10200 PLANKTON*#(1)

10200 A. Introduction

The term ‘‘plankton’’ refers to those microscopic aquatic forms having little or no resistance to currents and living free-floating and suspended in natural waters. Planktonic plants, ‘‘phytoplankton,’’ and planktonic animals, ‘‘zooplankton,’’ are covered in this section. The phytoplankton (microscopic algae) occur as unicellular, colonial, or filamentous forms. Many are photosynthetic and are grazed upon by zooplankton and other aquatic organisms. Other organisms occurring in the same environment are dealt with elsewhere: zoosporic fungi in Section 9610F; aquatic hyphomycetes in Section 9610G; and bacteria in Part 9000. The zooplankton in fresh water comprise principally protozoans, rotifers, cladocerans, and copepods; a greater variety of organisms occurs in marine waters.

1. Significance

Plankton, particularly phytoplankton, long have been used as indicators of water quality.[1-4] Some species flourish in highly eutrophic waters while others are very sensitive to organic and/or chemical wastes. Some species develop noxious blooms, sometimes creating offensive tastes and odors[5] or anoxic or toxic conditions resulting in animal deaths or human illness.[6] The species assemblage of phytoplankton and zooplankton also may be useful in assessing water quality.[7]

Because of their short life cycles, plankters respond quickly to environmental changes, and hence their standing crop and species composition are more likely to indicate the quality of the water mass in which they are found. They strongly influence certain nonbiological aspects of water quality (such as pH, color, taste, and odor), and in a very practical sense, they are a part of water quality. Certain taxa often are useful in determining the origin or recent history of a given water mass. Because of their transient nature, and often patchy distribution, however, the utility of plankters as water quality indicators may be limited. Information on plankton as indicators is interpreted best in conjunction with concurrently collected, physicochemical and other biological data.

Planktonic organisms predominate in ponds, lakes, and oceans. Potamoplankton develop in large rivers with slow-moving waters that approach lentic conditions. Because their origin can be uncertain and the duration of their exposure to pollutants unknown, plankters generally are less valuable as water quality indicators in lotic than in lentic environments.

2. References

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10200 B. Sample Collection

1. General Considerations

The frequency and location of sampling is dictated by the purpose of the study.[1] Locate sampling stations as near as possible to those selected for chemical and bacteriological sampling to insure maximum correlation of findings. Establish a sufficient number of stations in as many locations as necessary to define adequately the kinds and quantities of plankton in the waters studied. The physical nature of the water (standing, flowing, or tidal) will influence greatly the selection of sampling stations. The use of sampling sites selected by previous investigators usually will assure the availability of historical data that will lead to a better understanding of current results and provide continuity in the study of an area.

In stream and river work, locate stations upstream and downstream from suspected pollution sources and major tributary streams and at appropriate intervals throughout the reach under investigation. If possible, locate stations on both sides of the river because lateral mixing of river water may not occur for great distances downstream. In a similar manner, investigate tributary streams suspected of being polluted but take care in the interpretation of data from a small stream because much of the plankton may be periphytic in origin, arising from scouring of natural substrates by the flowing water. 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. The depth from which water is discharged from upstream stratified reservoirs also can affect the nature of the plankton.

Because water of rivers and streams usually is well mixed vertically, subsurface sampling, i.e., the upper meter or a composite of two or more strata, often is adequate for collection of a representative sample. There may be problems caused by stratification due to thermal discharges or mixing of warmer or colder waters from tributaries and reservoirs. Always sample in the main channel of a river and avoid sloughs, inlets, or backwater areas that reflect local habitats rather than river conditions. 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.

If it can be determined or correctly assumed that the plankton distribution is uniform and normal, use a scheme of random sampling to accomodate statistical testing. Include both random selection of sampling sites and transects as well as the random collection of samples at each selected site. On the other hand, if it is known or assumed that plankton distribution is variable or patchy, include additional sampling sites, collect composite samples, and increase sample replication. Use appropriate statistical tests to determine population variability.

In sampling a lake or reservoir use a grid network or transect lines in combination with random procedures. Take a sufficient number of samples to make the data meaningful. Sample a circular lake basin at strategic points along a minimum of two perpendicular transects extending from shore to shore; include the deepest point in the basin. Sample a long, narrow basin at several points along a minimum of 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. Sample a large bay along several parallel transects originating near shore and extending to the lake proper. Because many samples are required to appraise completely the plankton assemblage, it may be necessary to restrict sampling to strategic points, such as the vicinity of water intakes and discharges, constrictions within the water body, and major bays that may influence the main basin.

In lakes, reservoirs, and estuaries where plankton populations can vary with depth, collect samples from all major depth zones or water masses. The sampling depths will be determined by the water depth at the station, the depth of the thermocline or an isohaline, or other factors. In shallow areas of 2 to 3 m depth, 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 pyncocline. Depth intervals for sampling vary for estuaries of different sizes and depths, but use depths representative of the vertical range. Composite sampling above and below the pyncocline often is used. In marine sampling, the intent and scope of the study will determine the collection extent.

Over the continental shelf, take samples at stations approximately equidistant from the shore seaward. Take a vertical series from surface to near bottom at each station, gradually adding more stations across the shelf. It is important to sample the entire vertical range over a continental 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 and to deeper depths for zooplankton. Sampling depths vary, but often are at 10- to 25-m intervals above the thermocline, then at 100- to 200-m intervals below the thermocline to 1000 m, and thereafter at 500- to 1000-m intervals.

Samples usually are referred to as ‘‘surface’’ or ‘‘depth’’ (subsurface) samples. The latter are samples taken from some stated depth, whereas surface samples may be interpreted as samples collected as near the water surface as possible. A ‘‘skimmed’’ sample of the surface film plankton (neuston)[2] can be revealing; however, ordinarily do not include a disproportionate quantity of surface film in a surface sample because a neustonic flora[3] as well as plankton often are trapped on top or at the surface film together with pollen, dust, and other detritus. Various

methods have been used for sampling surface organisms.

Sampling frequency depends on the intent of the study as well as the range of seasonal fluctuations, the immediate meteorological conditions, adequacy of equipment, and availability of personnel. Select a sampling frequency at some interval shorter than community turnover time. This requires consideration of life-cycle length, competition, predation, flushing, and current displacement. Frequent plankton sampling is desirable because of normal temporal variability and migratory character of the plankton community. Daily vertical migrations occur in response to sunlight, and random horizontal migrations or drifts are produced by winds, shifting currents, and tides. Ideally, collect daily samples and, when possible, sample at different times during the day and at different depths. When this is not possible, weekly, biweekly, monthly, or even quarterly sampling still may 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 a station within an estuary includes a vertical series of samples taken from the surface, across the pyncocline, 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.

A useful series of monographs on oceanographic methodology has been published.[4-7] Representative taxonomic references for estuarine and marine phytoplankton include diatoms,[8-11] dinoflagellates,[12-14] coccolithophores,[15] and cyanophyceae[16] (cyanobacteria).

2. Sampling Procedures

Once sampling locations, depths, and frequency have been determined, prepare for field sampling. Label sample containers with sufficient information to avoid confusion or error. On the label indicate date, cruise number, sampling station, study area (river, lake, reservoir), type of sample, and depth. Use waterproof labels. When possible, enclose collection vessels in a protective container to avoid breakage. If samples are to be preserved immediately after collection, add preservative to container before sampling. Sample size depends on type and number of determinations to be made; the number of replicates depends on statistical design of the study and statistical analyses selected for data interpretation. Always design a study around an objective with a 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, and other significant observations. Engineer's field notebooks with waterproof paper are very suitable. Field data are invaluable when analytical results are interpreted and often help to explain unusual changes caused by the variable character of the aquatic environment. Collect coincident samples for chemical analyses to help define environmental variations having a potential effect on plankton.

a. Phytoplankton: In oligotrophic waters or where phytoplankton densities are expected to be low collect a sample of up to 6 L. For richer, eutrophic waters collect a sample of 0.5 to 1 L.

Because of their small size, nannoplankton and picoplankton can pass through collection

nets, making nets unsuitable for most phytoplankton sampling.

For qualitative and quantitative evaluations collect whole (unfiltered and unstrained) water samples with a water 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 close by dropping a weight, called a messenger, which slides down the supporting wire or cord and trips the closing mechanism. If possible, obtain composite samples from several depths or pool samples from one depth from several casts. The most commonly used samplers that operate on this principle are the Kemmerer,[17] Van Dorn[18] (Figure 10200:1), Niskin, and Nansen samplers.

Because these samplers collect whole water samples, all size classes of phytoplankton are collected. Different size categories of phytoplankton can be separated by subsequently filtering these whole water samples through netting of the appropriate mesh size. Select appropriate mesh sizes for concentrating the various size categories of phytoplankton typical of the aquatic system under study.[19,20]

The Van Dorn usually is the preferred sampler for standing crop, primary productivity, and other quantitative determinations because its design offers no inhibition to free flow of water through the cylinder. In deep-water situations, the Niskin bottle is preferred. It has the same design as the Van Dorn sampler except that the Niskin sampler can be cast in a series on a single line for simultaneous sampling at multiple depths with the use of auxiliary messengers. Because the triggering devices of these samplers are very sensitive, 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. Use polyethylene or glass sample storage bottles. Metallic ion contamination can lead to significant errors when algal assays or productivity measurements are made.

For shallow waters use the Jenkins surface mud sampler,[21] one of the bottle samplers modified so that it is held horizontally,[22] or an appropriate bacteriological sampler.[23]

For greater speed of collection and to obtain large, accurately measured quantities of organisms, use a pump. Diaphragm and peristaltic pumps are less damaging to organisms than centrifugal pumps.[24] Centrifugal pump impellers can damage organisms as can passage through the hose.[25] Lower a weighted hose, attached to a suction pump, to the desired depth, and pump water to the surface. The pump is advantageous because it supplies 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 TFE tubing.

To examine live samples fill containers partially and store in a refrigerator or ice chest in the dark, or preferably, hold at ambient temperature. Examine specimens promptly after collection.

If it is impossible to examine living material or if phytoplankton are to be counted later, preserve the sample. For a sample that will be preserved, fill the container completely. The most suitable phytoplankton preservative is Lugol’s solution, which can be used for most forms

including the naked flagellates. Unfortunately, acidic Lugol's solution (or formalin) dissolves the coccoliths of Coccolithophores, which are common in estuarine and marine waters.

Lugol's solution: 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 and buffered formaldehyde to a minimum of 2.5% final concentration after 1 h. 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.[26] Utermohl's[27] 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 allows preservation of Coccolithophores, but would be less effective for other flagellates.

Other acceptable preservatives are:

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. 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 must be added.[28]

''M^3'' 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 the iodine). Add 20 mL fixative to 1 L sample and store in the dark.

Glutaraldehyde: Preserve samples by adding neutralized glutaraldehyde to yield a final concentration of 1 to 2%.

Other commonly used preservatives include 95% alcohol, and 6-3-1 preservative, (6 parts water, 3 parts 95% alcohol, and 1 part formalin). Use equal volumes of preservative and sample.

To retain color in preserved plankton, store samples in the dark or add 1 mL saturated copper sulfate ($CuSO_4$) solution/L.

Most preservatives distort and disrupt certain cells,[29,30] especially those of delicate forms such as *Euglena, Cryptomonas, Synura, Chromulina,* and *Mallamonas*. Lugol's iodine solution usually is least damaging for these phytoflagellates. To become familiar with live specimens and preservation-caused distortions, use reference collection from biological supply houses or consult experienced co-workers.

b. Zooplankton: The choice of sampler depends on the type 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.

For collecting microzooplankton (20 to 200 μm) such as protozoa, rotifers, and immature microcrustacea, use the bottle samplers described for phytoplankton. The small zooplankters usually are sufficiently abundant to yield adequate samples in 5- to 10-L bottles; however, composite samples over depth and time are recommended. Water bottle samplers are suitable especially for discrete-depth samples. If depth-integrated samples are desired, use pumps or nets. The 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 for enumeration of these forms.

Bottle samplers usually are unsuitable for collecting larger zooplankton, such as mature microcrustacea, that, unlike the smaller forms, are much less numerous and are sufficiently agile to avoid capture. Although comparatively large water volumes, and consequently adequate numbers of microcrustacea, can be sampled with a pump, 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[31] operates on the same principle as the water bottle samplers but is generally larger (10 L). The larger size makes the Juday trap 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. Because it is constructed of metal it is unsuited if heavy metals analyses are required.

The Schindler-Patalas trap[32] (Figure 10200:2) usually is preferred to the Juday trap because it is constructed of clear acrylic plastic and 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 and thus 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, but the No. 20 mesh net is used most often.

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

The mesh size, type of material, orifice size, length, hauling method, type of tow, and volume sampled will depend on the particular needs of the study.[33,34] Type of netting and mesh size determine filtration efficiency, clogging tendencies, velocity, drag, and the condition of the sample after collection. Silk, formerly the common mesh material in plankton nets, is not recommended because of shrinkage of mesh openings and rotting with age. Nylon monofilament mesh is preferred because of its mesh size accuracy and durability. Nylon nets of different mesh sizes still are labelled by the silk rating system: characteristics of commonly used nylon plankton nets are listed in Table 10200:I. Finer mesh sizes clog more readily than coarser mesh; a compromise must be made between mesh size small enough to retain desired organisms

effectively and a size large enough to preclude a serious clogging problem. If clogging occurs, reduce its effects by decreasing the length of tow.

The maximum volume, V_M, of water that can be filtered through a net during a vertical tow can be estimated with the formula,

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

where:

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

This volume is a maximum because clogging of the net's meshes 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.[35,36] Keep net towing distance as short as practical to alleviate clogging. If the net has a pronounced green or brown color after towing, clogging probably has occurred.

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 flow reduction associated with the towing bridle).[37] Equip meter with lock mechanisms to prevent it turning in reverse or while in air. Record flow-meter readings before and after collecting sample. Calculate filtration efficiency, E, from:

$$E = V_A/V_M$$

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 because filtration efficiency is nonuniform during the tow.[34]

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 the filtration characteristics of conical nets 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 exemplifies a commonly used net with a truncated cone. For good filtration characteristics the ratio of filtering area of net 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 10200:3) 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 the 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 depth of the net 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 the vertical as possible by controlling the boat's speed null against the wind drift, or wherever feasible, do vertical hauls from an anchored boat.

Vertical and oblique tows collect a composite sample, whereas 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 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 samplers is available (see Figure 10200:4), use the Clarke-Bumpus sampler[38] 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 method of 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 by bucket and filter it through the appropriate mesh size. Select sample size based on concentration of zooplankters.

Give plankton nets proper care and maintenance. Do not let particulate matter 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. Periodically clean with a warm soap solution. Because nylon net material 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 growths of aquatic vegetation. To obtain an integrated sample for the entire water column in such areas, use a length of light-weight rubber or polyethylene tubing with netting attached over one end and a rope on the other.[39] Attach netting by tape or rubber bands that will stay in place in water, but can be removed easily after sampling. Use tubing of 5- to 10-cm diam and long enough to reach from the surface to the bottom. Lower the open end (the end with the rope attached) until it almost touches the bottom. Then pull this end up using the rope and keep the covered end above the water surface. When the open end is out of the water, let the end with the netting fall back into the water, pull the tubing into the boat, open end first, and let the water in the tube drain out 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 assure that all the zooplankton is collected. This method is not limited to areas with aquatic vegetation. It provides 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.

Preserve zooplankton samples with 70% ethanol or 5% buffered formalin. 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 preservative may cause distortion of 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.[40] Bouin's fixative produces reasonable results for soft-bodied microzooplankton.[41] This fixative is picric acid saturated in calcium carbonate-buffered formaldehyde containing 5% (v/v) acetic acid. 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 such as carbonated water, menthol-saturated water, or neosynephrine to prevent or reduce contraction or distortion of organisms, especially rotifers, cladocerans, and many marine invertebrates.[42,43] Adding a few drops of detergent prevents clumping of preserved organisms. 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.

3. References

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10200 H. Chlorophyll

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

The three methods for determining chlorophyll *a* in phytoplankton are the spectrophotometric,[3-5] the fluorometric,[6-8] and the 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 of the overlap of the absorption and fluorescence bands of co-occurring accessory pigments and chlorophyll degradation products.

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 does chlorophyll *a*. If these pheopigments are present, significant errors in chlorophyll *a* values will result. Pheopigments can be measured either by spectrophotometry or fluorometry, but in marine and freshwater environments the fluorometric method is unreliable when chlorophyll *b* co-occurs. Upon acidification of chlorophyll *b*, the resulting fluorescence emission of pheophytin *b* is coincident with that of pheophytin *a*, thus producing underestimation and overestimation of chlorophyll *a* and pheopigments, respectively.

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

Conduct work with chlorophyll extracts in subdued light to avoid degradation. Use opaque containers or wrap with aluminum foil. The pigments are extracted from the plankton concentrate with aqueous acetone and the optical density (absorbance) of the extract is determined with a spectrophotometer. The ease with which the chlorophylls are removed from the cells varies considerably with different algae. To achieve consistent complete extraction of the pigments, disrupt the cells mechanically with a tissue grinder.

Glass fiber filters are preferred for removing algae from water. The glass fibers assist in breaking 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 where these factors are irrelevant.

a. Equipment and reagents:

1) *Tissue grinder:**#(2) 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 having grooves in the TFE tip.

2) *Clinical centrifuge.*

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

4) *Filtration equipment,* filters, glass fiber†#(3) or membrane (0.45-µm porosity, 47-mm diam); vacuum pump; solvent-resistant disposable filter assembly, 1.0-µm pore size;‡#(4) 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. Samples on filters taken from water having pH 7 or higher may be placed in airtight plastic bags and stored frozen for 3 weeks. Process samples from acidic water promptly after filtration to prevent possible chlorophyll degradation from residual acidic water on filter. Use glassware and cuvettes that are clean and acid-free.

2) Place sample in a tissue grinder, cover with the 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. Use solvent sparingly and avoid excessive dilution of pigments. Steep samples at least 2 h at 4°C in the dark. Glass fiber filters of 25- and 47-mm diam§#(5) 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 (to minimize retention of extract in filter and filter holder, force 1 to 2 mL air through the filter after the extract), or by centrifuging in closed tubes for 20 min at 500 g. Decant clarified extract into a clean, calibrated, 15-mL, screw-cap centrifuge tube and measure total volume. Proceed as in 2, 3, 4, or 5 below.

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.* Addition of 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* by acidification, the absorption-peak-ratio (OD664/OD665) of 1.70 is used in correcting the apparent chlorophyll *a* concentration for pheophytin *a.*

Samples with an OD664 before/OD665 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 OD665 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 optical density (OD) at 750 and 664 nm. Acidify extract in the cuvette with 0.1 mL 0.1*N* HCl. Gently agitate the acidified extract and read OD at 750 and at 665 nm, 90 s after acidification. The volumes of extract and acid and the time after acidification are critical for accurate, consistent results.

The OD664 before acidification should be between 0.1 and 1.0. For very dilute extracts use cuvettes having a longer path length. If a larger cell is used, add a proportionately larger volume of acid. Correct OD obtained with larger cuvettes to 1 cm before making calculations.

Subtract the 750-nm OD value from the readings before (OD 664 nm) and after acidification (OD 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 = optical densities 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 optical density (OD) at 750, 664, 647, and 630 nm. Choose a cell path length or dilution to give OD664 between 0.1 and 1.0.

Use the optical density readings at 664, 647, and 630 nm to determine chlorophyll *a*, *b*, and *c*, respectively. The OD reading at 750 nm is a correction for turbidity. Subtract this reading from each of the pigment OD values of the other wavelengths before using them in the equations below. Because the OD of the extract at 750 nm is very 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 by 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]

$$\text{a) } C_a = 11.85(OD664) - 1.54(OD647) - 0.08(OD630)$$

$$\text{b) } C_b = 21.03(OD647) - 5.43(OD664) - 2.66(OD630)$$

$$\text{c) } C_c = 24.52(OD630) - 7.60(OD647) - 1.67(OD664)$$

where:

C_a, C_b, and C_c = concentrations of chlorophyll *a*, *b*, and *c*, respectively, mg/L, and OD664, OD647,

and OD630 = 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 and thus smaller samples can be used. Calibrate the fluorometer spectrophotometrically with a sample from the same source to achieve acceptable results. 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 continuous measurement of 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 1*a* and 2*a* above:

*Fluorometer,*i#(6) 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 1*b* above.

1) Calibrate fluorometer with a chlorophyll solution of known concentration 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 discussion under Section 10200H and ¶ 2*b* above.

1) Equipment and reagents—In addition to those listed under ¶ 1*a* and ¶ 2*a* above, pure chlorophyll *a*##(7) (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 ¶ 3*b*1). 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 fluorescence reading obtained before acidification by the reading 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 with the following equations:[8,24]

$$\text{Chlorophyll } a, \text{ mg/m}^3 = F_s \frac{r}{r-1}(R_b - R_a)\frac{V_e}{V_s}$$

$$\text{Pheophytin } a, \text{ mg/m}^3 = F_s \frac{r}{r-1}(rR_a - R_b)\frac{V_e}{V_s}$$

where:

F_s = conversion factor for sensitivity setting *S* (see ¶ 2*b*, above),
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**#(8) with output impedance of 36 ma/W at 675 nm and a high-sensitivity door. Place neutral density filter (40–60N) in the rear light path,††#(9) 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 through glass fiber filter‡‡#(10) or centrifuge. Measure fluorescence as described in Section 10200H.3 and estimate concentrations as in ¶ 3*c*. Because humic substances interfere, if they are present filter a sample portion (see Section 10200H.1*b*) and process filtrate with sample. Subtract filtrate (blank) fluorescence from that of sample.

6. References

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Endnotes

1 (Popup - Footnote)
* APPROVED BY STANDARD METHODS COMMITTEE, 1994.

2 (Popup - Footnote)
* Kontes Glass Co., Vineland, NJ 08360: Glass/glass grinder, Model No. 8855: Glass/TEE grinder, Model 886000; or equivalent.

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

4 (Popup - Footnote)
‡ Gelman Acrodisc or equivalent.

5 (Popup - Footnote)
§ GF/F or equivalent.

6 (Popup - Footnote)
¡ Model 10-005, Turner Designs, Sunnyvale, CA or equivalent.

7 (Popup - Footnote)
Purified chlorophyll *a*, Sigma Chemical Company, St. Louis, MO, or equivalent.

8 (Popup - Footnote)
** Hammamatsu Corp., Middlesex, NJ, or equivalent.

9 (Popup - Footnote)
†† If using Model 10-005, Turner Designs, or equivalent.

10 (Popup - Footnote)
‡‡ Whatman GF/F or equivalent.