases. Extrapolate graphically (or mathematically) to zero precipitate thickness and multiply the zero-thickness counting rate by 1000 to correct for ampule dilution. This represents the amount of activity added to each sample bottle used to determine the fraction of carbon-14 taken up in light and dark bottles.

c. Calculations:

1) Subtract the mean dark-bottle or time-zero sample count from the mean light-bottle counts for each replicate pair.

2) Determine the total dissolved inorganic carbon available for photosynthesis (carbonate, bicarbonate, and free CO_2) from pH and alkalinity measurements; make direct measurement of total CO_2 according to Section 4500-CO_2 or methods described in the literature.[27–30]

3) Determine quantity of fixed carbon as follows:

$$\text{mg fixed carbon/L} = \frac{\text{counting rate of filtered sample}}{\text{total activity added to sample}} \times \frac{300}{\text{volume filtered}} \times \text{mg/L initial inorganic carbon} \times 1.064\dagger$$

4) Integrate productivity for the entire depth of euphotic zone and express as grams fixed carbon per square meter per day [see 10200J.2*c*2)].

5) Using the solar radiation records and photosynthesis rates during incubation, adjust data to represent phytoplankton productivity for the entire photoperiod. If samples were incubated for less than the full photoperiod, apply a correction factor.

4. References

1. Gundersen, K. 1973. *In-situ* determination of primary production by means of the new incubator, ISIS. *Helgolander wiss. Meeresunters.* 24:465.
2. Burris, R.H., F.J. Eppling, H.B. Wahlin & P.W. Wilson. 1942. Studies of biological nitrogen fixation with isotopic nitrogen. *Proc. Soil Sci. Soc. Amer.* 7:258.
3. Neess, J.C., R.C. Dugdale, V.A. Dugdale & J.J. Goering. 1962. Nitrogen metabolism in lakes; I. Measurement of nitrogen fixation with N^{15}. *Limnol. Oceanogr.* 7:163.
4. Stewart, W.D.P., G.P. Fitzgerald & R.H. Burris. 1967. *In situ* studies on N_2 fixation using the acetylene reduction technique. *Proc. Nat. Acad. Sci.* 58:2071.
5. Stewart, W.D.P., G.P. Fitzgerald & R.H. Burris. 1970. Acetylene reduction assay for determination of phosphorus availability in Wisconsin lakes. *Proc. Nat. Acad. Sci.* 66:1104.
6. Goldman, C.R. 1968. Aquatic primary production. *Amer. Zoologist* 8:31.
7. Odum, H.T. 1957. Primary production measurements in eleven Florida springs and a marine turtle-grass community. *Limnol. Oceanogr.* 2:85.
8. Beyers, R.J. & H.T. Odum. 1959. The use of carbon dioxide to construct pH curves for the measurements of productivity. *Limnol. Oceanogr.* 4:499.
9. Gaarder, T. & H.H. Gran. 1927. Investigations of the production of plankton in Oslo Fjord. *Rapp. Proces-Verbaux. Reunions Cons. Perma. Int. Explor. Mer* 42:1.

† Correction for isotope effect

10. Steeman-Neilsen, E. 1952. The use of radioactive carbon (C-14) for measuring organic production in the sea. *J. Cons. Perma. Int. Explor. Mer* 18:117.
11. Williams, P.J. leB., R.C.T. Raine & J.R. Bryan. 1979. Agreement between the ^{14}C and oxygen methods of measuring phytoplankton production: Reassessment of the photosynthetic quotient. *Oceanol. Acta* 2:411.
12. Davies, J.M. & P.J. leB. Williams. 1984. Verification of ^{14}C and O_2 derived primary organic production using an enclosed system. *J. Plankton Res.* 6:457.
13. Ryther, J.H. 1956. Photosynthesis in the ocean as a function of light intensity. *Limnol. Oceanogr.* 1:61.
14. Fee, E.J. 1969. A numerical model for the estimation of photosynthetic production, integrated over time and depth, in natural waters. *Limnol. Oceanogr.* 14:906.
15. Steeman-Neilsen, E. 1964. Recent advances in measuring and understanding marine primary production. *J. Ecol.* 52(Suppl.):119.
16. Allen, M.B. 1956. Excretion of organic compounds by *Chlamydomonas. Arch. Mikrobiol.* 24:163.
17. Fogg, G.E. & W.D. Watt. 1965. The kinetics of release of extracellular products of photosynthesis by phytoplankton. *In* C.R. Goldman, ed. Primary Productivity in Aquatic Environments, Suppl. 18. Univ. California Press, Berkeley.
18. Wetzel, R.G. 1965. Necessity for decontamination of filters in C^{14} measured rates of photosynthesis in fresh waters. *Ecology* 46:540.
19. McAllister, C.D. 1961. Decontamination of filters in the C^{14} method of measuring marine photosynthesis. *Limnol. Oceanogr.* 6:447.
20. Carpenter, E.J. & J.S. Lively. 1980. Review of estimates of algal growth using ^{14}C tracer techniques. *In* P.G. Falkowski, ed. Primary Productivity in the Sea, Brookhaven Symp. Biol. No. 31. Plenum Press, New York, N.Y.
21. Strickland, J.D.H. & T.R. Parsons. 1968. A Practical Manual of Sea Water Analysis, Fish. Res. Board Can. Bull. No. 167. Queen's Printer, Ottawa, Ont.
22. Parsons, T.R., Y. Maita & C.M. Lalli. 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Pergamon Press, New York, N.Y.
23. Lasker, R. & R.W. Holmes. 1957. Variability in retention of marine phytoplankton by membrane filters. *Nature* 180:1295.
24. Holmes, R.W. & C.G. Anderson. 1963. Size fractionation of C^{14}-labelled natural phytoplankton communities. *In* C.H. Oppenheimer, ed. Symposium on Marine Microbiology. Charles C. Thomas, Springfield, Ill.
25. Arthur, C.R. & F.H. Rigler. 1967. A possible source of error in the C^{14} method of measuring primary productivity. *Limnol. Oceanogr.* 12:121.
26. Jitts, H.R. & B.D. Scott. 1961. The determination of zero-thickness activity in Geiger counting of C^{14} solutions used in marine productivity studies. *Limnol. Oceanogr.* 6:116.
27. Saunders, G.W., F.B. Trama & R.W. Bachmann. 1962. Evaluation of modified C^{14} technique for shipboard estimation of photosynthesis in large lakes, Publ. No. 8. Great Lakes Research Div., Univ. Michigan, Ann Arbor.
28. Dye, J.F. 1944. The calculation of alkalinities and free carbon dioxide in water by use of nomographs. *J. Amer. Water Works Assoc.* 36:859.
29. Moore, E.W. 1939. Graphic determination of carbon dioxide and the three forms of alkalinity. *J. Amer. Water Works Assoc.* 31:51.
30. Park, K., D.W. Hood & H.T. Odum. 1958. Diurnal pH variation in Texas bays and its application to primary production estimations. *Publ. Inst. Mar. Sci. Univ. Tex.* 5:47.