PART 10000

BIOLOGICAL EXAMINATION

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10010 Introduction ..... Michael K. Hein
10200 Plankton ..... Harold G. Marshall
10300 Periphyton ..... Robert G. Wetzel
10400 Macrophytes ..... Miles M. Smart
10500 Benthic Macroinvertebrates ..... Donald J. Klemm
10600 Fishes ..... Donald M. Baltz
10700 Benthic Meiofauna ..... Byron J. Adams
10750 Nematological Examination ..... Byron J. Adams
10900 Identification of Aquatic Organisms ..... Donald J. Reish

Summary of Major Changes since 1998

An introductory section entitled Benthic Meiofauna (10700) is new.
The section on Nematodes (formerly 10550) was renumbered 10750 and moved accordingly.

10010 INTRODUCTION*

Physical and chemical characteristics of water bodies affect the abundance, species composition, stability, productivity, and physiological condition of aquatic organism populations. Biological methods used for assessing water quality include the collection, counting, and identification of aquatic organisms; biomass measurements; measurements of metabolic activity rates; measurements of the toxicity, bioconcentration, and bioaccumulation of pollutants; and processing and interpretation of biological data.

Information from these methods may serve one or more of the following purposes:

1. To explain the cause of color, turbidity, odor, taste, or visible particulates in water;
2. To aid in the interpretation of chemical analyses, for example, in relating the presence or absence of certain biological forms to oxygen deficiency or supersaturation in natural waters;
3. To identify the source of a water that is mixing with another water;
4. To explain the clogging of pipes, screens, or filters, and to aid in the design and operation of water and wastewater treatment plants;
5. To determine optimum times for treatment of surface water with algicides and to monitor treatment effectiveness;
6. To determine the effectiveness of drinking water treatment stages, to aid in determining effective chlorine dosage within a water treatment plant, and to indicate treatment problems or deficiencies;
7. To identify the nature, extent, and biological effects of pollution;
8. To indicate the progress of self-purification in bodies of water;
9. To aid in determining the condition and effectiveness of unit processes and biological wastewater treatment methods in a wastewater treatment plant;
10. To document short- and long-term variability in water quality caused by natural phenomena and/or human activities;
11. To provide data on the status and trends of an aquatic system;
12. To correlate the biological mass or components with water chemistry or conditions. (NOTE: A statistical correlation may not always signify a cause-and-effect relationship, because of the presence of confounding variables or unknown covariates.)

The specific nature of a problem and the reasons for collecting samples will dictate which communities of aquatic organisms will be examined and which sampling and analytical techniques will be used.

The following communities of aquatic organisms are considered in specific sections that follow:

1. PLANKTON (Section 10200): A community of plants (phytoplankton) and animals (zooplankton), usually drifting or suspended in water, nonmotile or insufficiently motile to overcome transport by currents. In fresh water they generally are small or microscopic in size; in the marine or estuarine environment, large plankters are often observed.
2. PERIPHYTON (Section 10300): A community of microscopic plants and animals associated with the surfaces of submersed objects. Some are attached, some move about. Many of the protozoa and other minute invertebrates and algae found in the plankton also occur in the periphyton.
3. MACROPHYTES (Section 10400): Large plants of all types. They are sometimes attached to the bottom (benthic), sometimes free-floating, sometimes totally submersed, and sometimes partly emergent. Complex vascular plants usually have true roots, stems, and leaves. Macroalgae are simpler but may have stem- and leaf-like thalli.
4. MACROINVERTEBRATES (Section 10500): The invertebrates defined here are those retained by the US Standard No. 30 sieve (0.6-mm openings). They are generally bottom-dwelling organisms (benthos) that live at least part of their life cycles within or upon available substrates in lentic (standing) and lotic (flowing) bodies of water.
5. FISHES (Section 10600): Vertebrates of diverse morphology, ecology, and behavior, inhabiting (and generally limited to) aquatic systems. They have fins and gills.
6. BENTHIC MEIOFAUNA (Section 10700): The invertebrates defined here are those that pass through a US Standard No. 35 sieve (0.5-mm openings) and are retained on a No. 230 sieve (0.063-mm openings) or No. 325 sieve (0.044-mm openings). Benthic meiofauna include nematodes (Section 10750), express an extreme range of morphological and life history diversity, and have free-living, parasitic, or symbiotic trophic habits.

Large numbers of bacteria and fungi are present in the plankton and periphyton and constitute an essential element of the total aquatic ecosystem. Although their interactions with living and dead organic matter profoundly affect the larger aquatic organisms, techniques for their investigation are not included herein (see Part 9000).

Amphibians, aquatic reptiles, birds, and mammals are useful in monitoring long-term changes in water quality and the presence of toxic substances (see Section 8930). These organisms may be affected directly or indirectly by spills or other discharges of pollutants.

Field observations are indispensable for meaningful biological interpretations, but many biological factors cannot be evaluated directly in the field. These must be analyzed as field data or field samples within the laboratory. Because the significance of the analytical result depends upon the representativeness of the sample, attention is given to field methods as well as to associated laboratory procedures.

Before sampling begins, clearly define study objectives. For example, the frequency of a repetitive sampling program may vary from hourly, for a detailed study of diel variability, to every third month (quarterly) for a general assessment of seasonal conditions. The scope of the study must be adjusted to limitations in personnel, time, and budget. Before the development of a study plan, examine historic data for the study area and conduct a literature search to identify related work elsewhere.

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* Approved by Standards Methods Committee, 2001.
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Whenever practicable, biologists should collect their own samples. Much of the value of an experienced biologist lies in personal observations of conditions in the field and in the ability to recognize signs of environmental changes as reflected in the various aquatic communities. Detection of environmental changes also depends on the accurate and consistent identification of the organisms present. Parts 10600 (Fishes), 10750 (Nematological Examination), and 10900 (Identification of Aquatic Organisms) include basic keys, drawings of organisms, and selected references to assist the biologists in identifying the plants and animals collected in field surveys. However, these cannot fully replace examination by taxonomic experts for key groups.

The primary orientation of Part 10000 is toward field collection and associated laboratory analyses to aid in determining the status of aquatic communities under field conditions and to aid in interpreting the influence of past and present environmental conditions. Principal emphasis is on methods and equipment, rather than on interpretation or application of results. The complex interrelationships existing in an aquatic environment often require many different field and laboratory procedures; consequently, frequent cross-references between sections have been made.

Many other types of studies may be, and are being, conducted that are oriented more toward laboratory research. Such laboratory studies will develop further basic knowledge of community and/or organism responses under controlled conditions and will aid in predicting effects of future changes in environmental conditions on the aquatic communities. However, such studies are not within the scope of Part 10000.

10200 PLANKTON*

10200 A. Introduction

The term "plankton" refers to those 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. Most of these 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, have long 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 may be useful in assessing water quality.[7–13] Plankton also may be used as indicators of relative treatment efficiencies of water treatment plants and the probability of groundwater sources under the direct influence of surface water.[14–19]

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, oxygen concentration, 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.

Algal blooms frequently occur during the summer and early fall months. These are most common in eutrophic waters and may be associated with periods of reduced oxygen concentrations in the water, unpleasant ordors, and, at times, fish deaths. There are also cyanobacteria and dinoflagellate taxa (among others) that produce toxins that may be harmful to fish, and other animals in contact with waters containing the toxins. Several of these toxins have been found in seafood and drinking water, and have produced illness and fatalities in humans.[20–22]

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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

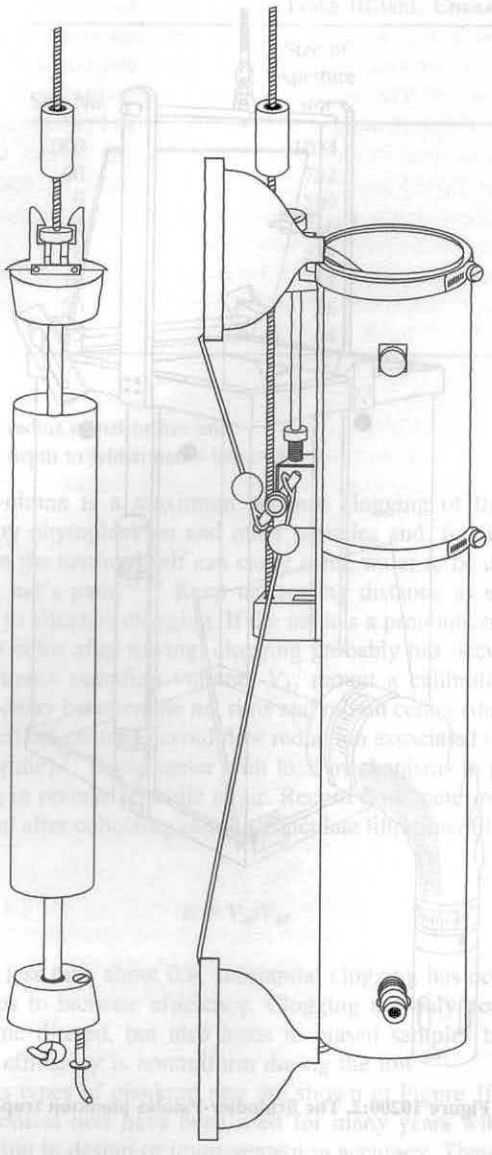

Figure 10200:1. Structural features of common water samplers, Kemmerer (left) and Van Dorn (right).

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, leaving sufficient air space to permit mixing the sample by shaking. 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.

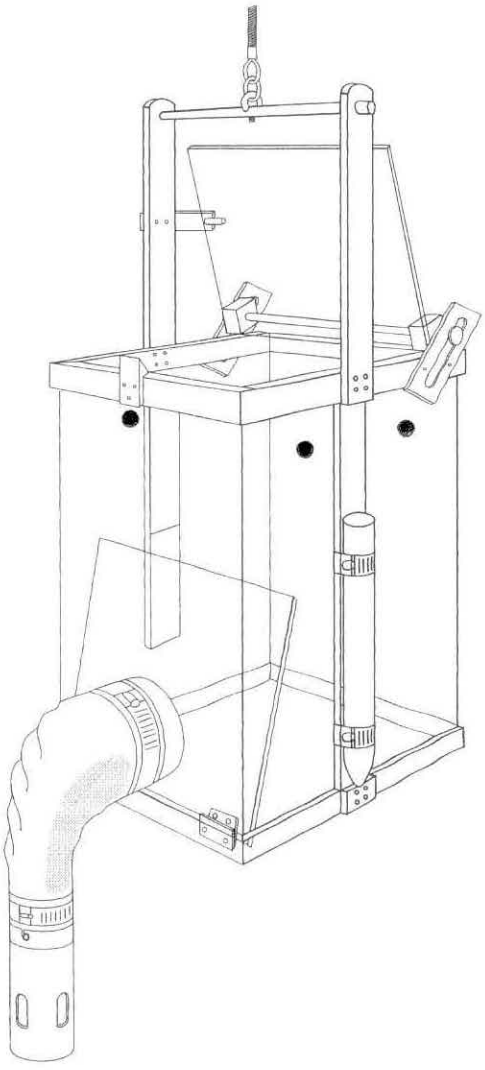

Figure 10200:2. The Schindler-Patalas plankton trap.

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:

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	Nannoplankton

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 ex-

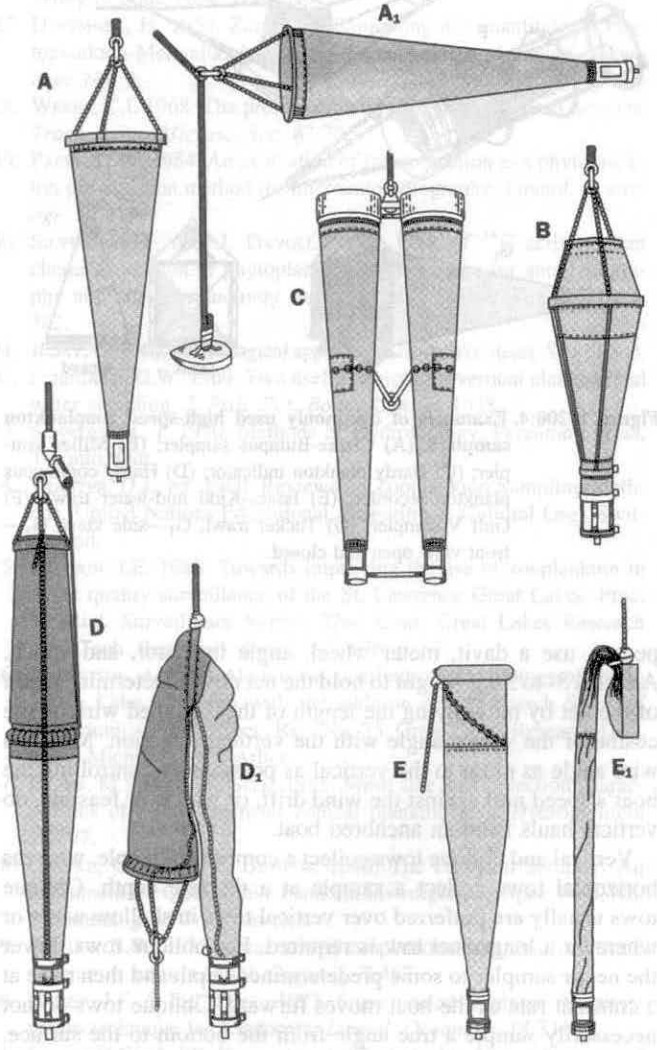

Figure 10200:3. Examples of commonly used plankton sampling nets. (A) Simple conical tow-net; A—rigged for vertical tows; A_1—for oblique or horizontal tows; (B) Wisconsin (Birge) tow-net with truncated cone to improve filtration efficiency; (C) Bongo net, can be fitted with flow meters and opening/closing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

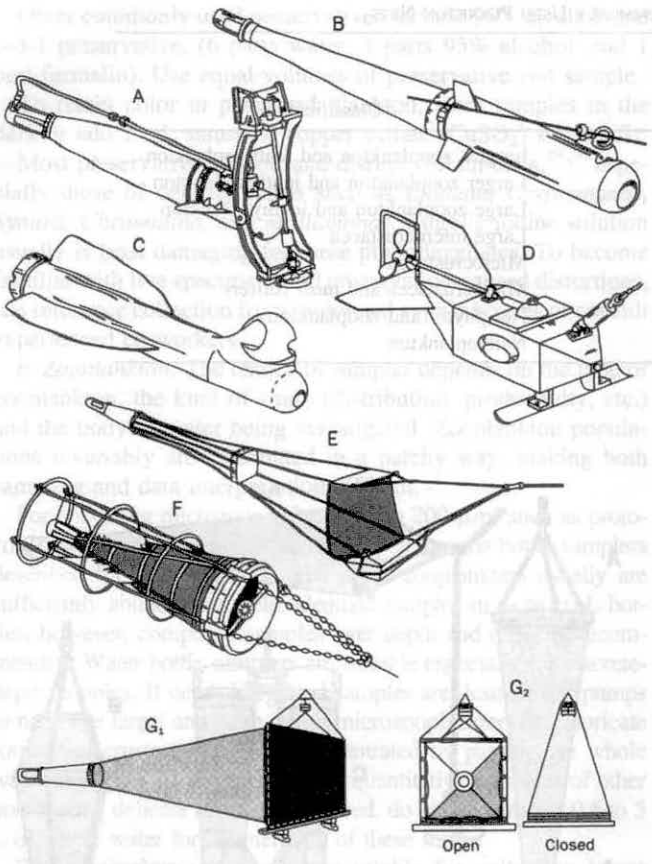

Figure 10200:4. Examples of commonly used high-speed zooplankton samplers. (A) Clarke-Bumpus sampler; (B) Miller sampler; (C) Hardy plankton indicator; (D) Hardy continuous plankton recorder; (E) Issacs-Kidd mid-water trawl; (F) Gulf V sampler; (G) Tucker trawl, G_1—side view, G_2—front view open and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

pected, 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.

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10200 C. Concentration Techniques

The organisms contained in water samples sometimes must be concentrated in the laboratory before analysis. Three techniques for concentrating phytoplankton, namely, sedimentation, membrane filtration, and centrifugation, are described below. A special technique for zooplankton also is given.

1. Sedimentation

Sedimentation is the preferred method of concentration because it is nonselective (unlike filtration) and nondestructive (unlike filtration or centrifugation), although many of the picoplankton, the smaller nannoplankton, and actively swimming flagellates (in unpreserved samples) may not settle completely. The volume concentrated varies inversely with the abundance of organisms and is related to sample turbidity. It may be as small as 1 mL for use with an inverted microscope or as large as 1 L for general phytoplankton and zooplankton enumeration.

Allow 1 h settling/mm of column depth. For a treated sample (10 mL liquid detergent/L) allow about 0.5 h settling/mm depth.[1] The sample may be concentrated in a series of steps by quantitatively transferring the sediment from the initial container to sequentially smaller ones. Use cylindrical settling chambers with thin, clear glass bottoms. Fill settling chambers without forming a vortex, keep them vibration-free, and move them carefully to avoid nonrandom distribution of settled matter. Carefully siphon or decant the supernatants to obtain the desired final volume (5 mL for diatom mounts). Store the concentrated sample in a closed, labeled glass vial.

2. Membrane Filtration

The filtration method permits use of high magnification for enumerating small plankters including flagellates and cyanobacteria. However, delicate forms such as "naked" flagellates are distorted by even gentle filtration. When populations are dense and the content of detritus is high, the filter clogs quickly and silt may crush the organisms or obscure them from view.

Pour a measured volume of well-mixed sample into a funnel equipped with a membrane filter having a pore diameter of 0.45 μm. Apply a vacuum of less than 50 kPa to the filter until about 0.5 cm of sample remains on filter. Break vacuum, then apply low vacuum (about 12 kPa) to remove remaining water but not to dry the filter.

For samples with a low phytoplankton and silt content the method does not require counting of individual plankters to assemble enumeration data and it 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, permitting the removal of the filter and transfer of plankton to the slide.[3,4]

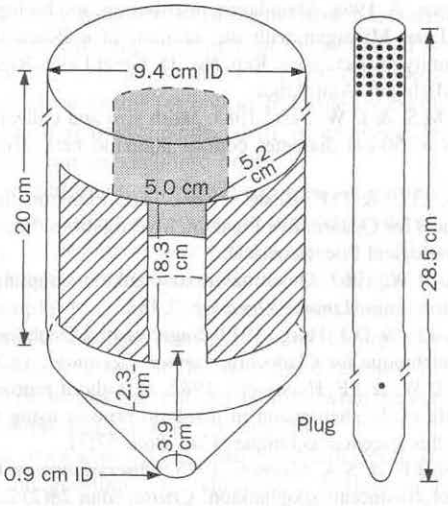

Figure 10200:5. Filter funnel for concentrating zooplankton. This device, originally designed for rotifers, can be modified for other zooplankters by changing the dimensions and mesh size. (After Likens and Gilbert.[5])

3. Centrifugation

Plankton can be concentrated by 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 it may be desirable to continue its use in existing programs to assure continuity with previously collected data. Although centrifugation accelerates sedimentation, it may damage fragile organisms.

4. Zooplankton Concentration

Zooplankton samples often need to be concentrated in the field, especially when large water bottles or pump methods of sampling are used. Moreover, samples obtained by nets or other methods sometimes need to be concentrated further for storage or preparation for examination. When only small volume reductions are needed, pour sample back into the bucket of traps or nets. In processing large volumes of water as with pump sampling, use larger plankton buckets or funnels with greater 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.[5] The volume of the apparatus and the mesh size depend on volume of water to be filtered and size of organisms to be retained. The mesh size of the filter funnel normally is the same as that of the net or other field sampling device.

5. References

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10200 D. Preparing Slide Mounts

1. Phytoplankton Semi-Permanent Wet Mounts

Agitate the settled sample concentrate and withdraw a subsample with an accurately calibrated pipet. Clean pipet regularly. To prepare wet mounts transfer 0.1 mL to a glass slide, place a cover slip over the sample, and ring the cover slip with an adhesive such as clear nail polish to prevent evaporation. For semipermanent mounts, add a few drops of glycerin to the slide. As the sample ages the water evaporates, leaving the organisms imbedded 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 Mounts

a. Membrane filter mounts: Place two drops of immersion oil on a labeled slide. Immediately after filtering place the filter on top of the oil with a pair of forceps and add two drops of oil on top of the filter. The oil impregnates the filter and makes it transparent. Impregnation time is 24 to 48 h. This procedure can be completed in 1 to 2 h by applying heat (70°C). Once the filter has cleared, place a few additional drops of oil on it and cover with a cover slip. The mounted filter is now ready for microscopic examination. 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. Sedimented slide mounts: Two techniques are available for making permanent, resin mounts of natural phytoplankton that has been deposited by sedimentation on a microscope slide or cover glass and dehydrated by ethanol vapor substitution.[2,3]

3. Diatom Mounts

Samples concentrated for diatom analysis by settling or centrifugation may contain dissolved materials, such as marine salts, formalin, and detergents, that will leave interfering residues. Wash well with distilled water before slide preparation. Transfer several drops of washed concentrate by means of 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 enough to cause boiling (use a large-bore pipet or dropper to prevent possible selective filtration, thus exclusion, of larger forms or those forming colonies or chains). If the cleaned material is very concentrated, improve distribution of diatoms by adding the drops to a cover glass already flooded with distilled water. Evaporate to dryness. Repeat addition and evaporation until a sufficient quantity of sample has been transferred to the cover glass, but avoid 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 hot plate at 300 to 500°C; alternatively, use a muffle furnace. This usually requires 20 to 45 min. Mount as described below.

Treat samples concentrated for diatom analysis by membrane filtration as described by Patrick and Reimer.[4] Mix equal volumes of conc nitric acid (HNO_3) and sample. CAUTION: *When working with conc HNO_3 wear safety goggles and an acid-resistant apron and gloves, and work under a hood.* 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 approximately one-third the original volume. Alternatively, let treated sample stand overnight. This cleaning process destroys organic matter and leaves only diatom shells (frustules). Cool, wash with distilled water, and mount as described above. Transfer cleaned frustules to a cover glass and dry as described above.

Place a drop of mounting medium in the center of a labeled slide. Use 25- by 75-mm slides with frosted ends. Using a suitable high-refractive-index microscopic mounting medium assures permanent, easily handled mounts for examination under oil immersion. Heat the 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 the slide to a cool surface and, during cooling (5 to 10 s), apply firm but gentle pressure to the cover glass with a broad, flat instrument.

4. Zooplankton Mounts

For zooplankton analyses, withdraw a 5-mL subsample from the concentrate and dilute or concentrate further as necessary. Transfer sample to a counting cell or chamber (see below) for analysis as a wet mount. Use polyvinyl lactyl phenol† for preparing semipermanent zooplankton mounts. The mounts are good for about a year, after which time the clearing agent causes deterioration of organisms. For long-term storage ring cover slip with clear lacquer (fingernail polish) to retard mountant crystallization. For permanent mounting, other mountants are available.‡

For the protozoan portion of the microzooplankton, a protargol staining procedure[6] not only provides a permanent mount but also reveals the cytological details often necessary for identification. This procedure is qualitative and is especially important in taxonomic studies of the ciliated protozoa.

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* Permount, Fisher Scientific Co., 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.

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10200 E. Microscopes and Calibrations

1. Compound Microscope

Use either a standard or an inverted compound microscope for algal identification and enumeration. Equip either type with a mechanical stage capable of moving all parts of a counting cell past the objective lens. Standard equipment is a set of 10× or 12.5× oculars and 10×, 20×, 40×, and 100× objectives. Use objectives to provide adequate working distance for the counting chamber. Magnification requirements vary with the plankton fraction being investigated, the type of microscope, counting chamber used, and optics. With standard objectives, the Sedgwick-Rafter chamber limits magnification to approximately 200× and the Palmer-Maloney cell limits magnification to approximately 500×. Inverted microscopes are limited in resolution by their optics. 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. When the limit is exceeded, empty magnification results. Empty magnification occurs where the image is larger but no greater resolution is achieved. Optics providing contrast enhancement such as phase contrast or differential interference contrast are useful.

2. Stereoscopic Microscope

The 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 for the study and counting of large plankters such as mature microcrustacea. Include 10× to 15× paired oculars in combination with 1× to 8× objectives. This combination of optics bridges the gap between the hand lens and the compound microscope and provides magnification ranging from 10× to 120×. Alternatively, use a good-quality zoom-type instrument with comparable magnification.

3. Inverted Compound Microscope

The inverted compound microscope often is used routinely for plankton counting in many laboratories.[1–3] This instrument is unique in that the objectives are below a movable stage and the illumination comes from above, thus permitting viewing of organisms that have settled to the bottom of a chamber. Place samples in a cylindrical settling chamber having a thin, clear glass bottom. Chambers of various capacities are available; the appropriate size depends on the density of organisms. After a suitable period of settling (see Section 10200C.1), count organisms in the settling chamber.

The major advantage of the inverted microscope is that by a simple rotation of the nosepiece a specimen can be examined (or counted) directly in the settling chamber at any desired magnification. Although not recommended, oil immersion objectives have some useful applications. No preparation or manipulation other than settling is required. Generally, examine a preserved sample. Techniques are available for samples with an abundance of organisms that tend to float.[4]

4. Epifluorescence Microscope

An epifluorescence microscope may be either standard or inverted. It uses incident light to excite electrons in intracellular compounds, such as pigments or absorbed stains, with the energy emitted during electron return to the ground state being measured as fluorescent light. The technique has been applied to the microscopic identification of chlorophyll-containing cells (autotrophs) and nonpigmented heterotrophic plankton; fluorescent stains such as primulin or proflavin also have been used to differentiate nannoplanktonic primary and secondary producers.[5–7] Excitation and emission wavelengths are unique for each pigment and stain and require distinct light filter combinations and light sources. Select the filter combinations for the particular application. Epifluorescence microscopy is particularly useful for the enumeration of picoplankton and heterotrophic flagellate populations common to most aquatic systems. Concentrate samples by membrane filtration. Use epifluorescence microscopy as a complementary procedure to standard light microscope counting techniques.

5. Microscope Calibration

Microscope calibration is essential. The usual equipment for calibration is a Whipple grid (ocular micrometer, reticle, or reticule) placed in an eyepiece of the microscope and a stage micrometer that has a standardized, accurately ruled scale on a glass slide. 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 outer dimensions of the grid are such that with a 10× objective and a 10× ocular, it delimits an area of approximately 1 mm^2 on the microscope stage. Because this area may differ from one microscope to another, carefully calibrate the Whipple grid 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

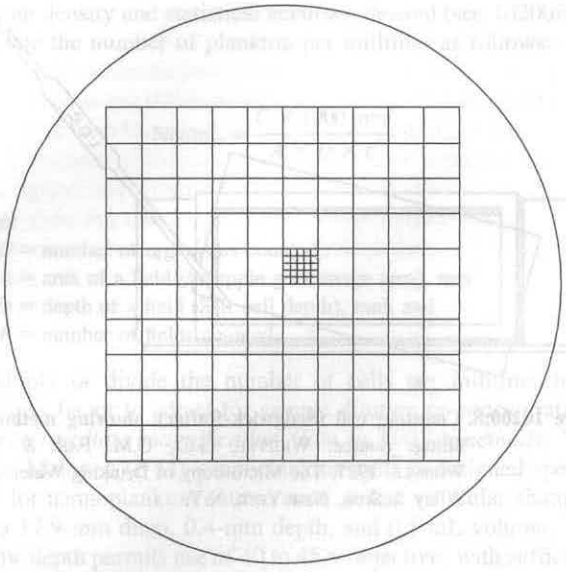

Figure 10200:6. Ocular micrometer ruling. A Whipple micrometer reticule is illustrated.

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. Should the width of the image of the Whipple grid be exactly 1 mm (1000 μm), the larger squares will be 1/10 mm (100 μm) on a side and each of the smaller squares 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 details for calibration are available.[8]

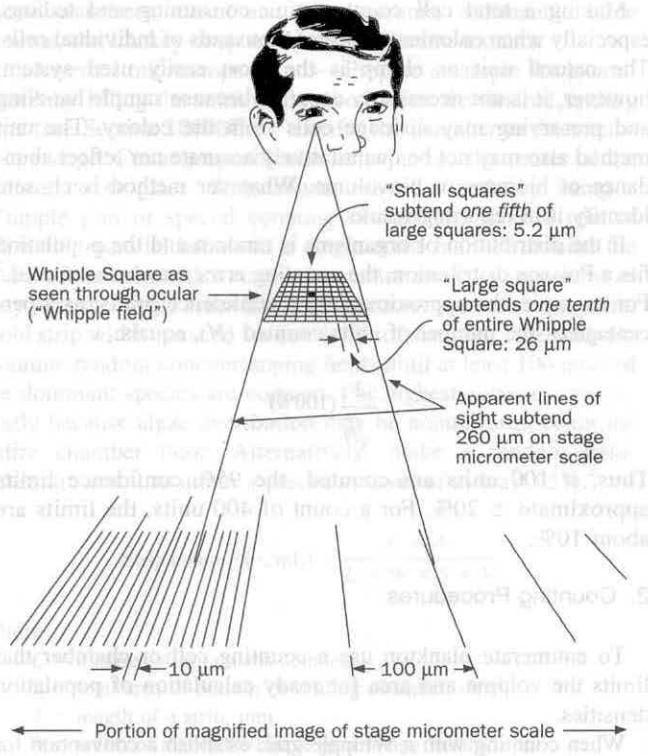

Figure 10200:7. Calibration of Whipple Square, as seen with 10× ocular and 43× objective (approximately 430× total magnification).

6. References

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10200 F. Phytoplankton Counting Techniques

1. Counting Units

Some phytoplankton are unicellular while others are multicellular (colonial). The variety of configurations poses a problem in enumeration. For example, should a four-celled colony of *Scenedesmus* (Plates 32, 34) be reported as one colony or four individual cells? Listed below are suggestions for reporting:

Enumeration Method	Counting Unit	Reporting Unit
Total cell count	One cell	Cells/mL
Natural unit count[1] (clump count)	One organism (any unicellular organism or natural colony)	Units/mL
Areal standard unit count*	400 μm^2	Units/mL

*Areal standard unit equals area of four small squares in Whipple grid at a magnification of 200.

Making a total cell count is time-consuming and tedious, especially when colonies consist of thousands of individual cells. The natural unit or clump is the most easily used system; however, it is not necessarily accurate because sample handling and preserving may dislodge cells from the colony. The unit method also may not be quantitatively accurate nor reflect abundance of biomass or biovolume. Whatever method is chosen, identify it in reporting results.

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 limits, as a percentage of the number of units counted (N), equals:

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

Thus, if 100 units are counted, the 95% confidence limits approximate ± 20%. For a count of 400 units, the limits are about 10%.

2. Counting Procedures

To enumerate plankton use a counting cell or chamber that limits the volume and area for ready calculation of population densities.

When counting with a Whipple grid, establish a convention for tallying organisms lying on an outer boundary line. For example, in 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 Section 10900).

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.

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 techniques for phytoplankton counting according to the magnifications provided.

a. Low-magnification (up to 200×) methods: The Sedgwick-Rafter (S-R) cell is a device commonly used for plankton counting because it is easily manipulated and provides reasonably reproducible data when used with a calibrated microscope equipped with an eyepiece measuring device such as the Whipple grid.

The greatest disadvantage associated with the cell is that objectives providing high magnification cannot be used. As a result, the S-R cell is not appropriate for examining nannoplankton. The S-R cell is approximately 50 mm long by 20 mm wide by 1 mm deep. The total area of the bottom is approximately 1000 mm^2 and the total volume is approximately 1000 mm^3 or 1 mL. Carefully check the exact length and depth of the cell with a micrometer and calipers before use.

1) Filling the cell—Before filling the S-R cell with sample, place the cover glass diagonally across the cell and transfer sample with a large-bore pipet (Figure 10200:8). Placing cover slip in this manner will help prevent formation of air bubbles in cell corners. The cover slip often will rotate slowly and cover the inner portion of the S-R cell during filling. Do not overfill because this would yield a depth greater than 1 mm and produce an invalid count. Do not permit large air spaces caused by evaporation to develop in the chamber during a lengthy examination. To prevent formation of air spaces, occasionally place a small drop of distilled water on edge of cover glass.

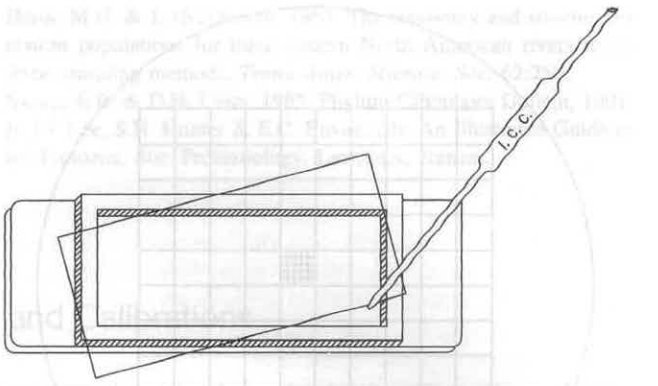

Figure 10200:8. Counting cell (Sedgwick-Rafter), showing method of filling. Source: WHIPPLE, G.C., G.M. FAIR & M.C. WHIPPLE. 1927. The Microscopy of Drinking Water. John Wiley & Sons, New York, N.Y.

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 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—A "strip" the length of the cell constitutes a volume approximately 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 from the following:

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

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.

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.

b. Intermediate magnification (low to 500×) methods: The Palmer-Maloney (P-M) nannoplankton cell[3] is designed specifically for nannoplankton enumeration. It has a circular chamber with a 17.9-mm diam, 0.4-mm depth, and 0.1-mL volume. 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 nannoplankton 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 causes serious underestimation of density.

Introduce sample with a pipet into one of the 2- by 5-mm channels on the side of the chamber with the cover slip in place. After a 10-min settling period count the plankters in random fields, with the number of fields depending on density and variety of plankton and the 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.

Another readily available chamber is the standard medical hemacytometer used for enumerating blood cells. It 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; the chamber is 0.1 mm deep. Introduce sample by pipet and view under 450× magnification. Count all cells within the grid. The chamber comes from the manufacturer with a detailed instruction sheet containing directions on calculations and proper usage. A disadvantage to these counting cells is that the sample must have a very high plankton density to yield statistically reliable data.

c. High-magnification methods: Examination of phytoplankton at high magnification requires the use of oil immersion objectives. Suitable procedures include using 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 Section 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 by using a Whipple grid or special counting oculars that have a pair of adjustable parallel hairs and a single cross hair. Determine the width of the strip with a stage micrometer and tally organisms as they pass the single cross hair that 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. For highest accuracy, particularly because algae distribution may be nonuniform, count the entire chamber floor. Alternatively, make a random field-minimum count to attain a precision level of at least 85%.[4]

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

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 Section 10200C.2 and prepare membrane filter as directed in Section 10200D.2*a*.

Examine samples, concentrated on unlined membrane filters and mounted in oil 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) such that 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 part or all of the Whipple grid. Examine 30 random microscope fields and record number of fields in which each species occurred. 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).

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	?

$$* F = \frac{\text{total number of species occurrences} \times 100}{\text{total number of fields examined}}$$

† N = number of organisms per field.

3) Sedimented slide mounts—Examine mounts prepared as directed in Section 10200D.2*b*.

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.

Prepare slides as directed in Section 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 Section 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 until at least 250 cells are counted. Available time and accuracy required dictate the number of cells 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 greater accuracy distinguish between living and dead diatoms at the species level.

6) Phytoplankton staining technique—Staining algae permits differentiation between "live" and "dead" diatoms.[6] This permits enumerating total phytoplankton in a single sample without sacrificing detailed diatom taxonomy. It also results in permanent reference slides. The procedure is most useful when diatoms are major components of phytoplankton and it is important to distinguish between living and dead diatoms.

Preferably preserve samples in Lugol's solution or alternatively in formalin (see 10200B.3). For analysis thoroughly mix the sample and filter a portion through a 47-mm-diam membrane filter (pore diam 0.45 or 0.65 μm). Use a vacuum of 16 to 20 kPa and never let sample dry. Add 2 to 5 mL aqueous acid fuchsin solution (dissolve 1 g acid fuchsin in 100 mL distilled water to which 2 mL glacial acetic acid has been added; filter) to the filter and let stand for 20 min. 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 for species identifications of diatoms 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.

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.
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† Permount, Fisher Scientific Co., or equivalent.

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10200 G. Zooplankton Counting Techniques

1. Subsampling

Count entire samples having low zooplankton numbers (<200 zooplankters) without subsampling. However, most zooplankton samples will contain more organisms than can be enumerated practically; therefore, use a subsampling procedure. Before subsampling, remove and enumerate all large uncommon organisms such as 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 the plankton by using a rubber bulb and clear acrylic plastic tube with fine mesh netting fitted on the end is convenient and accurate (Figure 10200:9). For picoplankton and the smaller microzooplankton, use sedimentation techniques described for concentrating phytoplankton. Transfer sample to a beaker or other wide-mouth vessel for subsampling with 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 with 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 the 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.

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 on the basis of 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, error about the 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 not suitable because of size. An open counting chamber 80 by 50

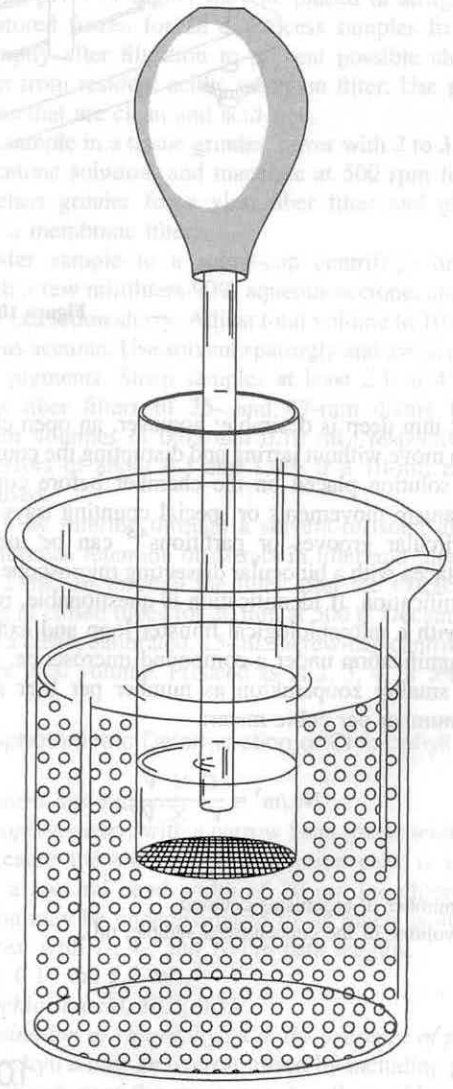

Figure 10200:9. A simple, efficient device for concentrating plankton. The tube is lowered into the beaker containing the sample. Water filtering into the tube is removed with the rubber bulb. The filter is nylon monofilament screen cloth that is glued to the bottom of the tube. The mesh size should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

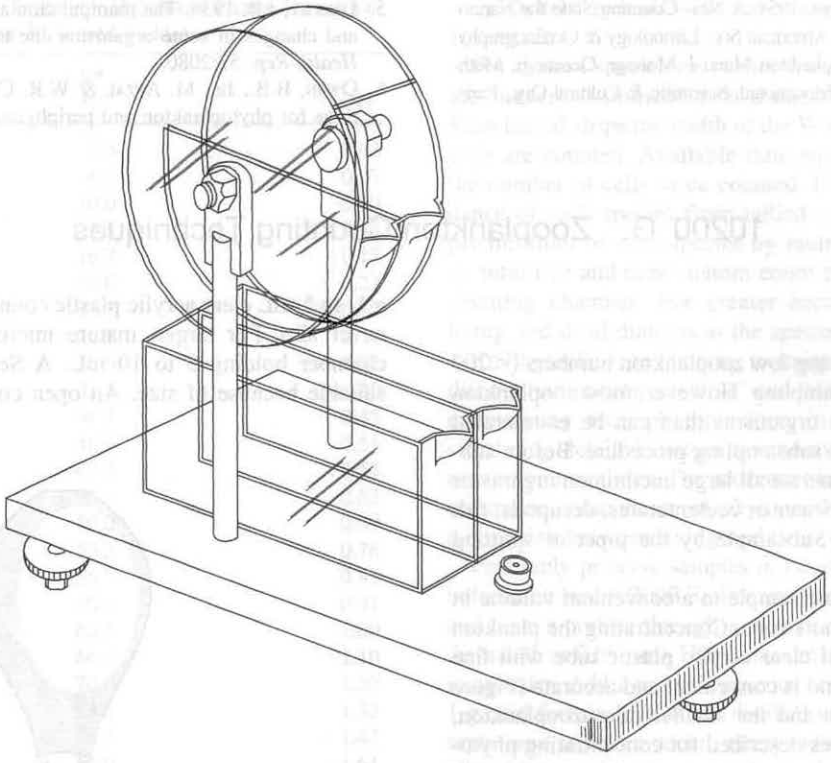

Figure 10200:10. The Folsom plankton splitter.

mm and 2 mm deep is desirable; however, an open chamber is difficult to move without jarring and disrupting the count. A mild detergent solution placed on the chamber before counting reduces organism movements or special counting trays with parallel or circular grooves or partitions[4,5] can be used. 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 smaller zooplankton as number per liter and larger forms as number per cubic meter:

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

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

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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

Chlorophyll can be extracted with several different solvents, including 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 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:** 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† or membrane (0.45-μm porosity, 47-mm diam); vacuum pump; solvent-resistant disposable filter assembly, 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. Samples on filters taken from water having pH 7 or higher may be placed in airtight plastic bags and stored frozen for 28 d. 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 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§ 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 magne-

* Kontes Glass Co., Vineland, NJ 08360: Glass/glass grinder, Model No. 8855: 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.
§ GF/F or equivalent.

sium 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.

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

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

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]

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

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

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

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,‖ 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 (CS2-64) 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 10200H and ¶ 2*b* above.

1) Equipment and reagents—In addition to those listed under 1*a* and 2*a* above, 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 ¶ 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** 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,†† 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‡‡ 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 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, ¶ 1*a* above:

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) for extending life of primary column.

4) *Reverse-phase HPLC column.*§§

5) *Fluorescence detector* capable of excitation at 430 ± 30 nm and measuring emission 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; methanol:reagent water: ion-pairing solution) and System B (80:20; methanol:acetone). 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]

‖ 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.
‡‡ 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.

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^{-1} cm^{-1}; ϵ_{647} for chlorophyll *b* in 90% acetone = 51.36 L g^{-1} cm^{-1}).[5] Prepare pheophytin *a* + *a'* and *b* + *b'* standards from the primary chlorophyll *a* and *b* standards by 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^{-1} cm^{-1}; 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^{-1} cm^{-1}).[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^{-1} cm^{-1}).[28] 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 system 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 system by preparing working standards from the primary standards (on day of use). Once retention times of the standards 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, overfilling 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 the 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 approximately 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 system with reagent water to avoid buildup of ion pairing agents.

5) Calculate individual pigment concentrations using the following formula:

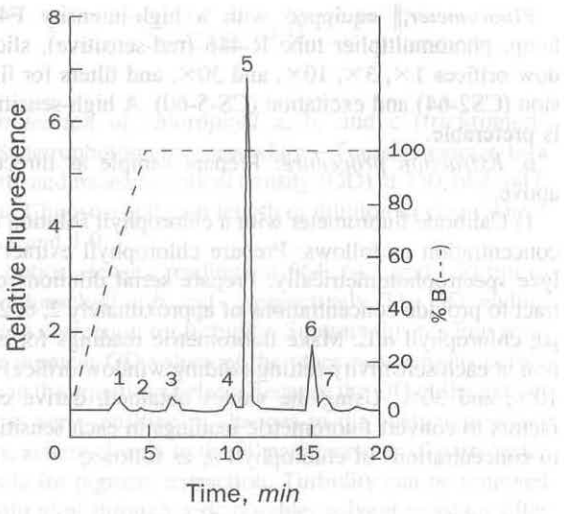

Figure 10200:11. Reverse-phase HPLC chromatogram for a fivefold dilution of EPA sample. Injection volume 100 μL; peaks detected by fluorescence spectroscopy (λ_{ex}: 400–460 nm; λ_{ex}: >600 nm). Peak identities are: 1—chlorophyllide *a;* 2—chlorophyll *c;* 3—pheophorbide *a;* 4—chlorophyll *b;* 5—chlorophyll *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).

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

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 for quantification of chlorophylls and their degradation products. Detect carotenoid pigments, which also are present in 90% acetone extracts but do not fluoresce, by 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 (*s/n* = 2) vary with fluorometer configuration and flow rate; however, they range from 10 to 100 pg per injection for most chlorophylls and their degradation products.[15,21,29] The accuracy of the HPLC method depends primarily on purity of pigment standards. Preferably measure absorption spectra (350 to 750 nm) of the standards and compare with published data. Pigment purity also can be assessed by HPLC analysis, providing there are no co-eluting contaminants with absorption and fluorescence bands overlapping those of the standards. HPLC and spectrophotometrically derived pigment concentrations for available EPA standards agree reasonably well (± 20%) if spectrophotometric results are corrected for the presence of pheopigments and the HPLC results

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} $L\ g^{-1}\ cm^{-1}$	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.

are expressed as pigment equivalents (e.g., chlorophyll *a* equivalents = chlorophyllide *a* + chlorophyll *a* + chlorophyll *a'*, provided that the 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, ¶ 1*a* above:

1) *High-performance liquid chromatographic pump* capable of gradient delivery of three different 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) for extending 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 flowthrough 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, v:v; methanol:0.5*M* ammonium acetate, pH 7.2); Eluent B (90:10, v:v; acetonitrile: water), 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 by thin-layer chromatography (TLC)[25] or preparative-scale HPLC. Determine concentration of all standards using a monochromator-based spectrophotometer in the appropriate solvents before calibration of the HPLC system. The recommended extinction coefficients for 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 concentrations of standards as follows:

$$C_i = \frac{(A_{\lambda max} - A_{750nm})}{E_{1cm} \times b} \times 1000$$

where:

C_i = individual pigment concentration, mg/L,
A = absorbance at specific wavelength,
E_{1cm} = weight-specific absorption coefficient, L g^{-1} cm^{-1},
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 system with Eluent A at a flow rate of 1 mL/min.

*** 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.

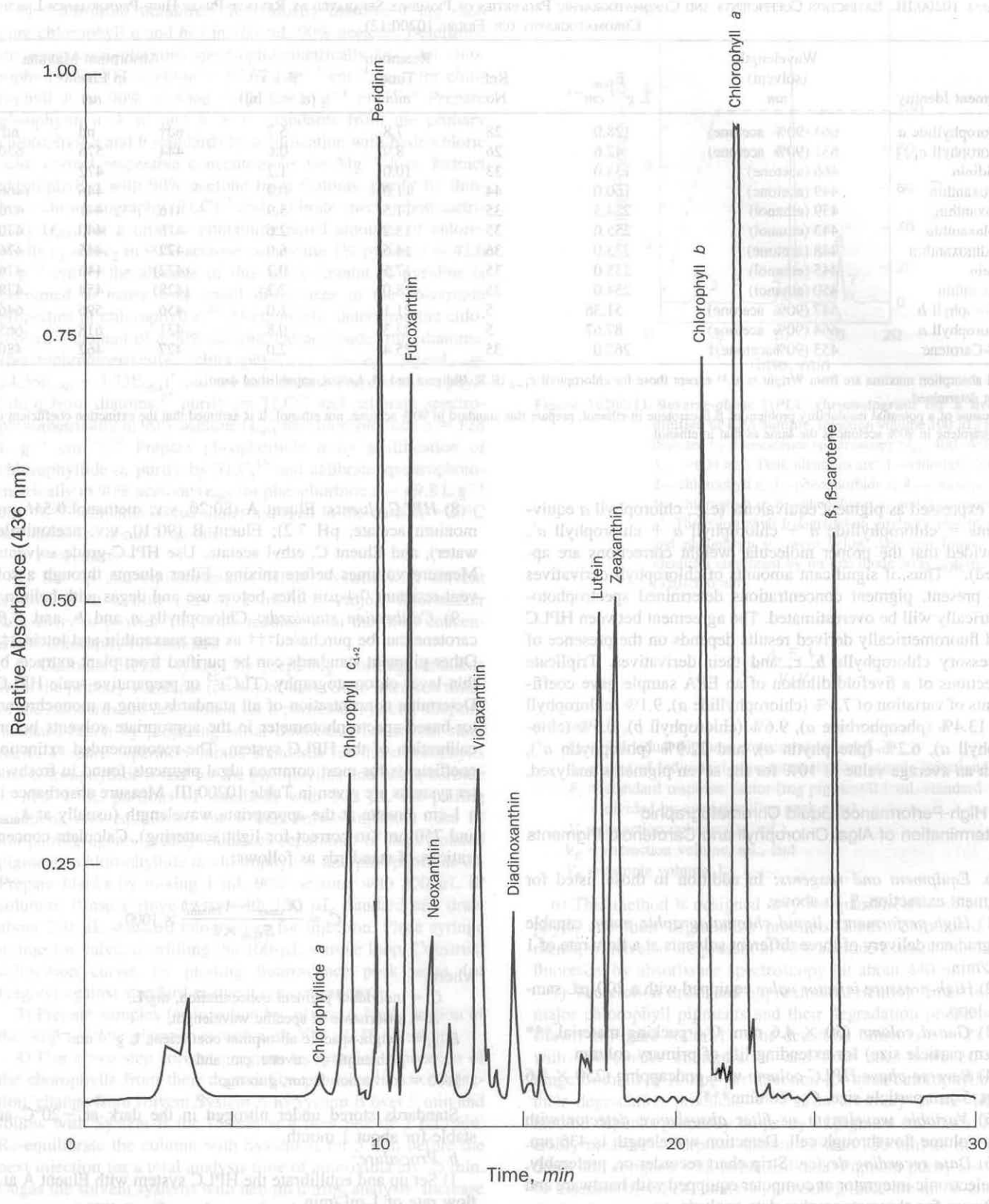

Figure 10200:12. Reverse-phase HPLC pigment chromatogram for a mixture of common algal pigments found in freshwater systems. For further data see Table 10200:III. Sample contained a natural extract with authentic known additions. The small unlabeled peaks are pigment degradation products.

TABLE 10200:IV. HPLC SOLVENT SYSTEM PROGRAM

Time	Flow Rate	Percentage of Eluent			
min	*mL/min*	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

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 affinity of pigments for the column in the loading step, resulting in an improved separation of more polar pigments). Rinse syringe twice with 300 μL standard and draw 500 μL standard into syringe for injection. Place syringe in injector valve, overfilling the 200 μL sample loop 2.5-fold. To check for possible interferences in the extraction solvent and/or filter, prepare a blank by extracting a glass fiber filter in 90% acetone; mixing 1 mL 90% acetone filter extract and 300 μL distilled water; and injecting into the HPLC system. Plot absorbance peak areas (or heights) against standard pigment concentrations. Calculate response factors as the slope of the regression between the weights of the injected standards (ng) and the areas of the parent pigment (plus areas of structurally related isomers if present). These isomers contribute to the absorption signal of the standards; 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 and 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 insure elution of most hydrophobic pigments. Degas solvent system with helium during analysis. Periodically flush HPLC system with distilled water to avoid accumulation of ion-pairing reagents.

5) Routinely determine peak identities by comparing retention times of sample peaks 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). Absorption maxima for most common pigments found in freshwater systems are given in Table 10200:II.

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

7) This method is designed for separation of 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 elsewhere.[33–37]

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10200 I. Determination of Biomass (Standing Crop)

Biomass is a quantitative estimate of the total mass of living organisms within 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 estimation of 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. All estimates of biomass can be affected by the presence of organic and inorganic detritus; ATP and DNA analyses include contributions from the bacterial flora.[4]

1. Chlorophyll *a*

Chlorophyll *a* is used as an algal biomass indicator.[5] Assuming that chlorophyll *a* constitutes, on the average, 1.5% of the dry weight of organic matter (ash-free weight) of algae, estimate the algal biomass by multiplying the chlorophyll *a* content by a factor of 67.

2. Biovolume (Cell Volume)

Plankton data derived on a volume-per-volume basis often are more useful than numbers per milliliter.[6] Determine cell volume by using the simplest geometric configuration that best fits the shape of the cell being measured (such as sphere, cone, cylinder).[7] Cell sizes of an organism can differ substantially in different waters and from the same waters at different times during the year; therefore, average measurements from 20 individuals of each species for each sampling period. Calculate the total biovolume of any species by multiplying the average cell volume in cubic micrometers by the number per milliliter.

Compute total wet algal volume as:

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

where:

V_t = total plankton cell volume, mm^3/L,
N_i = number of organisms of the *i*th species/L, and
V_i = average volume of cells of *i*th species, μm^3.

3. Cell Surface Area

An estimation of cell surface area is valuable in analyzing interactions between the cell and surrounding waters. Compute average surface area in square micrometers and multiply by the number per milliliter of the species being considered.

4. Displacement Volume

This method[8] measures an equivalent volume of liquid that is displaced by the sample. Displacement volume may be determined by several methods; for simple, direct measurement proceed as follows: Place sample in sieve of mesh size equal to or smaller than net used in capture; let sample drain and transfer to a measured volume of water in a graduated cylinder; measure the new volume containing sample plus known volume. The displacement volume equals the new volume minus original measured volume of water.

5. Gravimetric Methods

The biomass of the plankton community 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-pore-diam membrane or a prerinsed, dried, and preweighed glass-fiber filter. (Note that the small sample used in direct filtration may lead to error if not handled properly.) Cool sample in a desiccator and weigh. Obtain ash weight by igniting the dried sample at 500°C for 1 h. Cool, rewet ash with distilled water, and bring to constant weight at 105°C. The ash is rewetted to restore water of hydration of clays and other minerals; this may amount to as much as 10% of weight lost during incineration.[9] The ash-free dry weight is the difference between the dry weight and the weight of the ash residue after ashing. The ash-free dry weight is preferred to dry weight to compare mixed assemblages. The ash content may constitute 50% or more of the dry weight in phytoplankton having inorganic structures, such as the diatoms. In other forms the ash content is only about 5% of dry weight.

6. Adenosine Triphosphate (ATP)

Methods of measuring adenosine triphosphate (ATP) in plankton provide the only means of determining the total viable plankton biomass. ATP occurs in all plants and animals, but only in living cells; it is not associated with nonliving particulate material. The ratio of ATP to biomass varies from species to species, but appears to be constant enough to permit reliable estimates of biomass from ATP measurements.[10] 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 tris-hydroxymethylaminomethane in 3 L distilled water and adjust to pH 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 as:

$$F_S = \frac{C}{A_S}$$

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[10] and filter through a 47-mm 0.45-μm-porosity filter by applying a vacuum of about 30 kPa. (IMPORTANT: Break the 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 into a glass vial. Measure the light emission of the enzyme preparation (blank) for 2 to 3 min at sensitivity settings near that 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;

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

record output for 2 to 3 min, using the same time period for all samples. Determine the mean of areas under the curves obtained and correct by subtracting mean of areas under the curves obtained from blanks prepared as directed in Strickland and Parsons.[11]

c. Calculations: Calculate concentration of ATP as:

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

where:

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

If an ATP content of 2.4 μg ATP/mg dry weight organic matter is assumed,[12] total living plankton biomass (B), as dry weight organic matter, is given as:

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

7. References

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10200 J. Metabolic Rate Measurements

The physiological condition and the spectrum of biological interactions of the aquatic community must be considered for evaluation of the state of natural waters. Earlier, numbers, species composition, and biomass were the prime considerations. Recognition of the limitations of this approach 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 complex nature of the aquatic ecosystem. An indication of photosynthetic efficiency can be determined by the productivity index (mg C fixed/unit chlorophyll *a*).[1]

1. Nitrogen Fixation

The ability of an organism 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

Productivity is defined as 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 the 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 a sensitive property for detecting variations in the system. As CO_2 is removed during photosynthesis, the 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 of 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 an incubation period of several hours 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, summarized by the relationship:

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

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 on the assumption that one atom of carbon is assimilated for each molecule of oxygen 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 phosphorus-containing detergents.

If suitable opaque bottles are not available, make clear BOD bottles opaque by painting them black and wrapping 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 Van Dorn sampler* or equivalent, of 3- to 5-L capacity.

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

5) *Pyrheliometer.*

6) *Submarine photometer.*

7) *Thermometer.*

b. Procedure:

1) Obtain a profile of the input of solar radiation for the photoperiod with a pyrheliometer.

2) Determine depth of euphotic zone (the region that receives 1% or more of surface illumination) with a submarine photometer. Select depth intervals for bottle placement. The photosynthesis-depth curve will be approximated closely by placing 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 with probe or by titration and temperature and salinity to determine whether water is supersaturated with respect to 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 delivery tube of sampler to bottom of sample bottle and fill so that 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 for the chemical determination of initial dissolved oxygen (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 the clear bottles or DO to be depleted in the 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 of the concurrent use of oxygen in respiration, is somewhat less than the total (or 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:

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

where K is the photosynthetic quotient (PQ), ranging from 1 to 2, depending on the 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 carbon (12 g) fixed.

2) Productivity is defined as the rate of production and 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

A solution of radioactive carbonate ($^{14}CO_3^{2-}$) is added 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 carbon fixed is proportional to the fraction of radioactive carbon assimilated.

This procedure differs from the oxygen method in that it affords a direct measurement of 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 ¶s 2*a*1) and 2), above.

4) *Membrane-filtering device and 25-mm filters* with pore diameters 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). 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 a depth of about 1.4 cm conc HCl 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 having approximately 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 studies of fresh water use carrier-free radioactive carbonate and for studies of marine water 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]

b. Procedure:

1) Obtain a record of incident solar radiation for the photoperiod with a pyrheliometer.

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 approximately 10 μCi/L in relatively productive waters, to 100 μCi/L, or higher, in oligotrophic (open ocean) waters. To obtain statistical significance, have at least 1000 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 provided energy input is used as the basis for integrating incubation period to entire photoperiod. For incubation procedure, see ¶ 2*b*6) above.

5) After incubating remove sample bottles and immediately place in the dark. Filter unpreserved samples without delay. Avoid sample preservation to avoid lysing cells or determine extracellular products.

6) Filter two portions of each sample through a membrane filter, taking care that the largest pore size is consistent with quantitative retention of plankton. Although the 0.45-μm pore filter usually is adequate, determine the efficiency of sample retention immediately before analysis, with a wide range of pore sizes.[23,24] Apply approximately 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 will have different thicknesses ranging 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 with decreasing precipitate thickness. 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 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 the methods described in the literature.[27–30]

3) Determine quantity of carbon fixed by using the following relationship:

* Chelex 100 or equivalent.

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

4) Integrate productivity for the entire depth of euphotic zone and express as grams carbon fixed per square meter per day [see ¶ 2c2) above].

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.

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† Correction for isotope effect.

10300 PERIPHYTON*

10300 A. Introduction

1. Definition and Significance

Microorganisms growing on stones, sticks, aquatic macrophytes, and other submerged surfaces are useful in assessing the effects of pollutants on lakes, streams, and estuaries. Included in this group of organisms, here designated periphyton,[1,2] are the zoogleal and filamentous bacteria, attached protozoa, rotifers, and algae, and the free-living microorganisms that swim, creep, or lodge among the attached forms.

Unlike the plankton, which often do not respond fully to the influence of pollution in rivers for a considerable distance downstream, the periphyton show marked responses immediately below pollution sources. Examples are the beds of *Sphaerotilus* and other "slime organisms" commonly observed in streams below discharges of organic wastes. Because the abundance and composition of the periphyton at a given location are governed by the water quality at that point, observations of their condition generally are useful in evaluating conditions in bodies of water.

The use of periphyton in assessing water quality often is hindered by the lack of suitable natural substrata† at the desired sampling station. Furthermore, it often is difficult to collect quantitative samples from these surfaces. To circumvent these problems artificial substrata have been used to provide a uniform surface type, area, and orientation.[3]

2. References

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* Approved by Standard Methods Committee, 2001.
Joint Task Group: Robert G. Wetzel (chair), Hamish Duthie, Gordon Goldsborough, Michael K. Hein, Stanford Loeb, Alena Sládečková.

† Although the terms "substrate" and "substratum" often have been used interchangeably, technically it is more correct to use "substratum" in connection with periphyton. A substrate (plural substrates), in biochemical usage, is the substance acted upon by an enzyme and the source of energy. In contrast, a substratum (plural substrata) as defined in biological usage is the base or material on which a nonmotile organism lives or grows (i.e., the submerged surfaces used for periphyton colonization).

10300 B. Sample Collection

1. Station Selection

In rivers, locate stations a short distance upstream and at one or more points downstream from the suspected pollution source or intended study area in the areas of central mixing. In large rivers, sample both sides of the stream in main flow areas. Because the effects of a pollutant depend on the assimilative capacity of the stream and on the nature of the pollutant, progressive changes in water quality downstream from the pollution source may be caused entirely by dilution and cooling—as in the case of nutrients, toxic industrial wastes, and thermal pollution—or by gradual mineralization of degradable organic compounds. Cursory examination of shoreline and bottom periphyton growths on natural substrata downstream from an outfall may indicate conspicuous zones of biological response to water quality that will be useful in determining appropriate sites for sampling stations. When an intensive sampling program is not feasible, a minimum of three sampling stations, one in a reference area upstream from a pollution source and the others in the community downstream from the source, where complete mixing with the receiving water has occurred, will provide minimal data on the periphyton community.

In lentic waters (e.g., lakes, reservoirs, ponds) and other standing-water bodies where zones of pollution may be arranged concentrically, locate stations in areas adjacent to a waste outfall and in unaffected areas. Use control stations similar to the affected ones (e.g., similar in water depth and distance from shore).

2. Sample Collection

a. Natural substrata: Collect qualitative samples by scraping submerged stones, sticks, pilings, and other available substrata. Many devices have been developed to collect quantitative samples from irregular surfaces. Appropriate techniques for the removal of periphyton from both living and nonliving surfaces have been described.[1–4]

b. Artificial substrata: The most widely used artificial substratum is the standard, plain, 25- by 75-mm glass microscope slide, but other materials such as clear vinyl plastic also are suitable. Do not change substratum type during a study because colonization varies with substratum. In small, shallow streams and in the littoral regions of lakes and reservoirs where light penetrates to the bottom, place slides or other substrata vertically in frames anchored to the bottom. In large, deep streams or standing-water bodies where turbidity varies widely, place slides vertically with the slide face at right angles to the prevailing current. A floating

rack, as shown in Figure 10300:1,* is suitable. Expose several slides (minimally five; three for biomass, one for species, and one backup for each time interval) for each type of analysis to assure collecting sufficient material and to determine variability in results caused by normal differences in colonization of individual slides. In addition to effects of pollutants, length of substratum exposure and seasonal changes in temperature and other natural environmental conditions may have a profound effect on sample composition. No community on an artificial substratum is representative of the natural community.

Place, expose, and handle all artificial substratum samplers in conditions as nearly identical as possible, whether they are replicate samplers at a particular sampling location or samplers at different locations. Sampler type and/or construction cause changes in surrounding physical conditions that in turn affect periphyton growth. Variations of 10 to 25% between sample replicates are common. Therefore, to reduce sampling error and increase interpretive power, reduce the magnitude of all possible test variables and use sufficient replication.

c. Exposure period: Colonization on clean slides proceeds at an exponential rate for the first 1 or 2 weeks and then slows. Because exposures of less than 2 weeks may result in very sparse collections, and exposures of more than 2 weeks may result in loss of material due to sloughing, sample for 2 weeks during the summer. This exposure period precludes collecting sexually mature thalli of larger, slow-growing filamentous algae such as *Cladophora* and *Stigeoclonium*. To obtain optimum growth during the winter, use a longer exposure period. For the most exacting work, determine the optimum exposure period by testing colonization rates over a period of about 6 weeks.

Secondary problems associated with macroinvertebrate infestation and grazing may occur, often within 7 to 14 d. To reduce the confounding influence of grazing, increase substratum sampling area and expose for 7 to 10 d.

3. Sample Preservation

Preserve samples that are taken for counting and identification in 5% neutralized formalin, Lugol's iodine, or merthiolate (see Section 10200B.2). Gluteraldehyde (2 to 5%) also is an excellent preservative, and in some ways is superior because it impacts cell membranes less severely.

Preserve slides intact in bottles of suitable size or scrape into containers in the field. Air-dry slides for dry and ash-free dry weight in the field and store in a 3.0- × 7.7-cm glass bottle. Place slides for chlorophyll analyses in acetone or methanol in the field or collect and freeze with trichlorotrifluoroethane† (or alternative) or CO_2. Ethanol (95%) is an excellent solvent for chlorophyll extraction and yields greater extraction, is less toxic, and is less expensive than acetone or methanol.[5,6] The specific absorption coefficient for chlorophyll *a* in 95% ethanol is 83.4 L/(g·cm). Alternatively, hold on dry ice until returned to the laboratory. If samples are frozen in the field, remove from the substratum and concentrate (by filtration or other means) before freezing. Store all samples in the dark. For pollution and eutrophication studies using periphyton biota as indicators, do not preserve samples. Enclose substrata with periphyton in containers filled with water, and transport and analyze them immediately. Also see Section 10300E.

* Wildlife Supply Co., 301 Cass St., Saginaw, MI 48602, or equivalent.

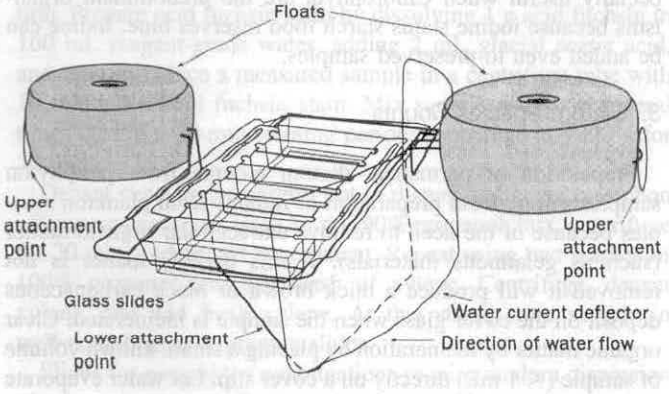

Figure 10300:1. Periphyton sampler. Floating sampler with upstream deflecting baffle and transparent, removable slide rack holding up to eight microscope slides.

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† Freon or equivalent.

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10300 C. Sample Analysis

1. Sedgwick-Rafter Counts

Remove periphyton from slides with a razor blade and rubber policeman. Disperse scrapings in 100 mL or other suitable volume of preservative with vigorous shaking, or use a blender. Transfer a 1-mL portion to a Sedgwick-Rafter cell, and make a strip count as described in Section 10200F.2*a*. If material in the Sedgwick-Rafter cell is too dense to count directly, discard and replace with a diluted sample.

Sedgwick-Rafter cells do not permit examination at magnifications higher than 200×. The Palmer cell,[1] a thinner version of the S-R cell, permits examination at 400 to 500× with a standard compound microscope.

Express counts as cells or filaments per square millimeter of substratum area, calculated as in ¶ C.2.

2. Inverted Microscope Method Counts

Using an inverted microscope for periphyton counts permits magnifications higher than those possible with the Sedgwick-Rafter cell. If an inverted microscope is not available, use one of the available alternative methods with a standard compound microscope.[2,3] Remove periphyton quantitatively from slides with a razor blade and policeman. Transfer a measured portion, after serial dilution if necessary, into a standardized plankton sedimentation chamber. After a suitable period of settling (see Section 10200C.1), count organisms in the settling chamber by counting all organisms within a known number of strips or random fields. Calculate algal density per unit area of substratum as follows:

$$\text{Organisms/mm}^2 = \frac{N \times A_t \times V_t}{A_c \times V_s \times A_s}$$

where:

N = number of organisms counted,
A_t = total area of chamber bottom, mm^2,
V_t = total volume of original sample suspension, mL,
A_c = area counted (strips or fields), mm^2,
V_s = sample volume used in chamber, mL, and
A_s = surface area of slide or substratum, mm^2.

Separation of periphyton from silt and detritus may be enhanced by adding a drop or less of a saturated iodine solution to the counting chamber just before counting. This method is especially useful when Chlorophyta are the predominant organisms because iodine stains starch food reserves blue. Iodine can be added even to preserved samples.

3. Diatom Species Counts

Preparation of permanent diatom mounts from periphyton samples differs from preparation of mounts from plankton samples because of the need to remove extracellular organic matter (such as gelatinous materials). If this organic matter is not removed it will produce a thick brown or black carbonaceous deposit on the cover glass when the sample is incinerated. Clear organic matter by incineration by placing a small, known volume of sample (< 1 mL) directly on a cover slip. Let water evaporate and ash at 525°C (not more) for 6 to 10 min. Mount cover slip for direct examination of diatom frustules. Alternatively, decompose organic substances by oxidation with ammonium persulfate

or with HNO_3 or 30% H_2O_2 and $K_2Cr_2O_7$ (see Section 10200D.3) before mounting sample. To oxidize with persulfate place a measured sample of approximately 5 mL in a disposable 10-mL vial. Let stand 24 h, withdraw supernatant liquid by aspiration, replace with a 5% solution of $(NH_4)_2S_2O_8$, and mix thoroughly. Do not exceed a total volume of 8 mL. Heat vial to approximately 90°C for 30 min. Let stand 24 h, withdraw supernatant liquid, and replace with reagent-grade water. After three changes of reagent-grade water, with a disposable pipet transfer a drop of the diatom suspension to a cover glass, evaporate to dryness, and prepare and count a mount as described for plankton (Section 10200). Count as least 500 frustules and express results as relative numbers or percentage of each species per unit area. Counts of more than 500 frustules may be needed, depending on the questions being addressed.[4]

4. Stained Sample Preparation and Counting

Staining periphyton samples permits distinguishing algae from detritus and "live" from "dead" diatoms. This distinction is especially important because periphyton often contains many dead diatoms of planktonic as well as periphytic origin.

In the first method, cells are exposed to a vital stain to evaluate the percentages of live, senescent, and dead algae, particularly diatoms, by estimating relative metabolic activities. The colorless tetrazolium violet is reduced in the cytochrome system of metabolically active cells to form violet-colored triphenylformazan. When cells are senescent or dead, the reaction fails.

Make tetrazolium violet solution by adding 2.0 g tetrazolium violet to 1.0 L water. The solution may be buffered to a pH of 7.5 to 7.7 with tris-hydroxymethyl amine. Add 1 mL tetrazolium violet solution to 9 mL sample and incubate 2 to 4 h at room temperature. Count diatom frustules and other cells (at least 300/sample) and place into the following categories: a) active: violet precipitate observed within the cell or mitochondria; b) senescent: chlorophyll present, but no violet precipitate; c) dead: no chlorophyll or violet precipitate present.

In the second method, all algal components of periphyton may be studied in one preparation, without sacrificing detailed diatom taxonomy.[5] This method yields permanent slides for reference collections.

Thoroughly mix preserved samples in the preservative solution. Prepare acid fuchsin stain by dissolving 1 g acid fuchsin in 100 mL reagent-grade water, adding 2 mL glacial acetic acid, and filtering. Place a measured sample in a centrifuge tube with 10 to 15 mL acid fuchsin stain. Mix sample and stain several times during a 20-min staining period; centrifuge at 1000 *g* for 20 min.

Decant stain, being careful not to disturb sediment, or siphon off supernatant. Add 10 to 15 mL 90% propanol, mix, centrifuge for 20 min, and decant supernatant. Repeat using two washes of 100% propanol and one wash of xylene. Centrifuge, decant xylene, and add fresh xylene. At this stage, store sample in well-sealed vials or prepare slides.

Slides for periphyton examinations require random dispersion of a known amount of xylene suspension. Use a microstirrer to break up clumps of algae before removing sample portion from xylene suspension. Count a number of drops of suspended sample into a thin ring of mounting medium* on a slide. Mix the xylene suspension and medium with a spatula until the xylene has evaporated. Warm the slide on a hot plate at 45°C and cover sample with a cover slip.

Count diatoms on the prepared slides using the magnification most appropriate to the desired level of taxonomic identification. Count strips or random fields. Calculate diatom density per unit area of substratum:

$$\text{Organisms/ area sampled}^2 = \frac{N \times A_t \times V_t}{A_c \times V_s \times A_s}$$

where the terms are as defined in 10300C.2.

5. Biovolume

Cell volume (biovolume) provides a much more accurate evaluation of cellular biomass because of the great differences in cell dimensions among species and sometimes seasonally within the same species under different growth conditions. Cell volumes, based on cell dimensions, are calculated for each species from formulas for solid geometric shapes that most closely match the cell shape. A comprehensive set of geometric shapes and mathematical equations for calculating biovolume of more than 850 pelagic and benthic freshwater and marine microalgal genera has been compiled.[6]

6. Dry and Ash-Free Weight

Collect at least three replicate slides for weight determinations.[7] Slides air-dried in the field can be stored indefinitely if protected from abrasion, moisture, and dust. Use slides expressly designated for dry and ash-free weight analysis.

a. Equipment:

1) *Analytical balance,* with a sensitivity of 0.1 mg.

2) *Drying oven,* double-wall, thermostatically controlled to within ±1°C.

3) *Electric muffle furnace* with automatic temperature control.

4) *Crucibles,* porcelain, 30-mL capacity.

5) *Single-edge razor blades or rubber policeman.*

b. Procedure:

1) Dry slides to constant weight at 105°C, and ignite for 1 h at 500°C. If weights are to be obtained from field-dried material, re-wet dried material with reagent-grade water and remove from slides with a razor blade or rubber policeman. Place scrapings from each slide in a separate prewashed, prefired, tared crucible; dry to constant weight at 105°C; cool in a desiccator and weigh; and ignite for 1 h at 500°C.

2) Re-wet ash with reagent-grade water and dry to constant weight at 105°C. This reintroduces water of hydration of clay and other minerals, which is not driven off at 105°C but is lost during ashing. If not corrected for, this water loss will be recorded as volatile organic matter.[8]

c. Calculations: Calculate mean weight from slides and report as dry weight [(crucible + sample weight at 105°C) minus (tare

* Naphrax®, Northern Biological Supply, 3 Betts Avenue, Martlesham Heath, Ipswich IP5 7RH, U.K., or equivalent.

weight of crucible)] per square meter of exposed surface. If 25- by 75-mm slides are used, then

$$g/m^2 = \frac{g/\text{slide (average)}}{0.003\ 75}$$

Calculate ash weight for sample [(crucible + sample weight at 500°C) minus (tare weight of crucible)]. Subtract ash weight from dry weight to obtain ash-free weight, and report as ash-free weight per square meter of exposed surface.

7. Chlorophyll and Pheophytin

The chlorophyll content of attached algal communities is a useful index of the phytoperiphyton biomass. Quantitative chlorophyll determinations require the collection of periphyton from a known surface area. Extract the pigments with aqueous acetone or methanol (see Section 10200H) and use a spectrophotometer or fluorometer for analysis. If immediate pigment extraction is not possible, samples may be stored frozen for as long as 28 d if kept in the dark.[9] The ease with which chlorophylls are removed from cells varies considerably with different algae; to achieve complete pigment extraction disrupt the cells mechanically with a grinder, blender, or sonic disintegrator, or freeze them. Grinding is the most rigorous and effective of these methods.

The Autotrophic Index (AI) is a means of determining the trophic nature of the periphyton community (see Section 10200H). It is calculated as follows:

$$AI = \frac{\text{Biomass (ash-free weight of organic matter), mg/m}^2}{\text{Chlorophyll } a, \text{ mg/m}^2}$$

Normal AI values range from 50 to 200; larger values indicate heterotrophic associations or poor water quality. Nonviable organic material affects this index. Depending on the community, its location and growth habit, and method of sample collection, there may be large amounts of nonliving organic material that may inflate the numerator and produce disproportionately high AI values. Nonetheless, the AI is an approximate means of describing changes in periphyton communities between sampling locations.

a. Equipment and reagents: See Section 10200H.

b. Procedure: In the field, place individual glass microscope slides used as substrata directly into 100 mL of a mixture of 90% acetone (water with 10% saturated $MgCO_3$ solution). Immediately store on dry ice in the dark. (NOTE: Vinyl plastic is soluble in acetone. If vinyl plastic is used as the substratum, scrape periphyton from it before solvent extraction.) If extraction cannot be carried out immediately, freeze samples in the field and keep frozen until processed.

Rupture cells by grinding in a tissue homogenizer and steep in acetone for 24 h in the dark at or near 4°C.

To determine pigment concentration, follow the procedures given in Section 10200H.

c. Calculation: After determining pigment concentration in the extract, calculate amount of pigment per unit surface area of sample as follows:

$$\text{mg chlorophyll } a/\text{m}^2 = \frac{C_a \times \text{volume of extract, L}}{\text{area of substrate, m}^2}$$

where C_a is as defined in Section 10200H.

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10300 D. Primary Productivity

The productivity of periphyton communities is a function of water quality, substrata, and seasonal patterns in temperature and solar illumination. Measurements of biomass accrual rates can be useful indicators of pollution and eutrophication, but periphyton biomass accrual is not a measure of productivity. Productivity may be estimated from the rate of oxygen evolution or carbon uptake by the community.[1]

1. Biomass Accumulation

a. Ash-free dry weight: The accumulation rate of organic matter on artificial substrata by attachment, growth, and reproduction of colonizing organisms has been used widely to estimate the productivity of streams and reservoirs.[2,3] To use this method, expose several replicate clean substrata for a predetermined period, scrape the accumulated material from the slides, and ash as described previously.

$$P = \frac{\text{mg ash-free weight/slide}}{tA}$$

where:

P = net productivity, mg ash-free weight/m^2/d,
t = exposure time, d, and
A = area of a slide, m^2.

Obtain estimates of seasonal changes in biomass of established communities by placing many replicate substrata at a sampling point and then retrieving a few at a time at regular intervals. Replace removed slides with new clean slides. The recommended collection interval ranges from 2 to 4 weeks for a year or longer.[2] Gain in ash-free weight per unit area from one collection period to the next is a measure of net production.

b. ATP estimates: Measurement of adenosine triphosphate (ATP) has been used in recent years to estimate microbial biomass in water. This technique is applicable to periphyton.[4] It provides an additional tool for assessing the magnitude and rate of biomass accumulation on substrata in natural waters. At present, the procedure should be limited to communities colonizing artificial substrata.

1) Equipment and reagents—See Section 10200I.6*a*.

2) Procedure—Either scrape periphyton from an exposed artificial substratum or, if standard glass microscope slides are used, place them in polyethylene slide mailers containing preheated (99°C) Tris buffer. Immerse in a boiling water bath for 10 min to extract ATP. If samples are not assayed immediately, freeze at −25°C; they may be stored in a freezer for up to several months. Complete analysis as directed in Section 10200I.6*b*. Slides exposed in waters containing high turbidity may collect substantial amounts of particulates including clays. ATP sorbs to these materials; the sorption results in a quenching effect.

3) Calculations—See Section 10200I.6*c*.

2. Standing Water Productivity Measured by Oxygen Method

Hourly and daily rates of oxygen evolution and carbon uptake by periphyton growing in standing water can be studied by confining this community briefly in bottles, bell jars, or other chambers. In contrast, the metabolism of organisms in flowing water is highly dependent on current velocity and cannot be determined with precision under static conditions. Productivity estimates for flowing waters and those for standing waters present different problems; therefore, separate procedures are given.

Productivity and respiration of epilithic and epipelic periphyton in littoral regions of lakes and ponds can be determined by inserting transparent and opaque bell jars or open-ended plastic chambers into the substratum along transects perpendicular to the shoreline.[5,6] Chambers are left in place for one-half the daily photoperiod. The DO concentration in a chamber is determined at the beginning and end of the exposure period. Gross productivity is the sum of the net gain in DO in the transparent chamber and the oxygen used in respiration. Values obtained are doubled to estimate productivity for the entire photoperiod. Alternatively, determine the proportion of the incubation period of the total insolation during the photoperiod more accurately by measuring the insolation of the incubation period as a percentage of the total daily insolation. Both these methods assume that photosynthesis is proportional to irradiance (i.e., not light saturated and no photoinhibition).

Failure to account for changes in DO in chambers caused by phytoplankton photosynthesis and respiration may cause serious errors in the estimates of periphyton metabolism. It is essential that these values be obtained at the time the periphyton is studied by using the light- and dark-bottle method (see Section 10200J).

a. Equipment and reagents:

1) *Clear and darkened glass or plastic* chambers,* approximately 20 cm in diameter and 30 cm high, with a median lateral port, sealed with a serum bottle stopper for removal of small water samples for DO analyses or for the insertion of an oxygen probe. Fit the chamber with a small, manually operated, propeller-shaped stirring paddle.

2) *Dissolved oxygen probe, or equipment and reagents required for Winkler dissolved oxygen determinations:* See Section 4500-O.

b. Procedure: At each station place both a transparent and an opaque chamber over the substratum at sunrise or mid-daylight and leave in place for one-half the daily photoperiod. In extremely productive environments or to define the hourly primary productivity changes throughout the day, use incubation periods shorter than one-half the photoperiod. The minimum incubation period giving reliable results is 2 h. Determine DO concentration at the beginning of the incubation period.

Include a set of Gaarder-Gran light- and dark-bottle productivity and respiration measurements with each set of chambers to obtain a correction for phytoplankton metabolism. Incubate for the same time period as the chambers. See Section 10200J.

At end of exposure period, carefully mix the water in the chambers and determine DO concentration.

* Plexiglas or equivalent. Normal glass is opaque to UV-A and UV-B radiation and could have differential effects on photosynthesis of periphyton, whereas plastic of this type is UV transparent.[7]

c. Calculations: When the exposure period is one-half of the photoperiod, calculate gross primary productivity of the periphyton community as:

$$P_G = \frac{2[V_c(C'_{fc} - C'_{ic}) + V_o(C'_{io} - C'_{fo})]}{A}$$

where:

P_G = gross production, mg $O_2/m^2/d_{12h}$,
V_c = volume of clear chamber, L,
C'_{fc} and C'_{ic} = final and initial concentrations, respectively, of DO in the clear chamber, mg/L, corrected for phytoplankton metabolism,
V_o = volume of opaque chamber, L,
C'_{io} and C'_{fo} = initial and final concentrations, respectively, of DO in the opaque chamber, mg/L, corrected for phytoplankton metabolism, and
A = substratum area, m^2.

Correct for the effects of phytoplankton metabolism in the overall oxygen change in the clear chamber by the following equations:

$$C'_{fc} = C_{fc} - C_{flb}$$

$$C'_{ic} = C_{ic} - C_{ilb}$$

$$C'_{fo} = C_{fo} - C_{fdb}$$

$$C'_{io} = C_{io} - C_{idb}$$

where:

C_{fc} = final DO concentration in clear chamber, mg/L,
C_{flb} = final DO concentration in light bottle, mg/L,
C_{ic} = initial DO concentration in clear chamber, mg/L,
C_{ilb} = initial DO concentration in light bottle, mg/L,
C_{fo} = final DO concentration in opaque chamber, mg/L,
C_{fdb} = final DO concentration in dark bottle, mg/L,
C_{io} = initial DO concentration in opaque chamber, mg/L, and
C_{idb} = initial DO concentration in dark bottle, mg/L.

Calculate periphyton community respiration by:

$$R = \frac{24V_o(C'_{io} - C'_{fo})}{tA}$$

where:

R = community respiration, mg $O_2/m^2/d_{24h}$, and
t = length of exposure, h.

Determine the net periphyton community productivity (P_N) as the difference:

$$P_N = P_G - R$$

If the incubation time is different from one-half the photoperiod, modify the daily gross production calculation as follows:

$$P_G = \frac{t_p[V_c(C'_{fc} - C'_{ic}) + V_o(C'_{io} - C'_{fo})]}{tA}$$

where:

t_p = length of the daily photoperiod, h.

Community respiration and net production calculations for incubation periods other than one-half the photoperiod are not changed.

3. Standing Water Productivity Measured by Carbon-14 Method

The approach is similar to that described above for the oxygen method. Transparent and opaque chambers are placed over the substratum, carbon-14-labeled Na_2CO_3 is injected into the chamber by syringe, mixed well, and allowed to incubate with the periphyton for one-half the photoperiod. The concentration of dissolved inorganic carbon available for photosynthesis is determined by titration. At the end of the incubation period, the periphyton is removed from the substratum and assayed for carbon-14.[5]

a. Equipment and reagents:

1) *Incubation chamber:* See ¶ 2*a* above.

2) *Special equipment and reagents:* See Section 10200J.

3) *Carbon-14-labeled solution of sodium carbonate,* having a known specific activity of approximately 10 μCi/mL.

4) *Other equipment and reagents:* See Section 4500-CO_2.

b. Procedure: At each station place a transparent and opaque chamber over the substratum and add approximately 10 μCi carbon-14/L of chamber volume. Mix water in the chambers well, taking care to avoid disturbing the periphyton. Determine concentration of dissolved inorganic carbon as described in Section 2320. At end of exposure period, remove surface centimeter of periphyton and sediment enclosed in the chamber, freeze, and store frozen in a vacuum desiccator.

Immediately before analysis, expose sample to fumes of HCl for 10 to 15 min to drive off all inorganic carbon-14 retained in the periphyton. Combust sample (or portion) by the Van Slyke method[6] or oxidize by heating in a closed system. Collect all CO_2 for radioassay either by flushing CO_2 into a two-vial train of ethanolamine (2-aminoethanol) or alternative CO_2 absorber, such as methoxyethanol (1:7)[8] or flushing CO_2 produced by combustion into a gas-flow counter or electrometer. Alternatively, extract known amounts of periphyton biomass with a tissue solubilizer,† using, for example, 1.0 mL in closed vials at 60°C for 48 h.[9] Radioassay subsamples (100-μL) by liquid scintillation.

c. Calculations:

$$P_N = {}^{12}\text{C available} \times \frac{{}^{14}\text{C assimilated} \times \text{conversion factors}}{{}^{14}\text{C available (added)}}$$

$$P_N = \frac{(a)(b)(d)(e)}{(c)}$$

where:

† Beckman BTS-450 or equivalent.

P_N = net primary productivity per unit area of substratum per unit time, mg C/m^2/d,

a = ^{12}C available = dissolved inorganic carbon, mg ^{12}C/L = (total alkalinity − phenolphthalein alkalinity) × 0.240[6] = mg ^{12}C/L,

b = ^{14}C assimilated = [(radioactivity of sample in light chamber × k_1) − (background activity of dark chamber × k_2)] × (isotope effect, 1.06). Express radioactivity as disintegrations per second (dps), i.e., counts per second corrected to 100% radioassay counter efficiency.

k_1 = correction factor to convert individually different light-chamber volumes to 1 L,

k_2 = correction factor to convert individually different dark-chamber volumes to 1 L,

1.06 = isotope effect to correct for slightly greater mass of ^{14}C than of ^{12}C, which results in a 6% slower assimilation rate,

c = ^{14}C available = ^{14}C activity added = (μCi ^{14}C added) × (disintegrations of ^{14}C/s/μCi) = 3.7 × 10^4 μCi ^{14}C added, mL,

d = a dimensional factor to convert area of substratum sampled to m^2, and

e = factor to expand incubation period to the total daylight period. After integration by planimetry or electronic digitizer of the total amount of insolation for the day, determine percentage of total represented by the incubation period.

4. Flowing Water Productivity Measured by Oxygen Method

Primary productivity of the periphyton community in a stream or river ecosystem can be related to changes in DO. These changes are the integrated effects of photosynthesis, affected by light levels and turbidity, that occur during the photoperiod by stream phytoplankton, periphyton, and the submerged portions of macrophytes. Respiration results from metabolism of plant communities, aquatic animals, and attached and free-floating microbial heterotrophs. Water depth, turbulence, and water temperature all influence the process of reaeration. Oxygen also can enter by accrual of groundwater and surface waters. Daily fluctuations in photosynthetic production of oxygen are imposed on the relatively steady demand of respiratory activity. However, this latter process may fluctuate greatly in streams receiving a significant load of organic wastes, particularly under intermittent loads such as oxygen demand from urban stormwater runoff. Respiration rates also may vary diurnally under certain conditions, but the factors involved are not well understood.

The rate of change in stream DO (q) in grams per cubic meter per hour is represented by the following function of the photosynthetic rate (p), respiration (r), reaeration (d), and accrual from groundwater inflow and surface runoff (a):[10]

$$q = p - r + d + a$$

If the equation is multiplied through by depth in meters (z), the resulting values are in terms of grams oxygen per square meter per hour. Figure 10300:2 illustrates this conceptual relationship between q, primary productivity, and respiration of the stream plant community.

The procedure measures the time-variable oxygen concentrations in a stream over a 24-h period. Compensations are made for oxygen changes due to physical factors (accrual and reaeration) and the rate of oxygen change due to biological activity that is separated into components due to respiration and primary production. The metabolic rates are the sum of the activity of the entire stream community. Planktonic productivity and respiration can be separated from overall community activity by the use of the light- and dark-bottle oxygen technique (see Section 10200J). However, in most small streams planktonic production is insignificant. The component of production and respiration due to macrophytes is very difficult to separate from periphytic metabolic activity in systems where vascular plants are common.

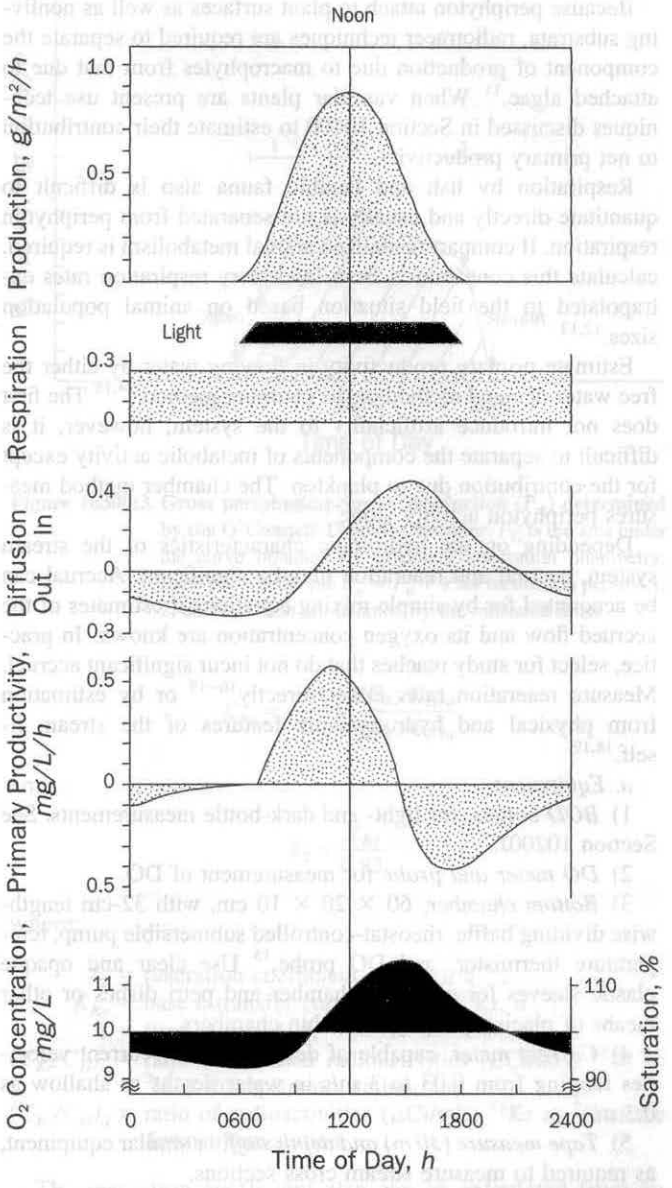

Figure 10300:2. Component processes in the oxygen metabolism of a section of a hypothetical stream during the course of a cloudless day. Production, respiration, and diffusion are given on an areal basis. The combined effect of these rate processes for a stream 1 m deep is given in mg/L/h (q). The actual oxygen values that would result in a stream with a long homogeneous community are given in the lowermost curve. Source: ODUM, H.T. 1956. Primary production in flowing waters. *Limnol. Oceanogr.* 1:102.

Because periphyton attach to plant surfaces as well as nonliving substrata, radiotracer techniques are required to separate the component of production due to macrophytes from that due to attached algae.[11] When vascular plants are present use techniques discussed in Section 10400 to estimate their contribution to net primary productivity.

Respiration by fish and benthic fauna also is difficult to quantitate directly and usually is not separated from periphyton respiration. If compartmentalized animal metabolism is required, calculate this contribution from laboratory respiration rates extrapolated to the field situation based on animal population sizes.[12,13]

Estimate primary productivity in flowing water by either the free water demand method or the chamber method.[14,15] The first does not introduce artificiality to the system; however, it is difficult to separate the components of metabolic activity except for the contribution due to plankton. The chamber method measures periphyton activity alone.[16–20]

Depending on the hydrologic characteristics of the stream system, accrual and reaeration may be significant. Accrual can be accounted for by simple mixing equations if estimates of the accrued flow and its oxygen concentration are known. In practice, select for study reaches that do not incur significant accrual. Measure reaeration rates either directly[16–19] or by estimation from physical and hydrodynamic features of the stream itself.[18,19]

a. Equipment:

1) *BOD bottles,* for light- and dark-bottle measurements. See Section 10200J.

2) *DO meter and probe* for measurement of DO.

3) *Bottom chamber,* 60 × 20 × 10 cm, with 32-cm lengthwise dividing baffle, rheostat-controlled submersible pump, temperature thermistor, and DO probe.[15] Use clear and opaque plastic sleeves for covering chamber and petri dishes or other means of placing periphyton within chambers.

4) *Current meter,* capable of detecting water current velocities ranging from 0.03 to 3 m/s in water depths as shallow as 0.3 m.

5) *Tape measure (30 m) and depth staff,* or similar equipment, as required to measure stream cross sections.

6) *Fluorometer,* capable of detecting fluorescent dye concentration at 0.5 to 100 μg/L (required only if direct measurement of reaeration is made).

7) *Liquid scintillation counter,* capable of sensitive detection of ^{85}Kr and ^{3}H (required only if direct measurement of reaeration is made).

b. Procedure:

1) Light- and dark-chamber method—Grow samples of typical periphyton communities on artificial substratum or collect natural material. Transfer identical portions to both clear and opaque chambers, taking care to use sufficient periphyton to make the ratio of chamber volume to periphyton area equivalent to the ratio of stream volume to periphyton substratum area. Measure current in the stream and match the circulation rate in the clear and opaque chambers to the current. Measure DO concentrations initially in both clear and opaque chambers and after 1 to 3 h to estimate the rate of oxygen increase or decrease. Make concurrent measurements of phytoplankton activity using light- and dark-bottle techniques as described in Section 10200J.2. Incubate light and dark bottles for the same time interval as the chambers.

Make several measurements during the photoperiod to define daily primary productivity. In addition, collect sufficient natural substratum samples of the study reach to estimate periphyton biomass (see Section 10300C). At end of incubation period harvest enclosed periphyton and determine ash-free biomass (see Section 10300C.6).

2) Free-water diurnal curve methods—Measure, hourly or continuously, DO concentration and water temperature for a 24-h period at one or two stations, depending on stream conditions, precision desired, and availability of equipment. If similar conditions exist for some distance upstream from the reach being studied, diurnal measurements of DO at a single station are sufficient to estimate productivity. Where upstream conditions are significantly different from those in the reach being studied, make measurements at the upstream and downstream limits of the reach.

If the single-station method is used, measure depth at several points along the study reach to define average depth. Map and/or make physical surveys to estimate magnitude of possible sources of accrual via effluents or tributary streams and springs. If the two-station method is used, measure the wetted cross-sectional stream area as well as current velocity at several points to define flow (in cubic meters per second) and average cross-sectional area. Correct for phytoplankton activity by light- and dark-bottle measurements (see Section 10200J.2).

3) Direct measurement of reaeration[17]—Under special circumstances it may be desirable to estimate reaeration directly although the results may not be more accurate than those of the empirical formulations usually used. The tracer gas technique is satisfactory, but is difficult and requires sophisticated equipment not routinely available. Use this method with care and with full recognition of its restrictions. Depending on stream flow, release 10 to 250 μCi ^{85}Kr with 5 to 125 μCi ^{3}H at the upstream end of the reach together with sufficient fluorescent dye to produce a concentration of 10 μg/L when completely mixed across the river cross section. Make fluorometric measurements at the downstream end of the reach until the dye peak appears, then collect water samples to measure the $^{85}Kr/^{3}H$ ratio by liquid scintillation techniques. Record time of travel for the dye peak from the injection point.

c. Calculations:

1) Chamber method—Calculation is analogous to that used for the bell jar technique discussed in Section 10300D.2.

$$P_n = \frac{V_c(C'_{fc} - C'_{ic})B}{tW_c}$$

where:

P_n = hourly rate of net primary production, mg $O_2/m^2/h$,
V_c = volume of clear chamber, L,
B = average periphyton biomass estimated for the study reach, mg/m^2,
t = incubation period, h,
W_c = total biomass of periphyton contained in clear chamber, mg,
C'_{fc} = final oxygen concentration in clear chamber, corrected for phytoplankton metabolism, mg/L:
$C'_{fc} = C_{fc} - C_{flb}$

C_{fc} = final DO in clear chamber,
C_{flb} = final DO in light bottle, and
C'_{ic} = initial oxygen concentration in clear chamber corrected for light-bottle measurement, mg/L:
$C'_{ic} = C_{ic} - C_{ilb}$
C_{ic} = initial DO in clear chamber, and
C_{ilb} = initial DO in light bottle.

$$r = \frac{V_o(C'_{io} - C'_{fo})B}{tW_o}$$

where:

r = hourly periphyton respiration rate, mg $O_2/m^2/h$,
V_o = volume of opaque chamber, L,
B = average periphyton biomass for the study reach, mg/m^2,
W_o = total biomass of periphyton contained in opaque chamber, mg,
C'_{io} = initial oxygen concentration in opaque chamber, corrected for phytoplankton respiration, mg/L:
$C'_{io} = C_{io} - C_{idb}$
C_{io} = initial DO in opaque chamber, mg/L,
C_{idb} = initial DO in dark bottle, mg/L, and
C'_{fo} = final oxygen concentration in opaque chamber, mg/L:
$C'_{fo} = C_{fo} - C_{fdb}$
C_{fo} = final DO in opaque chamber, mg/L, and
C_{fdb} = final DO in dark bottle, mg/L.

For each pair of chamber measurements,

$$P_g = P_n + r$$

where:

P_g = hourly gross periphytic primary production, mg $O_2/m^2/h$.

P_G is the area under the curve of primary production per hour through the photoperiod, mg $O_2/m^2/d$ (see Figure 10300:3). Also,

$$R = \left(\frac{\sum_{1}^{n} r_n}{n} \right) \times 24$$

where:

R = total periphyton community respiration, mg $O_2/m^2/d$, and
n = number of observations.

Thus,

$$P_N = P_G - R$$

where:

P_N = net periphytic production, mg $O_2/m^2/d$.

2) Free water methods

a) Calculation of reaeration or diffusion for both the single and upstream-downstream methods—Calculate k_2 from radiotracer data as follows:

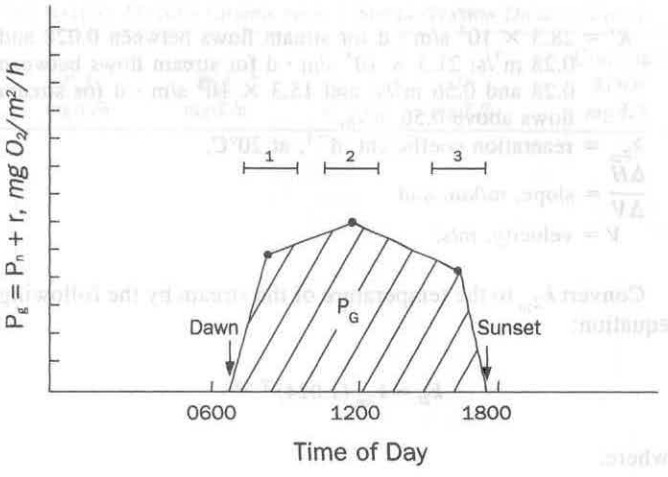

Figure 10300:3. Gross periphytic primary production (P_G) determined by the O'Connell-Thomas Chamber. P_G is the area under the curve obtained by graphical integration planimetry. Each point is the run $P_g = P_n + r$ for incubation periods 1, 2, and 3, which are denoted by the indicated lines.

$$K_{Kr} = \frac{-1}{t} \ln \frac{(C_{Kr}/C_H)_d}{(C_{Kr}/C_H)_u}$$

and

$$k_2 = \frac{K_{Kr}}{0.83}$$

where:

k_2 = reaeration coefficient (base e), d^{-1},
K_{Kr} = base e transfer coefficient for ^{85}Kr, d^{-1},
T = time of travel, d,
$(C_{Kr}/C_H)_u$ = ratio of released radioactivities (μCi/mL) ^{85}Kr to ^{3}H at the upstream station, and
$(C_{Kr}/C_H)_d$ = ratio of radioactivities (μCi/mL) ^{85}Kr to ^{3}H at the downstream station.

The reaeration coefficient also can be calculated from an equation relating the rate of energy dissipation in a stream to k_2.[17,18]

$$k_2 = K \frac{\Delta h}{t}$$

where:

K = escape coefficient,
Δh = change in water surface elevation in a stream reach, and
t = time of flow through a stream reach.

This can be expressed in terms of hydrodynamic and physical data:

$$k_{2_{20}} = K' \frac{\Delta H}{\Delta X} \times V$$

where:

K' = 28.3×10^3 s/m · d for stream flows between 0.028 and 0.28 m^3/s; 21.3×10^3 s/m · d for stream flows between 0.28 and 0.56 m^3/s; and 15.3×10^3 s/m · d for stream flows above 0.56 m^3/s,
$k_{2_{20}}$ = reaeration coefficient, d^{-1}, at 20°C,
$\frac{\Delta H}{\Delta V}$ = slope, m/km, and
V = velocity, m/s.

Convert $k_{2_{20}}$ to the temperature of the stream by the following equation:

$$k_{2t} = k_{2_{20}} (1.024)^{(T-20)}$$

where:

k_{2t} = k_2 at ambient water temperature, d^{-1}, and
T = ambient water temperature, °C.

Convert to D in mg/L/h:

$$D = \frac{k_{2t}(C_s - C)}{24}$$

where:

C_s = oxygen concentration at saturation at ambient stream temperatures, mg/L, and
C = measured oxygen concentration, mg/L.

b) Single-station method—Calculation of primary productivity and respiration from diurnal oxygen and temperature measurements at a single station is summarized in Figure 10300:4 and Table 10300:I.

Tabulate hourly DO measurements and temperatures. Determine C_s (DO of air-saturated H_2O at each temperature from Table 4500-O:I) and compute uncorrected DO consumption, milligrams per liter per hour, for each period:

$$\Delta DO_{\text{hours 1 to 2}} = DO_{\text{hour 2}} - DO_{\text{hour 1}}$$

Plot on the half hour, as shown in Figure 10300:4b.

Calculate the net primary production and respiration of phytoplankton as shown in Section 10200J. Determine the 24-h average hourly plankton respiration, $(\sum_1^n r_p)/n$ in milligrams per liter per hour every half hour. Calculate the hourly net phytoplankton production and tabulate for the approximate hours during the photoperiod. Plot as shown on Figure 10300:4c.

Calculate and tabulate k_{2t} and substitute D for each C_s, as outlined in ¶ a), above. Plot as shown in Figure 10300:4c.

Correct each ΔDO for diffusion and phytoplankton metabolism:

$$\Delta DO_{\text{corrected, mg/L/h}} = \Delta DO_{\text{uncorrected}} - D - P_p - R_p$$

Plot each point as shown in Figure 10300:4d.

Gross primary productivity of the benthic and attached algal populations is computed as the area under the curve in Figure 10300:4d from sunrise to sunset. This is primary production in grams per cubic meter per day. Multiply by the average depth

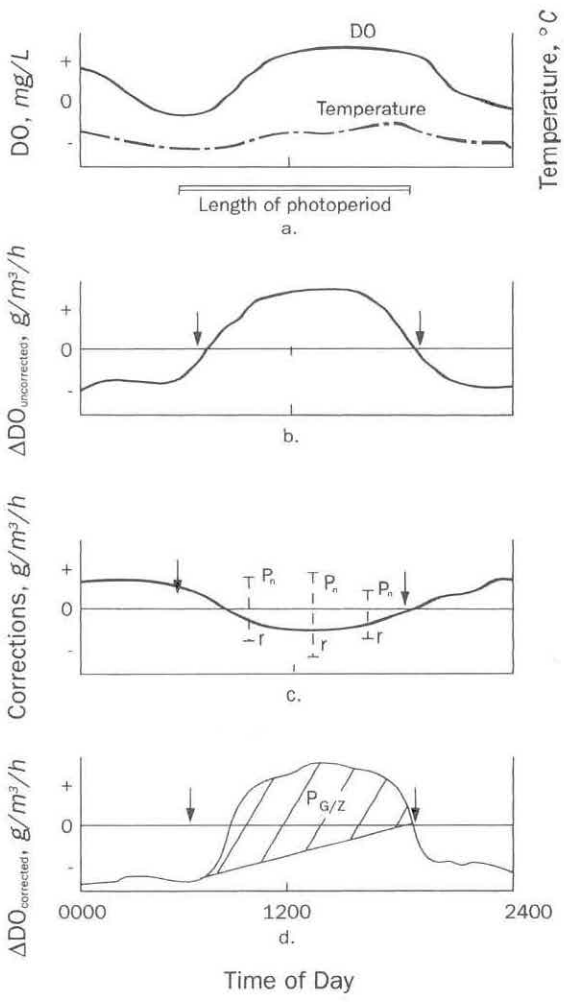

Figure 10300:4. Calculation of gross primary production at a single station. P_g, g $O_2/m^2/h$ = area of corrected rate of change curve integrated for the length of the photoperiod multiplied by average water depth, z, for the reach in meters.

for a reach, z meters, to obtain P_G in grams per square meter per day. Calculate community respiration:

$$R = 24\ z\ F$$

where:

R = community respiration, $g/m^2/d$,
z = depth, m, and
F = average hourly ΔDO for the dark period (without regard to sign), mg/L/h.

Calculate net primary productivity P_N as:

$$P_N = P_G - R$$

c) Upstream-downstream method—Calculation of primary productivity and respiration for a stream reach from upstream and downstream pairs of diurnal curves of oxygen and water temperature is summarized in Figure 10300:5 and Table

TABLE 10300:I. SAMPLE CALCULATION LEDGER FOR COMPUTATION OF CORRECTED RATE OF OXYGEN CHANGE FROM A SINGLE-STATION DIURNAL CURVE

Time *h*	DO *mg/L*	Water Temp. *°C*	C_s* *mg/L*	Uncorrected ΔDO† *mg/L/h*	P_p‡ *mg/L/h*	R_p§ *mg/L/h*	k_2 d^{-1}	D *mg/L/h*	Corrected ΔDO‖ *mg/L/h*
Midnight									
0030									
0100									
0230									
.									
.									
.									
Noon									
1230									
1300									
.									
.									
.									
Midnight									

* DO concentration at 100% saturation for a given water temperature, from Table 4500-O:I.
† Hourly rate of change of DO. For example, for noon to 1300, $DO_{1200-1300} = DO_{1300} - DO_{1200}$; plot at 1230.
‡ Phytoplankton net production.
§ Phytoplankton respiration rate.
‖ $\Delta DO_{corrected} = \Delta DO_{uncorrected} - D - P_p - R_p$

10300:II. Alternatively, calculate as below, with oxygen change expressed as the difference between stations rather than as change per hour. The calculations are analogous. Multiply the area under a curve of oxygen change between two stations, corrected for diffusion and plankton metabolism and expressed in milligrams per liter, by the discharge in cubic meters per hour, and divide by the water surface area between the two stations. This, multiplied by 24, yields gross primary productivity in grams per square meter per day.

To compute gross primary productivity by this method, tabulate upstream and downstream DO and average water temperature for the reach at each hour. Calculate ΔDO between upstream and downstream stations for each hour as

$$\Delta DO = DO_{downstream} - DO_{upstream}$$

Tabulate C_s and determine the planktonic activity. Correct for planktonic respiration by relating average hourly dark bottle DO change to the time of travel in the stream reach; correct for planktonic production by the hourly change in DO in the light bottle times the time of travel (see Table 10300:II).

TABLE 10300:II. SAMPLE CALCULATION LEDGER FOR COMPUTATION OF CORRECTED RATES OF OXYGEN CHANGE FROM THE UPSTREAM-DOWNSTREAM DIURNAL CURVES OF OXYGEN CONCENTRATION AND TEMPERATURE

Time *h*	DO *mg/L*		Uncorrected ΔDO *mg/L*	Water Temp. *°C*	C_s* *mg/L*	P_p† mg/L	R_p‡ *mg/L*	k_2 d^{-1}	Corrected ΔDO‖ *mg/L*
	Upstream	Downstream							
Midnight									
0100									
0200									
.									
.									
.									
Noon									
1300									
.									
.									
.									
Midnight									

* DO concentration at 100% saturation for a given water temperature, from Table 4500-O:I.
† Change in oxygen concentration in the light bottle per hour multiplied by travel time between the upstream and downstream station.
‡ Change in oxygen concentration in the dark bottle multiplied by travel time between the upstream and downstream station.
‖ $\Delta DO_{corrected} = \Delta DO_{uncorrected} - D - P_p - R_p$

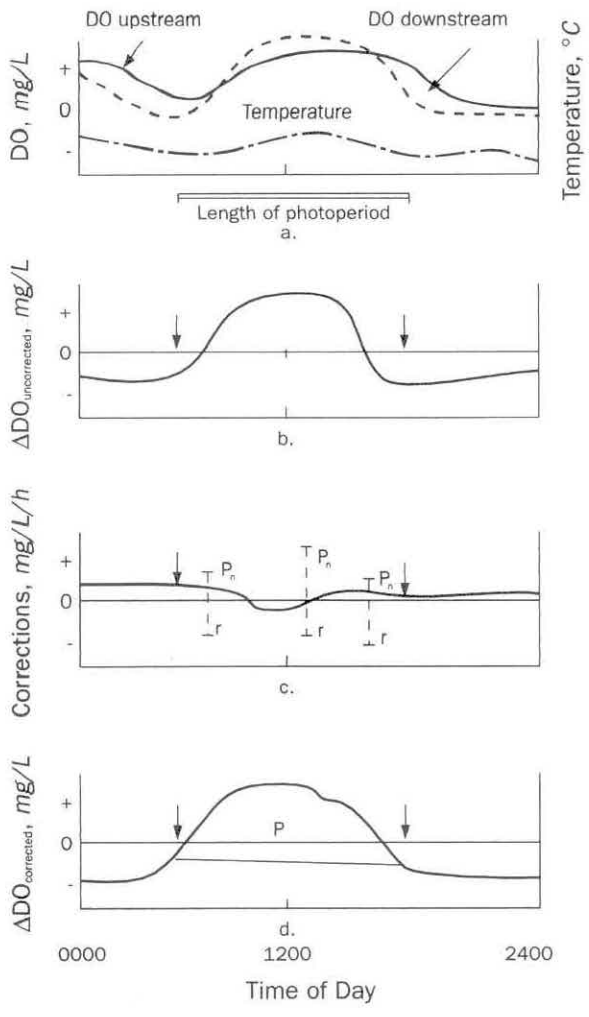

Figure 10300:5. Calculation of gross periphytic primary productivity from upstream-downstream diurnal curves. *P* is the area under the corrected rate of change graph.

Calculate or tabulate k_2 and convert it to the total oxygen diffusion for the reach. Because diffusion, *D*, is expressed as milligrams per liter per hour, multiply it by the travel time to obtain the diffusion correction.

Correct each hourly upstream-downstream ΔDO as shown in Table 10300:II. Integrate the area under this ΔDO curve from sunrise to sunset to give *P* as in Figure 10300:5d.

$$P_G,\ \text{g/m}^2\text{/d} = \frac{Q}{A}\,P$$

where:

Q = flow, m^3/h, and
A = reach area, m^2 (average reach width × reach length).

$$\text{Respiration, } R,\ \text{g O}_2\text{/m}^2\text{/d} = \frac{\Delta\text{DO}_{\text{dark}} \times Q \times 24}{A}$$

and

$$\text{Net production } P_N = P_G - R$$

Metabolism is thus estimated[21,22] with the difference in the upstream-downstream data by the graphical technique in Figure 10300:5 as:

$$\text{Net metabolism} = \Delta\ \text{DO}_{\text{light}} - \text{reaeration}$$

$$\text{Dark metabolism} = \Delta\ \text{DO}_{\text{dark}} - \text{reaeration}$$

Gross community primary productivity (GPP) then equals net metabolism minus respiration (light), and community respiration (CR_{24}) is average night respiration scaled for 24 h.

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10300 E. Interpreting and Reporting Results

Although several systems have been developed to organize ıd interpret periphyton data, no single method is universally :cepted. The methods may be qualitative or quantitative. Qualative methods deal with the taxonomic composition of the ɔmmunities in zones of pollution, whereas quantitative methods :al with community structure using diversity indices, similarity dices, and numerical indices of saprobity.

. Qualitative Methods (Indicator Species and ommunities)

The saprobity system developed by Kolkwitz and Marsson is widely used method of interpreting periphyton data. This heme divides polluted stream reaches into polysaprobic, α and mesosaprobic, and oligosaprobic zones, and lists the characristics of each. The system has been refined[1,2] and enlarged by erdingstad[3,4] and Sládeček.[5–7]

Evaluation of the saprobity system requires microscopic evalıtion of living indicator biota, particularly for the sensitive ssile protozoans. Glass slides and other transparent substrata e advantageous because they permit direct microscopic examation and identification. Removal of periphyton from slides ıd preservation for subsequent examination may be acceptable r diatoms, but observation of preserved material is not accept-ıle for most flagellated organisms.

Quantitative Methods

These methods use cell counts or biomass estimations per unit ea of substratum and numerical indices of pollution or water ıality. Considerable data on cell densities and species compoion of periphyton collected on glass slides in polluted rivers in ıgland are available.[8]

Other indices include the Shannon-Wiener,[9] Simpson's,[10] and nkham-Pearson.[11] The saprobity system[12] also may be used ıere code numbers assigned for the saprobial value and the abundance of individual species are used to calculate a Mean Saprobial Index. Results also may be expressed by the truncated-log normal distribution of diatom species[13,14] as well as the Autotrophic Index (AI).[15]

Multivariate techniques provide an excellent way to analyze and present periphyton community composition data with respect to pollution.[16–19] The importance of replication and statistical analysis, particularly in the use of multivariate techniques, has been noted.[20]

3. Water Quality Applications

Qualitative analyses of periphyton communities can be used for indication of pollution, eutrophication, and hygienic problems in the monitoring of drinking water quality.[21] Water quality surveillance can be assisted by bioassays on different types of artificial substrata in which changes and differences in species composition are determined.[22] In addition to the indicator value of individual species, the rates of biomass accrual during periphytic colonization on exposed artificial substrata can serve as a further criterion of water quality. Simple screening assays of periphyton are useful for the classification of biological stability of water in treatment and distribution systems.[23]

In wastewater treatment, qualitative periphyton analyses coupled with saprobiological evaluations may be used for classification of waste treatment plant efficiency and for monitoring of treatment plant effluents.[24] The utilization of periphyton growing on exposed artificial substrata for the reduction of nutrients in water supplies also has been proposed for water management practices.[25]

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10400 MACROPHYTES*

10400 A. Introduction

1. General Discussion

Macrophytes consist principally of aquatic vascular flowering plants, but also include the aquatic mosses, liverworts, ferns, and the larger macroalgae. Like other primary producers, macrophytes respond to the quality of the water in which they grow. The use of biota, including macrophytes, is an increasingly important and recognized technique for assessing aquatic habitats.[1–4] Macrophytes often constitute a dominant factor in the habitat of other aquatic organisms.

Freshwater macrophytes range in size from the tiny watermeal (*Wolffia* spp.), about the size of a pinhead, to plants such as the cattail (*Typha* spp.), up to 4 m high. Higher aquatic plants often are abundant, occur clustered in high numbers, many in almost pure stands, covering extensive areas of shallow lakes, reservoirs, marshes, and canals. In marine water, the intertidal rockweeds (*Fucus* spp. and *Ascophyllum* spp.) and offshore kelps (*Fucus* spp. and *Macrocystis* spp.) are conspicuous. Vascular marine or estuarine plants, such as the eelgrass (*Zostera* spp.) and the marshgrass (*Spartina* spp.), are essential to the aquatic ecosystem.

Three growth forms of macrophytes generally are recognized: floating, submersed, and emersed. Floating plants may be rooted or free-floating; their principal foliage or crown floats freely on the water surface. All or most of the foliage of submersed plants grows beneath the water surface: nearly all submersed vascular plants have roots. Growing tips of submersed plants may emerge to flower and some species can produce floating leaves. Emersed plants have their principal foliage in the air at or above the water surface; they are attached by roots to the bottom mud. In some cases the same species may grow as a floating or emersed type, or as a submersed or emersed type. Submersed and emersed vascular plants typically are rooted to the bottom but they may be found detached and floating.

The distribution and abundance of higher plants is subject to considerable spatial and temporal variation. Among the many factors that determine their presence, density, and morphology are sediment type, water turbidity, water current, nutrient concentrations, water depth, shoreline disturbance, herbivore grazing, and human activities. Zonation in the littoral zone of lakes and shallow, slow-moving streams is common. Emergent macrophytes generally are found in the most shallow portion of the littoral zone. During periods of low water level they may occupy the terrestrial as well as the aquatic habitat. The depth of inhabitation seldom exceeds 1 m. Floating-leaved plants commonly are found in the shallower littoral areas in water depths between 1 and 3 m. Submersed plants may occur from the edge of the shore to the interface of the littoral and profundal zones, but rarely extend beyond a depth of 10 m because of limitation of underwater light.

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10400 B. Preliminary Survey

1. General Considerations

A macrophyte survey includes species identification, location, assessment of health, and quantity. More detailed studies may involve functioning of aquatic plants in nutrient and heavy metal uptake and turnover, use of plants as indicator organisms, and effects of plants on water quality conditions.

Several sampling protocols are required to meet diverse survey needs. The usefulness of a given study and the appropriate types of statistical analyses are determined and fixed initially.

To develop a good sample design, determine what information is desired, prevailing environmental conditions, the life and growth form of the species being sampled, and the methods for obtaining reproducible data that are comparable to other or future studies. In defining reporting requirements, consider matters such as the use of scientific names; the selection of appropriate descriptors such as frequency, density, biomass, cover, diversity, productivity, and outer limit of vegetation growth; and the use of proper statistical techniques.

2. Pre-Field Investigations

During pre-field investigations assemble maps, charts, aerial photographs, taxonomic keys, and past reports. Maps, charts, and aerial photographs help determine access routes, project size, plant community distribution, habitat characteristics that may influence plant distribution, and sampling obstacles or hazards. These items also provide a base for doing field work and reporting results. They may be available locally from municipal engineering departments, zoning boards, planning commissions, drainage districts, and land conservation commissions. At the state level information may come from natural resource agencies, natural history survey organizations, universities, and transportation departments. At the federal level, the Geological Survey, Natural Resources Conservation Services, Bureau of Land Management, Forest Service, Park Service, Fish and Wildlife Service, Tennessee Valley Authority, Bureau of Indian Affairs, Army Corps of Engineers, Environmental Protection Agency, and the National Oceanic and Atmospheric Administration have many maps, charts, and aerial photographs available. A final source is private map companies that publish hydrographic maps for fishermen and recreational boaters. Maps, charts, and photographs in digital form from the sources listed above, including private companies, are becoming increasingly common.

Past reports provide historical records useful for planning sampling logistics and interpreting results. Comparable studies taken at different times provide a dynamic look at the vegetation. An often-overlooked resource is a herbarium storing scientific plant specimens. These generally are located in universities, natural history museums, and research botanical gardens.

3. Field Reconnaissance

Sampling efficiency is improved and a sampling scheme can be refined during a field reconnaissance. It provides an opportunity to learn the species of plants present and to sketch their distribution. The species-area curve technique frequently is used to determine the likelihood of finding more species during a preliminary survey. A field reconnaissance allows the investigator to answer logistical questions that are the bane of all sampling efforts.

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Also see Section 10400A.3.

10400 C. Vegetation Mapping Methods

Mapping vegetation stands may be necessary. Do this during the preliminary survey.[1]

1. Baseline Method

Vegetation maps constructed using the baseline method or the basepoint-stadia rod-alidade method generally are limited to pure stands of floating or emersed littoral macrophytes in all bodies of water. In clear water, the outline of pure stands of submersed vegetation can be determined by using a viewing box (usually a wooden or plastic box with a watertight glass lens) from the surface or by underwater observation by a diver using a snorkel or SCUBA (self-contained underwater breathing apparatus). The baseline method and the basepoint-stadia rod-alidade method provide accurate maps of vegetation in areas up to 1×10^5 m^2 where most of the vegetation outline is visible. The baseline method uses intercepting lines from each end of a predetermined base line to closely spaced markers (i.e., chaining pins) around the stand. By presetting the map scale, the ratio between the length of the base line and its reduction on the map (drawn on a plane table) can be determined. The basepoint-stadia rod-alidade method is a modification of the baseline method in that the distance between the vegetational outline and the base point is determined with an alidade, range finder, or portable Loran-C unit. One unit on the stadia rod, as viewed between the cross-hairs of the alidade, is equivalent to a distance of 3.05 m between the stadia rod and the alidade. Chaining pins are not required when this method is used. In practice, more readings closely spaced along the vegetational outline usually are taken using the basepoint-stadia rod-alidade method.

It is not necessary to measure all distances and angles; some can be determined trigonometrically. After all angles and distances are calculated, fill in irregularities through inspection and use of other maps and photographs.

The technology for using global positioning systems (GPS), especially when linked to geographical information systems (GIS) and remote sensing, is evolving rapidly.[2] These systems have wide applicability to mapping aquatic vegetation, thereby rendering older surveying techniques obsolete or at least time-consuming and tedious.

2. Line Intercept Method

The line intercept method is preferable for mapping mixed stands and/or large areas. Select sampling points at equal intervals along a base line. Choose interval length by the degree of accuracy desired: the closer the sampling points, the more accurate the map. Run transects perpendicularly from the base line to the boundary of the plant stand. Use an intercept line (transect line) of plastic-coated wire rope to prevent stretching. If line flotation is a problem, use lead weights applied at regular intervals to sink the line and act as interval markers on the rope to designate sampling units. Use 0.5-m segments (in dense vegetation) to 5-m sampling units (in sparse vegetation) for determining plant species that vertically intercept the line at each segment. During underwater surveys, record data with a wax or soft lead pencil on a writing board constructed of plastic overlays. Construct the vegetation map by placing points where plant species are found within an outline map (or aerial photograph) of the sample area. Determine the area that a single species or total vegetation occupies by planimetry or digitization and computer calculation.

Determine frequency from line intercept or quadrat data, or with a set sampler consisting of a 2-cm steel tube, 2 m long, to which five 0.75-cm by 25-cm steel rods are attached on 40-cm centers. Record vegetation touching each of the five points within 2.5 cm of the distal tip. If more than one plant species is touching, record the plant nearest the tip. If no plant is touched, record bare ground.

3. Remote Sensing

a. General considerations: Remote sensing techniques are used to detect, assess, and monitor aquatic macrophytes. These techniques include analog aircraft and satellite serial photography, digital aircraft and satellite multispectral scanning in the visible, infrared, and thermal bands; microwave techniques, primarily side-looking airborne radar (SLAR); and shuttle imaging radar (SIR). They provide a synoptic view of large areas, and allow quick surveys to further delineate areas of interest and repeated viewing at relatively low cost.

In selecting remote sensing system(s) consider expected results, time for project completion, and available resources. The larger the area, the greater the advantage of remote sensing. Remote sensing also lends itself to studies over time, because each image is a historical record.

For determining general associations of a widespread macrophytic growth, ground resolutions of between 30 and 80 m are recommended. Widespread multitemporal coverage is available at scales ranging from 1:100 000 to 1:1 000 000 at a reasonable cost in the form of paper prints, transparencies, and digital formats. Landsat Multispectral Scanner (MSS) and Thematic Mapper (TM) have been used to identify areas with emergent vegetation or topped-out submergent vegetation. Surface roughness is a requisite for an imaging return with SLAR and SIR. Landsat provides limited capability for species discrimination, but availability, cost, and repeat cycles make it useful in determining presence of large populations over time. For a detailed vegetation survey, including discrete species identification, use much larger-scale imagery, (1:10 000 or greater). High-altitude aerial coverage available through the National High Altitude Mapping Program (NHAP) and other sources at original scales of approximately 1:60 000 to 1:120 000 provides both good initial areal coverage and the capacity for enlargement up to five times.[3]

After determining scale, select film/filter or sensor combinations. These include black and white imagery, color infrared, and black and white-infrared imagery; color infrared film used with a yellow filter is widely applicable, but other combinations also are useful.[4–7] For detailed flight planning consider growth stage of plants, water depth and clarity, tidal conditions, cloud cover, and sun angle.[3,8,9] Available resources ultimately determine remote sensing activities.

b. Aerial photography:

1) Equipment—For all-format aerial photography, use a good-quality 35-mm single-lens reflex (SLR) camera with man-

ual or through-the-lens metering or any good 70-mm camera system, preferably with a motor drive. Intervalometers providing for exact exposure intervals for stereo photography are available for both systems. A 28-mm-focal-length lens with a 35-mm SLR camera gives wide coverage at low altitudes; 40-mm and 80-mm lenses are successful with 70-mm camera systems. When photographing with black-and-white or color film, use a skylight or haze filter. When photographing with color infrared film below 1700 m, use a Wratten 12 filter; at altitudes above 1700 m use a Wratten 15 filter. Time of year and condition of vegetation also may influence the choice of yellow/orange filter. In turbid waters, color film* provides better water penetration and is more useful in mapping submersed vegetation than is color infrared film.[4] Color infrared† yields more detail, is more useful in mapping emergent vegetation or wetlands, and may provide more detail in clear, nonturbid waters. Video cameras (camcorders) and digital cameras also are being used to obtain aerial photos of vegetation.

Preferably mount camera in the belly of a high-wing, single-engine aircraft for low-altitude, small-format photography. Belly mounts require special aircraft modification, but provide a stable platform, protection for the camera, and good access. Alternatively, camera may be mounted on the aircraft door.

2) Procedures—Because sun angle is critical in obtaining high-quality aerial photography, schedule flight for a time when solar altitude is between 40 and 68 degrees.[7]

Set camera at designated ISO reading for the film (assume 100 to 125 for color infrared film) and shoot in the automatic exposure mode. At a typical airspeed of about 190 km/h (approximately 120 mph) a shutter speed of 1/250 or 1/500 is adequate. Determine proper f-stop from an aerial exposure computer; in general, f 5.6 to 11 (f 8 is optimum) gives acceptable color exposure. Proper exposure of color infrared film depends on such factors as time of day and year, altitude, humidity, and type of landscape.

Process film through the manufacturer, a photo laboratory, or aerial photography service. Development of color infrared film is available solely through the manufacturer. Once an aerial photograph has been developed, it can be scanned into a digital format for computer analysis.

c. Fathometry: Recording fathometers are best applied in water more than 1 m deep where the instruments can determine accurately the height and distribution of subsurface macrophytes.

A recording fathometer can be mounted on most boats and can accurately determine one-dimensional (percent cover) and two-dimensional (percent vertical area) profiles of submersed vegetation. Fathometry is especially useful for determining the outer edge of plant growth. Make linear and planimetric measurements on chart tracings that provide permanent records for ready comparison over time. Calculate percent cover by dividing the linear measurement for a macrophyte species or community by the total chart paper length for any given transect. Dividing the area of the tracing with macrophytes by the total water area gives percent vertical area. Use a fathometer accurate to the nearest 0.1 m.

Mount the transducer for the recording fathometer with brackets on the boat's transom. Keep speed and recorder speed constant to produce tracings of similar length and resolution. Only a few minutes are required to replicate transects several kilometers long. Unless gross morphological differences exist, species discrimination on the chart tracings is difficult or impossible. Mark boundaries of monotypic colonies and community types with a fixed line on the chart tracings. Dense vegetation mats that reach the surface may impede boat movement, prevent the transducer signal from reaching the hydrosoil, and merge tracings of macrophytes with the transducer line. Tracing patterns from water less than 1 m deep may be difficult to interpret.[10] Location of transects or points along a transect also can be determined using GPS techniques.[2]

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* Kodak Ektachrome Professional film type 5036 or equivalent.
† Kodak Aerochrome Infrared film type 2443 or equivalent.

10400 D. Population Estimates

1. Sampling Design

The design of a sampling program depends on study aims, collection methods, variation and distribution of vegetation, personnel and funds available, and accuracy expected. Variation in space usually is not random; distribution is determined by water depth, shoreline activity, sediment type, or other factors. The parametric statistic for estimating the true population mean assumes that the population being sampled has a normal distribution and that all sample units have the same probability of being selected. Avoid fixed sampling stations in sampling programs to determine population means, unless they are chosen at random at the beginning of the study. Because normally distributed plant populations may not be a characteristic of contiguous plant communities, use parametric statistics with caution.

The simple random sampling design is best applied to homogeneous, noncontiguous plant communities. The number of stations required to obtain an estimate of the true population mean with a predetermined level of confidence and permissible error can be determined by applying the data from a pilot study to the following equation:

$$N = \left(\frac{t \times S}{d \times \bar{x}}\right)^2$$

where:

N = number of sampling stations,
t = Student's t at a given probability level; because N is unknown, set $t = 2.0$; t is approximately equal to 2.0 for $N > 30$,
S = standard deviation,
$\bar{x}$ = estimator of true population mean usually determined by conducting a pilot study; and
d = permissible error of the final mean; $d = 0.1$ is recommended for vegetation studies ($\pm$ 10%).

An estimate of sampling program cost may be obtained as the sum of initial fixed cost (such as cost of equipment purchase) and variable cost (cost per sample multiplied by number of samples).

Apply stratified random sampling to populations having many homogeneous stands. This design is best applied to populations with obvious gradients and, in practice, to gain precision by the minimized variance within strata. Determine placement of strata by a pilot study. To maximize precision, place stratum boundaries around homogeneous areas; generally, the fewer strata, the greater precision. Allocate sampling in stratified random sampling design according to:

$$\frac{N_i}{N} = \frac{W_i S_i}{\Sigma(W_i S_i)}$$

where:

N_i = number of samples in stratum i,
N = total number of samples,
W_i = a weight reflecting the size (number of quadrats, for example) of stratum i, and
S_i = standard deviation of sampled characteristic within stratum i.

Means for population measurement taken along randomly placed stations on a transect line do not represent large areas of lake populations unless the transect line is placed randomly. Randomization is important; arbitrarily placed transect lines within a sampling area may or may not reflect the true variation of the vegetation within.[1]

2. Collection Methods

a. Field inventory/reconnaissance:

1) Manual collection—If water depth, clarity, temperature, flow, and other circumstances permit, collect specimens by hand. Under ideal conditions, manual collection by wading, snorkeling, or with SCUBA in deeper water habitats permits a detailed and comprehensive evaluation of the macrophyte community.

2) Drag chains—Construct drag chains by welding sharpened U-shaped hooks to a short length (0.6 to 1.0 m) of medium-weight chain. Attach chain to a rope and pull it through the water. Attach a float to the end of the rope to prevent its loss if the chain is snagged and/or dropped. The drag chain can be used readily from a slow-moving or stationary boat and is most efficient in collection of submersed macrophyte species with tall growth forms.

3) Rakes and tongs—Rakes with various handle lengths and oyster tongs may be useful in collecting macrophytes. A rope may be attached to the rake handle for sampling in deep water or to facilitate sampling over a wider radius.

4) Grab samplers—Devices developed for sampling benthic organisms, such as the Ekman, Ponar®*, and similar grab samplers (see Section 10500B.3), may be used to collect macrophytes. The light weight of the Ekman grab makes it preferable for the rapid and numerous samplings often required for survey inventories.

5) Recording fathometers—Use to determine height and distribution of subsurface macrophytes. Species with similar morphology usually cannot be distinguished from chart tracing; use supplemental methods to identify species.

b. Quantitative sampling: Numerical data collected to describe vegetation commonly include such measures of abundance as density, frequency, cover, and biomass/standing crop.[2-4] Collect these data from plots or quadrats or, less frequently, by plotless sampling techniques. The choice of analytical method depends on vegetation density and types, water depth, flow, height of vegetation in the water column, and nature of the sediment.

1) Line intercepts[5]—This plotless sampling technique entails use of a weighted nylon or lead core line laid along the bottom between two known points or oriented by a compass reading or GPS unit. For dense floating mat vegetation, a floating line may be laid on top of the mat. A surveyor measures the linear distance occupied by various species that underlie the transect line. Express these as a percentage of the total line length for individual species as well as for all species combined. If frequency data are

* Registered trademark of Morris & Lee, Inc. d/b/a Wildlife Supply Co., Buffalo, NY.

desired, mark the line in increments (e.g., 1 m) and treat species presence/absence in a manner similar to data from quadrat sampling. The line intercept has been used to characterize and map aquatic communities[6] and to correlate distribution of macrophytes with selected environmental factors.[7] Line transects also are useful for determining patterns of plant distribution.[8] In aquatic environments, the line-intercept method is time-consuming and may require a diver equipped with SCUBA. Problems arise in determining whether a plant underlies the transect line.[9]

2) Belt transects—This technique is similar to the line transect and is useful for biomass or density determinations. Data are collected along a fixed line, but from a two-dimensional plot or belt. The belt can be treated as a series of contiguous quadrats or quadrat location may be selected on the basis of a fixed or random interval or water depth. Use floating or sinking frames.

3) Quadrats—Quadrats can be used for such population and community estimates as frequency, cover, density, and biomass. Quadrats can be any two-dimensional shape but are typically round or rectangular. The sampling area of quadrat samplers can be of any size, but typically varies from 0.1 to 1 m^2.

With the exception of frames, most sampling devices described have been used to obtain estimates of above-ground biomass (standing crop).[10] Above-ground biomass generally is used because of the difficulty in collecting underground plant parts, such as rhizome and roots. Without the underground parts, however, the data are of limited value for estimates of primary production.

a) Manual samplers—These are relatively simple devices for sampling macrophytes, such as cutting shears. Although they can be used in deep water and manipulated by a diver, they work best in shallow water. They are relatively inexpensive and can be constructed easily or purchased from commercial sources.

Frames are suitable for sampling in shallow water. For sampling short, erect plants, use a square sinking frame constructed of metal. For dense or tangled vegetation, a square assembly frame with pins or wing nuts at the corners or a fixed-corner three-sided frame may be useful. Decide whether to include only macrophytes rooted within the frame or also overlapping plants. In deep water, difficulty and bias may occur in sampling tall submersed vegetation. For macrophytes forming a dense floating mat, use a floating frame constructed of wood or PVC pipe.

Box samplers are useful for sampling where water is shallow and the bottom consists of unconsolidated sediments. The sampler consists of an open-ended box with a metal cutting flange at the bottom and lateral handles; a sampler constructed of 7-mm plexiglass with dimensions 0.5 m × 0.5 m × 0.6 m and aluminum cutting flange and corner reinforcements weighs about 12 kg. With modifications, a box sampler can be used in deep water.[11]

Benthic dome (BeD) samplers[12] may be used for sampling in deep flowing waters. The sampler consists of a plastic dome with a stainless steel circular collar that can be pushed into the substrate. It weighs approximately 11 kg and has a sampling area of 0.25 m^2.

Various samplers[13,14] developed for macroinvertebrate sampling also may be used to collect macrophytes. These include the Surber sampler, suitable for shallow rivers with moderate current [see Section 10500B.3*b*1)]; the stovepipe (cylindrical) sampler, suitable for wadable waters with unconsolidated sediment bottoms [see Section 10500B.3*c*4)]; and the Ekman grab sampler, best suited for soft sediment bottoms with short, erect vegetation [see Section 10500B.3*a*6)].

b) Mechanically operated samplers—Mechanical sampling devices are costly and complex, and require a floating platform with winches, cables, and booms. The samplers described below are useful in deep water. They may decrease sample collection time, increase accuracy of above-ground and total biomass estimates, and be subject to less bias than many manual methods.

CAUTION: *Use extreme caution for safe operation.*

The Louisiana box sampler is an open-ended 35-cm-high box made of sheet metal or similar material that samples a 61- × 61-cm quadrat (sampling area = 0.37 m^2).[15] It can be used from a V-hulled or pontoon boat and is hoisted above the water with a cable and boom. A quick-release mechanism lets the sampler fall free through the water column. Aquatic vegetation is trapped against the bottom and severed by cutting edges along the base of sampler. A nylon net sack over the top retains severed plant fragments. A diver inserts a horizontal cutting blade in a slot at the level of the substrate before the sampler is hoisted to the surface. Manual insertion of the cutting plate by a diver makes use of the Louisiana box sampler comparatively efficient. In soft sediments, the sampler may penetrate too deeply and require lifting before the cutting plate can be inserted. Rocks, stumps, roots, and other debris may prevent complete closure of the cutting door.

The Osborne sampler[16] is a stainless steel box having outside dimensions of 50 cm × 50 cm × 60 cm high and a sampling area of 0.25 m^2. The sampler weighs 110 kg and is operated by winch and cable from a pontoon boat. After hoisting and suspending the sampler alongside the pontoon boat, a quick-release mechanism allows free-fall through the water column. Tempered steel blades along the bottom edge of the sampler cut vegetation during the descent. A wire mesh screen fastened to the top prevents loss of plant fragments. A hinged slotted door is closed with a lift cable and the sampler is winched to the pontoon boat platform for removal of macrophytes and sediments. Because the sampler penetrates and collects sediments, the sample includes roots and rhizomes and can be used to estimate total biomass as well as above-ground biomass. Efficient operation and accurate biomass estimates require an unconsolidated substrate free of rocks and other debris.

The Waterways Experiment Station (WES)[17] sampler is made of perforated stainless steel and operated from a pontoon boat with an overhead beam that allows it to be hydraulically raised and lowered through a circular opening in the pontoon's platform. Two types are available: one is cylindrical with a sampling area of 0.28 m^2 and the other is square with sampling area of 0.39 m^2. Rotating cutting blades at the base of each sampler sever vegetation as the samplers are lowered. The bottom cutting plate of each is closed hydraulically. A major advantage of the WES sampler is its capability to obtain plant samples from any depth. The Louisiana Box and Osborne samplers, once released, free-fall to the substrate, whereas the hydraulic operation of the WES sampler controls its descent. The size and weight of the trailer and pontoon boat for the WES sampler restrict its use in certain water bodies and require an improved ramp for launching. Although the WES square sampling head is reported to provide a more accurate estimate of above-ground biomass than the circular one, a substantial underestimate of actual above-ground biomass still is reported.[17]

3. Sample Preparation and Analysis

a. Biomass:

1) Fresh weight (wet weight)—Wash samples free of silt and debris, place in a nylon bag (mesh size 0.75 cm) and spin in a garment washer at 560 rpm for 6 to 7 min to remove excess moisture. Weigh sample to nearest 0.1 g.

2) Dry weight—Dry subsample (not less than 10%) in a forced-air oven at 105°C for 48 h or until a constant weight is achieved. The coefficient of variation for a series of subsamples *should not exceed 10%*. Calculate dry weight by dividing dry weight of subsample by fresh weight of subsample times fresh weight of sample.

3) Ash-free dry weight—Transfer dried subsample to a covered and preweighed crucible. Ignite at 550°C for 6 h. The amount of ash is the weight of material remaining after combustion. Calculate ash-free dry weight by determining the ratio between ash and dry weight times dry weight of sample (see Section 10300C.6).

b. Chlorophyll content: Extract fresh plant material with 90% acetone made basic with $MgCO_3$. Grind the plant material and centrifuge at 2500 rpm for 10 to 15 min. Wash residue with acetone and add filtered washings to extract. Dry overnight in a container with anhydrous Na_2SO_4. Dilute with 90% acetone and water. Determine chlorophyll content (see Section 10200H).

c. Carbon content: Most plants (entire) contain 46 to 48% carbon on a dry-weight basis. A factor (46.5%) can be used to calculate carbon content and make comparisons.

d. Caloric content: Determine energy content by bomb calorimetry.

e. Species identification:

1) Sample preparation—Use fresh specimens for identification wherever possible. Avoid immature plants or plants lacking flowers. Because aquatic plants contain from 80 to 95% water and have less supportive tissue than terrestrial forms, a different procedure is required for drying, preserving, and mounting them. Collect plants during peak growth when flowers and/or fruits are present, if practical. Collect the entire plant (stems, rhizomes, leaves, roots, flowers, and fruits).

After collection, either press plants in the field[18–20] or wrap specimen in several layers of paper and submerge in water. Label wrapped specimens with date and location of collection on an index card and place sample and card in a plastic bag. Preferably use an ice chest containing crushed ice for storage in the field. Press plants as soon as practical. They can be kept for several days under refrigeration at 4°C.

Clean plant of all silt and residue. Prepare a mount by centering the plant on 100% rag herbarium paper. Place emergent plants immediately on paper because they take on a natural posture. Place a limp plant in a shallow pan of water and slide the herbarium paper under it; with a slow motion, raise the paper at a 30° angle while keeping the plant centered. Leaves and stems should lie flat on the paper. Drain off excess water, cover with wax paper to prevent plant from sticking to blotters, and place in a plant press between paper and blotters. Place plant press in a dryer. Dry plants at room temperature, but change blotters at least every other day until the plant is sufficiently dry for permanent mounting.

To prepare a wet mount place specimen in an airtight glass vessel filled with 1 part 10% formalin, 3 parts water, and a trace of powdered copper sulfate. Plants will remain lifelike and retain their color for many years in this condition.

2) Identification—A stereomicroscope is needed to identify many plants, especially aquatic grasses and sedges. Observe vegetative and floral structures by dissecting them, under magnification, with forceps and fine needle probes.

Preferably identify to species. Numerous references are available to assist in identifying aquatic macrophytes (see Section 10900E.4).

3) Plant label—An important part of the species collection is the label that identifies the plant, the collector, the location of the collection, and the date of the collection.[20] Attach label to the sheet with the mounted plant. The mounted plant is a permanent record that is most useful when placed in an herbarium where it can be utilized by others.

4. Data Presentation

Express fresh weight (wet weight), dry weight, and ash-free dry weight as grams or kilograms per square meter. Data are best expressed as ash-free dry weight of total biomass. Determine significant digits for dry weight and ash-free dry weight from the accuracy of the scale used to obtain fresh weight: do not use more significant digits than those used for expressing fresh weight. Report pigment as grams chlorophyll per gram dry plant matter and caloric value as gram calories per gram dry plant matter.

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10400 E. Productivity

1. General Discussion

The complexity and heterogeneity of form, function, phenology, and distribution of aquatic macrophytes have resulted in diverse ways of determining their productivity. These methods can be grouped broadly as biomass methods, based on biomass or on changes in biomass, and metabolic methods, based on estimates of inorganic carbon or oxygen exchange resulting from photosynthesis. The biomass methods generally are simpler than the metabolic methods and require little specialized equipment or expertise and fewer assumptions. Biomass methods integrate responses to environmental conditions and may provide estimates of above-ground production only. They are best used for long-term comparisons (several months to a year) because they are easily confounded by seasonal changes. Biomass methods are insensitive to losses due to fragmentation, herbivory, and secretion or leaching of dissolved organic compounds. In contrast, metabolic methods provide instantaneous measures of photosynthesis and thus reflect responses of plants to different environmental conditions. Metabolic methods for estimating plant productivity also can provide insight into factors controlling distribution and success. The principal drawbacks of the metabolic methods are that they require specialized equipment and assumptions that may be tenuous and are based on photosynthetic rates (typically measured over a period of minutes to hours) and require extrapolation to net assimilation over longer periods.

In choosing a method consider why macrophyte production is of interest and the use for the data, the habit (growth form and phenology) and habitat of the population, and the cost and effort required to obtain the desired information. The common methods are listed in Table 10400:I and are described below.

2. Biomass Methods

a. Biomass harvest methods: Biomass measurements vary from a simple, one-time sample of maximum biomass to complicated evaluations of seasonal biomass dynamics by methods originally intended for grassland plants.[1–3] These methods are applicable mainly to emergent and submersed macrophytes. Preferably evaluate productivity of floating plants by using growth rates, permanent quadrats, and random samples[4] or by the turnover and metabolic methods discussed in ¶s 2*b* and 3, below.

1) Above-ground biomass measures—Peak above-ground biomass is measured by the above-ground biomass (usually as ash-free dry weight per unit area) at the time of apparent maximum above-ground biomass (usually time of flowering). This single measurement does not account for biomass carried over from the previous season, losses of material before the peak, or growth after the peak, and therefore generally underestimates net above-ground annual production (NAAP) to an extent depending on the relationship of annual turnover to maximum biomass.[5] The method provides a reasonable estimate of NAAP for many submersed species.

The seasonal biomass accumulation method[6] is a modification of the peak above-ground biomass method that considers only the positive changes in live material. Live above-ground biomass is determined at the beginning of the growing season and at the time of maximum above-ground biomass; the NAAP is calculated as the difference. This method accounts for yearly carryover of living material, but in some cases this will result in a further underestimate of NAAP relative to the peak above-ground biomass.[2]

When recruitment is continuous during the growing season, the biomass peak is less well-defined and greater losses may occur before the seasonal maximum. Under such conditions,

TABLE 10400: I. METHODS USED TO DETERMINE MACROPHYTE PRODUCTION*

Method	Plant Habit					
	Emergent		Floating		Submersed	
	Deciduous	Evergreen	Deciduous	Evergreen	Deciduous	Evergreen
Biomass harvest:						
Above-ground biomass	+	–	+	–	+	–
Biomass dynamics	+	–	–	–	–	–
Biomass tagging:						
Turnover, growth increment, and summed shoot maximum	+	+	+	+	+	+
Cohort	+	–	+	–	+	–
Below-ground biomass	+	+	+	+	+	+
Oxygen measurement:						
Light and dark bottle	–	–	–	–	+	+
Open system	–	–	–	–	+	+
Radiocarbon incorporation	–	–	–	–	+	+
Inorganic carbon exchange:						
Continuous CO_2 exchange	+	+	+	+	+	+
Discrete inorganic C measurement	–	–	–	–	+	+
Potentiometric C flux	–	–	–	–	+	+

*+ designates applicable method: – designates method not commonly applied. Evergreen implies retention of substantial above-ground biomass year round: deciduous implies that 10% or less of seasonal maximum biomass is present year round.

above-ground biomass methods yield poor estimates of the NAAP.[3]

2) Biomass dynamics methods—These methods are applicable to emergent plants when dead material remains near the site of decomposition.

The Smalley method[7] estimates net production on the basis of samplings of live and dead material (per unit area) at regular 3- to 6-week intervals. Net production equals the increase in material between samplings: a decrease in live and dead material indicates no net production; an increase in live material and decrease in dead material indicates production equal to the increase in live material; and a decrease in live material and increase in dead biomass with a negative sum indicates no production, while a positive sum indicates production. The method underestimates production if dead material from other areas is present or if new growth is undetected when mortality is high. It is sensitive to sampling frequency[3] and requires a homogeneous area large enough to accommodate replicate sampling. A modification of this method can be used where above-ground biomass varies little from year to year; in this case net above-ground production is assumed to equal the summed losses of dead material.[8]

More complex procedures based on harvests from a series of paired plots have been proposed.[3,9,10]

b. Biomass tagging methods:

1) Biomass turnover and growth increment methods—Leaf turnover and biomass marking studies involve marking individual plant leaves to follow production, growth, and loss over the year or growing season. For plants with large, long-lived leaves and basal growth, e.g., *Vallisneria, Zostera,* macroalgae, determine short-term growth of individual leaves. Use these methods for studies of populations (species) rather than communities. Marking methods are particularly useful for evergreen plants, where there may be little seasonal change in biomass, and for plants where the ratio of production to biomass is either very much greater than, or very much less than, one. Turnover measures estimate production and biomass loss and are considered better than harvest techniques, both with respect to accuracy and for the additional phenological information. Tagging methods require major efforts in regular censusing and SCUBA diving for sampling deep, submersed populations.

Where leaves or plant parts are about the same size seasonally, the method is relatively simple, requiring only periodic censusing of plant parts. These methods are not appropriate for species with much branching or different rates of leaf production and loss per branch, and are confounded by intense grazing or by sloughing of newly produced parts.

a) Biomass turnover—To determine turnover time of leaves and shoots, where individuals are easy to distinguish, or where vegetative spread is insignificant or easy to account for and leaf size is fairly constant, choose 10 to 50 individual plants and mark each leaf of each plant. If all new leaves are initiated to the inside of older leaves tag only the newest leaves initially. Mark new leaves or stems with anything that does not interfere with normal growth and is not easily lost, e.g., staples, hole punches, plastic bird rings, fishing line, indelible markers. At regular intervals, revisit plant, tag new leaves, and record the number of new leaves and total number of leaves present. Because new leaves may not be fully expanded at a sampling visit, use a convention concerning the developmental stage of leaves.[11] The sampling interval (weeks to months) depends on research needs and the likelihood of losing the youngest tagged leaf. Compute the annual leaf turnover for each individual plant as the number of new leaves produced annually divided by the maximum number of leaves present at any time in the season. Because the turnover rate is the ratio of production to biomass, calculate NAAP by multiplying the turnover ratio by the maximum above-ground biomass. If vegetative spread is common, modify this method so

that all stems or leaves of a species within plots of a given area are tagged.[12,13] Revisit plots at regular intervals, tag new leaves, and record total number of leaves present. Calculate production as above. This method will not account for changes in plant size (increase in weight between years), for mortality, or for recruitment into the population, because only plants initially present that survive the season are included.

b) Growth increment measurements—If leaf size changes throughout the growing season or if several types of above-ground parts are present, use methods that account for such differences. Such methods frequently are used for seagrasses.[14–16] Mark each leaf of every plant within a quadrat at a set level above the bottom, relative to a stationary stake or frame. If plants are not buried deeply, use a set distance above the base (i.e., rhizome or root-shoot interface). After a predetermined time, remove all leaves or shoots at the level of the stake or at the set distance. Weigh unmarked leaves produced during the interval. Remove and weigh growth on older leaves (the portion below the marking, but above the ground or base). The combined weights are the net leaf production during the interval. These data also can be used to calculate relative growth rates as [(ln Δ weight)/Δ time]. Make detailed measurements of leaf growth rates by marking plants at the reference level more frequently, e.g., every other day.[17]

A modification of this method[18] permits computation of the growth rate of individual leaves and production of different plant parts.

c) Summed shoot maximum method—Where nondestructive measures are required, determine shoot size and number and estimate production.[3] Choose permanent quadrats of a size allowing easy enumeration of plant parts. Label every stem in each quadrat. On regular sampling visits label and count new stems. Develop length:weight regressions using plants collected from areas near but outside the quadrats. This procedure is required only once or twice during the year, depending on the characteristics of study species. Estimate net above-ground (shoot) primary productivity using the number of new parts produced and their weights (based on length:weight regressions). Estimate production per unit area as the mean leaf turnover, multiplied by maximum shoot mass.

For certain species, more complex variations of this method have been used.[19,20]

2) Cohort methods—Cohort methods often are used to determine net production of aquatic plants subject to substantial biomass loss before the seasonal maximum biomass is attained. They are useful for species in which groups of individuals, or subunits, initiated at the same time (cohorts), can be identified, that is, for plants where shoots emerge only during one time, or several discrete times, during an annual cycle.

The Allen curve method has been adapted for aquatic macrophytes. It provides an estimate of net production from tables of the numbers and weights of all individuals. It is particularly appropriate for populations for which shoot death and initiation occur throughout the growing season. The method can account for periods of negative production during the year.

Tag all members of a cohort (all leaves or shoots) shortly after initiation or emergence. This sampling is usually the maximum number of individuals present at any time in the cohort, because mortality will decrease the number thereafter.[21] Some new individuals (but still members of the same cohort) may appear by the second sampling visit.[3] Record the number of individuals and their mean weight (dry weight per leaf or shoot). Determine weight from size:weight regressions constructed from data for plants outside the study plot, but using members of the same cohort. Alternatively, harvest adjacent plots of cohort members to estimate the average weight per individual. Repeat for several replicate plots. Revisit plots regularly and record number and mean weight of individuals. Visit frequently enough to minimize potential for loss of young stems or leaves before they have been counted. Plot values (see Figure 10400:1) and determine total area beneath the curve by planimetery, digitally, or gravimetrically, to estimate net annual above-ground production. Repeat if more than one cohort emerges per year; net above-ground annual production then is the sum of the areas beneath several curves.[3,21] If negative production occurs during the year add this loss back to yield net production. Losses usually can be avoided by initiating studies after winter senescence.[21]

An adaptation of the cohort production method[22] recognizes the hierarchical structure of many aquatic macrophytes. This method may be used when differential turnover rates for plant subunits would confound the simple cohort method.

c. Estimates of below-ground production: The below-ground portions of aquatic macrophytes often comprise a substantial portion of the plant's biomass and net production, and may have important ecosystem implications because of metabolic activity and decay.[5] The amount of below-ground biomass varies dramatically between species, within a species, and seasonally for populations of the same species growing in different habitats.

1) Peak biomass—The maximum below-ground biomass is commonly taken as the net annual below-ground production. This may substantially over- or underestimate actual production, depending on biomass turnover. For many submersed nonevergreens, it provides a reasonable estimate of production, especially where shoot turnover approaches 1 and little below-ground biomass overwinters. For most floating-leaved and emergent macrophytes, it will overestimate biomass.

2) Seasonal biomass accumulation—A more appropriate, but more difficult, method involves repeated sampling and determination of live below-ground biomass at regular intervals during the year. For marsh plants, use sediment cores (25 cm deep, 6- to 10-cm diam) taken at regular intervals (2 to 4 weeks).[23,24] Sieve the cores to remove below-ground biomass and determine weight of live portion. Live material usually can be distinguished by its light color, texture, and turgor. Stains also may be used; chlorazol black stains dead material[25] and tetrazolium stains live material. Use the seasonal change in live biomass as an estimate of production. As for shoots, the loss of plant material before the maximum biomass is attained and any production after the maximum biomass is attained are not accounted for.

3) Root turnover measures—For many applications, the most reasonable way to determine below-ground production is by extrapolation from shoot production or turnover. For many plants, production and turnover of below-ground biomass is directly related to growth and turnover of above-ground plant parts. However, the seasonal translocation of carbohydrate reserves between the below-ground and above-ground portions by most aquatic macrophytes requires that these turnover-based estimates be used only for long-term, annual, production estimates, unless morphological observations indicate otherwise.

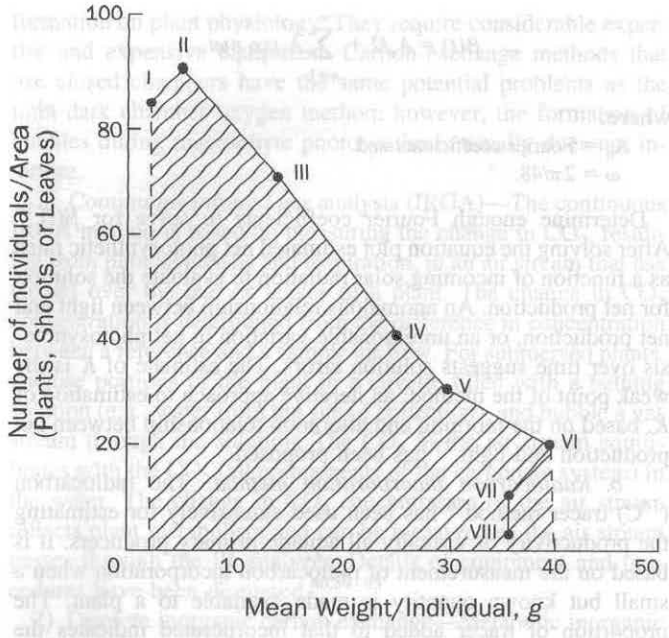

Figure 10400:1. Allen curve for a cohort of a population of aquatic macrophytes. Sample data are indicated by Roman numerals. Net annual above-ground production is proportional to the shaded area.

The establishment of a constant relationship between shoot and below-ground turnover[18,26] justifies such extrapolations.

3. Metabolic Methods

Metabolic methods estimate macrophyte productivity on the basis of short-term measures of inorganic carbon and oxygen exchange resulting from macrophyte photosynthesis and respiration. These short-term (usually < 3 h) measures must be integrated over time and space and converted to an estimate of organic matter production. Metabolic methods give short-term assessment of processes related to productivity that reflect a metabolic response to existing environmental conditions and they provide information concerning factors governing plant productivity.

The photosynthetic inorganic carbon budgets of aquatic macrophytes are more complex than those of terrestrial plants. When complex carbon or oxygen exchange patterns occur, a combination of metabolic methods may be used,[27,28] or a biomass method may be preferable.

a. Oxygen measurement: Determining changes in dissolved oxygen (DO) resulting from photosynthesis and respiration is the most common metabolic method for estimating macrophyte productivity. Changes in DO can be determined for plants in chambers (in the field or laboratory) or for the whole system when macrophyte metabolism dominates the oxygen dynamics. Measure DO concentrations with polarographic oxygen electrodes or by titration (Section 4500-O). For the most part, the methodology is similar to that described for phytoplankton and periphyton; however, several additional considerations warrant attention.

Measurements of net oxygen release to water or of changes in the utilization of inorganic carbon by photosynthesis of submersed macrophytes assume acquisition from, or release to, the surrounding water. Many rooted submersed plants do not release dissolved oxygen at rates proportional to photosynthesis.[29–32] Some oxygen diffuses internally from leaves to sites of high respiratory demand in rooting tissues. Additionally, some oxygen is released from leaves and is utilized by epiphytic bacteria and from roots and is utilized by rhizospheric bacteria.[33]

1) Light and dark bottle (chamber) method—This method is similar to those described for other primary producers, where portions of plants or the above-ground portions of several plants are enclosed in either clear or opaque containers. The change in oxygen per unit plant per unit time is an expression of plant photosynthesis (or respiration in the dark bottle) that is used to calculate productivity during the incubation period. Daily or longer-term production is estimated, using additional information on environmental conditions during the incubation period and during the period over which a production estimate is needed, and the relationship between the response of the plant segment incubated and the response of the entire plant (or population) to those environmental conditions.[34–38] See Sections 10200J.2 and 10300D.2 for procedural details. Mixing to simulate natural water movement is desirable.[39–46] Equipment modifications have been discussed,[47–53] as have specific analytical requirements.[34,54–57]

Determining productivity for periods longer than the test hours requires including respirations during the dark (24-h net production = daytime net production minus nighttime respiration or daytime gross production minus 24-h respiration) and extrapolating from the incubation period to these longer periods. Estimate net photosynthesis during the daylight period from productivity measurements in 3- to 4-h intervals from dawn to dusk daily, or by multiplying the production measured during a 3- to 4-h midday incubation by the fraction of total daily light received during the incubation period. For monthly or yearly periods there are several methods for extrapolating.[58,59] Most estimates of macrophyte production by these methods rely on a single midday incubation conducted at weekly-to-monthly intervals. There are three ways to estimate production for longer periods. First, measure productivity at 3- to 4-h intervals from dawn to dusk, on a clear day, several times during the growing season, to establish a relationship between midday photosynthesis and total daily photosynthesis. Estimate daily production on the basis of midday incubation at regular intervals (weekly to monthly), using the relationship between light during the incubation period to daily light and photosynthesis during the incubation period to daily photosynthesis as determined above. Estimate production on intervening days, when photosynthesis is not measured, on the basis of the available light on those days. Second, by using experimental evidence (P-I curves from plants incubated at different depths) or literature values, establish a relationship between light and productivity and a midday incubation (weekly to monthly) as a scaling factor. Carefully estimate production on the basis of available light for any day.[60,61] Third, use more involved modeling that accounts for growth characteristics and environmental conditions to provide estimates of macrophyte production.[62,63]

The epiphyton associated with a macrophyte may influence the determination of photosynthesis and respiration,[64–68] but the

respective contributions to photosynthesis cannot be resolved by the oxygen method.[66] Alternatively, use the ^{14}C method for estimating macrophyte photosynthesis.

Short-term measures of macrophyte production using the light-dark bottle method do not account for the loss of organic substances that may be as much as 10% of the recently fixed carbon.[69] Other losses of fixed carbon, as well as sloughing, grazing, and fragmentation, also are unaccounted for. Further, the light-dark bottle oxygen method cannot predict the allocation of photosynthate to below-ground growth or reproduction.[5,70] The inability to account for such factors is a shortcoming of all metabolic methods, but oxygen methods provide reasonable estimates of short-term biomass net accumulation for some plants.[38,57]

2) Open-system oxygen method—In flowing-water systems where macrophytes dominate primary production, analysis of diurnal oxygen curves can be used to estimate macrophyte production.[39,71] The change in the oxygen content of a parcel of water is the result of both community metabolism and oxygen diffusion across the water surface (see Section 10300D.4). Entry of groundwater or surface runoff is assumed negligible during measurements. Criteria[72] for determining the suitability of a system for open-system monitoring include extent of uniform area, high biological activity, water depth and residence time (influencing observable changes in water chemistry), turbulence (influencing spatial variation for monitoring and gaseous exchange with the atmosphere), and uniformity of the channel (allowing the calculation of production per unit area and providing uniform residence time). The accuracy and sensitivity of several methods have been compared.[73]

The single-station method (Section 10300D.4) is used most commonly. In addition to the radiotracer method presented, the reaeration coefficient can be determined by direct methods or calculations based on physical parameters.[73–75] To use a single station assume stream homogeneity above the region of measurement.

To use an automated two-station system, calculate a continuous function (Fourier series) to determine an exact solution to the oxygen mass balance equation, rather than the approximate finite difference solution.[76] This system models net productivity so that the oxygen concentrations at the downstream station can be predicted accurately from upstream values. Additionally, it provides detailed information, such as hourly variations in net production and seasonal changes in community photosynthetic characteristics.[77]

For analytical details, see Section 10300D.4*a* and *b*.

Determine production from the change in DO between the two stations:

$$dc/dr = K(c_s - c) + \beta(t)$$

where:

dc/dr = change in DO concentration between stations,
K = reaeration coefficient,
c_s = saturation DO concentration, mg/L,
c = DO concentration, mg/L,
$\beta(t)$ = net productivity, and
t = flow time between stations.

For short time intervals, write t as a Fourier cosine series:

$$\beta(t) = A_0/2 + \sum_{n=1}^{\infty} A_n \cos n\omega t$$

where:

A_n = Fourier coefficients and
$\omega = 2\pi/48$.

Determine enough Fourier coefficients to solve for $\beta(t)$.[76] After solving the equation plot estimated net photosynthetic rates as a function of incoming solar radiation to evaluate the solution for net production. An anomalous relationship between light and net production, or an unreasonable variation in net photosynthesis over time suggests solution errors. The estimate of K is the weak point of the method; an iterative approach to estimation of K, based on the morning and afternoon relationship between net production and light,[73] has been proposed.

b. Radiocarbon incorporation method: The radiocarbon (^{14}C) tracer method[78] has been used extensively for estimating the productivity of virtually all aquatic primary producers. It is based on the measurement of radiocarbon incorporation when a small but known quantity is made available to a plant. The proportion of tracer added to that incorporated indicates the fraction of stable carbon that is incorporated. This method is more sensitive than oxygen methods and thus can be used where photosynthetic rates are very low or where carbon consumption is excessive.

Although this method directly measures the incorporation of external inorganic carbon, the relationship of this incorporation to net or gross photosynthesis is not without controversy.[79] The general consensus is that the ^{14}C method for macrophytes provides a rate of carbon incorporation between net and gross photosynthesis, but closer to net photosynthesis.[35,38,47,52,57,80] The primary drawback of the ^{14}C method is that it provides no measure of dark-period respiration. If the photoassimilation of ^{14}C is assumed to estimate net photosynthesis for macrophytes (gross photosynthesis minus respiration), then respiration during the dark period must be measured separately to account for carbon lost at night in the calculation of 24-h net production (24-h net primary production = gross photosynthesis − 24-h respiration, or here, 24-h NPP = daytime ^{14}C uptake − nighttime respiration).[57] As noted for the oxygen method, respiration usually is assumed to be identical during the day and night for macrophytes.[38,57]

Portions of CO_2 from photorespiration and mitochondrial respiration are stored internally in gas lacunal spaces and are intensively recycled.[81] Some CO_2 from rooting tissues and rhizosphere bacterial metabolism can diffuse internally in these gas channels to photosynthetic tissues.[82] Thus, rates of incorporation of $^{14}CO_2$ from the water may not be proportional (underestimate) to true rates of carbon fixation.

For procedural details see above for the light and dark bottle oxygen method and Section 10300D.3. Analysis of organic fractions into which carbon 14 may be incorporated has been described[30,64,83] as have procedures for carbon-14.[84–88]

c. Inorganic carbon exchange methods: Changes in the inorganic carbon concentration in air or water surrounding aquatic macrophytes as a result of photosynthesis and respiration can be determined by several methods. These methods provide a highly sensitive, direct measure of photosynthetic carbon uptake, thus requiring few assumptions, and can provide additional in-

formation on plant physiology. They require considerable expertise and expensive equipment. Carbon-exchange methods that use closed chambers have the same potential problems as the light-dark chamber oxygen method; however, the formation of bubbles during macrophyte photosynthesis usually does not interfere.

1) Continuous infrared gas analysis (IRGA)—The continuous IRGA method is based on measuring the change in CO_2, resulting from photosynthesis and respiration, in an air stream that has passed over the leaf or leaves of a plant. The change in CO_2 concentration is determined from the difference in concentration between a reference and a sample air flow. For submersed plants, enclose portions of the plant in a cuvette filled with a bathing solution (e.g., water from the site of collection), and bubble a gas stream through the solution. The CO_2 in the air stream equilibrates with the CO_2 (all components of the carbonate system) in the water. The change in CO_2 concentration of the air stream reflects plant metabolism and can be measured as the air stream passes through the IR analyzer. Details of equipment and procedures have been discussed.[80,89–95]

2) Discrete inorganic carbon exchange—Metabolic inorganic carbon exchange with the air or water also can be measured by determining the change in inorganic carbon in sealed containers after a discrete incubation period, using IRGA,[68,92,96–98] gas chromatography,[99] or a total organic carbon analyzer operating in the inorganic carbon mode (see Section 5310). The change in CO_2 in the air, for emergent and floating plants, or the change in dissolved inorganic carbon (DIC) in the water, for submersed plants, is determined for subsamples of the incubation medium. This method is analogous to the light and dark bottle oxygen method, except that changes in the CO_2 or DIC concentrations are determined, rather than the change in DO concentration.[38] This method requires more expensive equipment than the oxygen method does; however, in low-DIC waters it is much easier to measure the change in DIC resulting from plant metabolism than the corresponding change in DO (due to the relative abundance of DO and DOC and the high sensitivity of the DIC method). See references cited above for procedural details.

The change in CO_2 in the light is net photosynthesis. A darkened chamber can be used to determine respiration. Extrapolation to estimate production and additional methodological considerations are covered in the discussion for the light and dark bottle oxygen method. As for other carbon exchange methods, the exchange of CO_2 with the atmosphere, as well as marl formation, can result in errors in the determination of carbon exchange.

3) Potentiometric measurement of inorganic carbon flux—The physical-chemical relationships of the carbonate buffer systems in natural waters dictate a definable relationship between DIC (or alkalinity) and pH. On the basis of such relationships, changes in the pH of the water resulting from plant-mediated changes in DIC concentrations (due to photosynthesis and respiration) can be used to estimate the inorganic carbon exchange for submersed macrophytes. The method presented here is based on the estimation of the DIC or total carbon (C_T) determined by a Gran titration.[100] This approach requires only a good pH meter capable of readings to 0.1 pH unit, an electrode, and careful laboratory procedure. However, it is not suitable for waters of very high or low pH, and has limitations similar to those of the oxygen method. While it has been applied primarily to laboratory studies,[101–103] it is adaptable for field use.

To measure inorganic carbon flux,[104] incubate and collect sample as described for the light and dark bottle method. Use 125-mL gastight bottle or polypropylene syringe for sampling. Determine temperature of a 2.0- to 100-mL water sample and add sample to a titration vessel containing a magnetic stir bar, or add stir bar to collection bottle for direct titration. Assure that titration vessel is partially sealed around the outlet to provide protection from the atmosphere. Record initial pH. Stir sample and use a 0.5- to 5-mL-capacity piston syringe-buret to titrate stepwise with HCl or other appropriate acid of known normality. Use acid of such normality that the total solution volume is not changed by more than 10% by the end of titration. Record pH and volume of titrant added at three points between pH 7.6 and 6.7 (or lower if necessary) and another three points between pH 4.4 and 3.7.

Calculate net photosynthesis by the change in carbon in the light bottle or container, and respiration by the change in carbon in the dark container as follows: Calculate F_2 from pH readings in the lower pH range as

$$F_2 = [\text{antilog}\,(a - \text{pH})] \times [(V_s + v)/V_s]$$

where:

F_2 = antilogarithmic Gran functions for pH change with titrant additions,
a = any convenient number above the pH range, e.g., 5,
V_s = sample volume, mL, and
v = titrant volume, mL.

Plot F_2 against v and fit with a straight line, locating the intersection of the line on the v axis (v_2). Calculate F_1 from pH readings in the higher pH range as

$$F_1 - [\text{antilog}\,(b - \text{pH})] \times (V_s + v) \times (v_2 - v)$$

where:

F_1 = antilogarithmic Gran functions for pH change with titrant additions and
b = any convenient number above the pH range, e.g., 8.

Plot F_1 against v, locating the intersection of the best-fitting straight line with the v axis (v_1). Then,

$$V_1 = v_1 \times \frac{1000}{V_S} \times n$$

$$C_T = V_2 - V_1 = \frac{1000}{V_S} \times n \times (v_2 - v_1)$$

$$V_2 = v_2 \times \frac{1000}{V_S} \times n$$

where:

V_1 = acidity, meq/L,
V_2 = total alkalinity, meq/L,
C_T = total DIC, mmol/L, and
n = normality of the acid titrant.

Use of antilog paper simplifies this procedure. The method can be used for water samples with an initial pH of less than 7 if an equal amount of NaOH is added to all samples.[101,104]

A formulation of a relationship of pH to C_T for a water of constant alkalinity and changing pH (e.g., as a result of photosynthesis or respiration) eliminates the need for titrations to determine the change in C_T in an incubation vessel resulting from macrophyte photosynthesis, as only the initial and final pH would be required.[104]

4. Data Presentation

Express seasonal and annual rates of macrophyte production in units of carbon or dry weight per unit area of colonization or littoral region. Occasionally, energy units (kcal) may be used but are not recommended. Net annual above-ground production, expressed as grams C per square meter per year or grams dry weight (or ash-free dry weight) per square meter per year is used often but variously underestimates total net annual production. Express the results of short-term (minutes, hours, days) measurements of production or photosynthesis as carbon fixed per unit shoot dry weight or per unit chlorophyll *a*. For emergent plants, report as carbon uptake per unit leaf or shoot surface area. The value of data collected depends on clear statements of how the production values are calculated and expressed (e.g., dried at 105°C, dry weight or ash- free dry weight, per unit area colonized or per area lake bottom, etc.) and on the provision of ancillary information to allow the data to be re-expressed and compared with values from other studies (chlorophyll content, ash content, above:below ground weight, etc.).

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10500 BENTHIC MACROINVERTEBRATES*

10500 A. Introduction

1. Definition

Benthic macroinvertebrates are animals inhabiting the sediment, or living on or in other available bottom substrates of freshwater, estuarine, and marine ecosystems. During all or part of their life cycles, these organisms may construct attached cases, tubes, or nets that they live on or in; roam freely over rocks, organic debris, and other substrates; or burrow freely in substrates. Although they vary in size from small forms, difficult to see without magnification, to other individuals large enough to see without difficulty, macroinvertebrates are considered historically by definition to be visible to the unaided eye and retained on a U.S. Standard No. 30 sieve (0.595-mm or 0.600-mm openings).[1]

The standard sieve for collecting freshwater, estuarine, and marine benthic macroinvertebrates is the U.S. Standard No. 30 sieve; however, some estuarine and marine programs use the U.S. Standard No. 50 sieve (0.300-mm openings) or the U.S. Standard No. 35 sieve (0.500-mm openings). For all aquatic assessment programs, use of the No. 30 sieve to collect benthic fauna of freshwater, estuarine, and marine habitats or from any water transport system is recommended. To accommodate some old historical databases and if the data-quality objectives of the study permit, a U.S. Standard No. 28 sieve (1.0-mm openings) might be utilized. To obtain a more representative sample of the benthos that would include smaller forms or early life-stages, and other taxa of macroinvertebrates, a U.S. Standard No. 60 sieve (0.250-mm openings) may be used.

The standardization of bioassessment for species composition, taxa richness, diversity, evenness, trophic levels, and major taxonomic spatial and temporal patterns may be enhanced significantly by the conventional use of a U.S. Standard No. 30 sieve.

The major macroinvertebrates found in freshwater are flatworms, annelids, mollusks, crustaceans, and insects. The major macroinvertebrate groups included in estuarine and marine waters are bryozoans, sponges, annelids, mollusks, roundworms, cnidarians (coelenterates), crustaceans, insects, and echinoderms.

2. Response to Environment

The species composition and population or species density (numbers of individuals per unit area) of macroinvertebrate communities in streams, lakes, estuaries, and marine waters can be uniform from year to year in unperturbed environments. However, life-cycle dynamics produce variations in species composition and abundance either temporally or spatially.

Most aquatic habitats, particularly free-flowing streams and waters with acceptable water quality and substrate conditions, support diverse macroinvertebrate communities in which there is a reasonably balanced distribution of species among the total number of individuals present. Such communities respond to changing habitats and water quality by alterations in community structure (invertebrate abundance and composition). However, many habitats, especially disturbed ones, may be dominated by a few species.

Macroinvertebrate community responses to environmental changes are useful in assessing the impact of municipal, industrial, oil, and agricultural wastes, and impacts from other land uses on surface waters. Four types of environmental changes for which patterns of macroinvertebrate community structure change have been documented are: increased inorganic nutrients, increased organic loading, substrate alteration, and toxic chemical pollution. Inorganic nutrients and severe organic pollution usually result in a reduction in the variety of macroinvertebrates to only the most tolerant ones and a corresponding increase in density of those tolerating the polluted conditions, usually associated with low dissolved oxygen concentration. In some cases severe organic pollution, siltation, or toxic chemical pollution may reduce or even eliminate the entire macroinvertebrate community from an affected area. Not all cases conform to those described because conditions may be mediated by other environmental (biological, chemical, and physical) conditions.

Assessing the impact of a pollution source generally involves comparing macroinvertebrate communities and their physical habitats at sites influenced by pollution with those collected from adjacent unaffected sites. This can include a gradient away from point sources of contamination. The procedure includes sampling and analyzing types of communities from different sites and subsequently determining whether the presumed pollution-affected community differs from the presumed nonaffected community. The basic information required for most community structure analyses is a count of individuals per species. From the

* Approved by Standard Methods Committee, 2001.
Joint Task Group: Donald J. Klemm (chair), David C. Beckett, Peter M. Chapman, Philip A. Lewis, Morris H. Roberts, Jr., Don W. Schloesser, William T. Thoeny.

count data, communities can be characterized and compared according to community structure, density, diversity, community metrics, pollution indicators, or other analyses,[1] including various statistical methods (see 10500D). Biomass and productivity estimates also can be determined with the organisms collected.[1–4] Equally desirable is a characterization of the dissolved oxygen concentration, substrate, water depth, type of sediment, grain size of the sediment, total organic carbon (TOC), and other site- and situation-specific characteristics.

While the following macroinvertebrate methods traditionally are used for sampling and quantifying benthic invertebrate communities, other methods also are being evaluated in an effort to develop and implement narrative biological criteria for surface waters.[5] Not discussed here are EPA-developed rapid bioassessment techniques[6,7] and Environmental Monitoring and Assessment Program (EMAP) protocols for field operations and methods and laboratory methods for sampling macroinvertebrates and assessing the ecological conditions of wadeable streams.[8–10]

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10500 B. Sample Collection

1. General Considerations

Before conducting a benthic survey, determine specific data quality objectives (DQOs) and define clearly the information sought. DQOs are qualitative and quantitative statements developed to specify the quality of data needed to support specific decisions and conclusions about the information sought. Discussion with water chemists, hydrologists, limnologists, and individuals from other disciplines will be helpful. Ultimate selection of a methodology will depend on whether the habitat to be studied is a stream, lake, reservoir, or marine area. For example, to determine whether the macroinvertebrate community downstream from a discharge is damaged, only a few sampling stations upstream and downstream from the discharge are needed. However, if the objective is to delimit the extent of damage from a discharge or series of discharges, it is necessary to have reference stations upstream from all discharges, to bracket each discharge with stations, and to establish stations downstream. In marine waters, it may be necessary to sample a nearby estuary or, for open ocean waters, to sample some distance from the discharge point.

Characterize the physicochemical properties of faunal sampling station substrate and overlying water. Measure such properties as sediment size class distribution (sand, silt, and clay); organic content and toxic pollutant concentrations; temperature, salinity, hardness, alkalinity, dissolved oxygen, total organic carbon (TOC), ammonia, sulfides, and nutrient (total and dissolved) concentrations; biochemical oxygen demand; water depth; and velocity of flowing streams.

After gaining a thorough understanding of the factors involved with a particular body of water, select specific areas to be sampled. There is no set number of sampling stations that will be appropriate to monitor all possible waste discharges. No water quality survey is routine, nor can one be conducted totally on a "cookbook" basis. However, if some basic rules such as the following are adhered to, a sound survey can be designed:

1. Always establish a reference station(s) upstream or at a point remote from all wastewater discharges of concern. Because most surveys are made to determine the damage that pollution causes to aquatic life, this will be the basis for comparison of the biota in polluted and unpolluted areas. Preferably have at least *two reference stations, one well away from, or upstream from,*

the discharge and the other directly above, or in the immediate vicinity of, the effluent discharge, but not subject to its influence. Whenever it is feasible, use reference stations having physicochemical characteristics similar to those of the substrate and overlying water of the receiving area.

2. Locate a station immediately downstream or in the affected area in the immediate vicinity of each discharge.

3. If the discharge does not mix completely on entering the body of water, but channels along one side or disperses in a specific direction, locate stations in the left-bank (looking upstream), midchannel, and right-bank sections of the stream, and in concentric arcs in lakes and oceanic waters, or any other configuration that will meet study objectives.

4. Establish stations at various distances downstream from the last discharge of concern to determine the linear extent of damage. In the marine environment, an estuary nearby may be sampled or in open ocean waters samples may be taken in a nearby area comparable with respect to currents, depth, sediment characteristics, and salinity.

5. To permit comparison of macroinvertebrate communities, be sure that all sampling stations are ecologically similar. For example, select stations that are similar with respect to bottom substrate (e.g., sand, gravel, rock, mud, organic content), depth, presence of riffles and pools, stream width, gradient, flow velocity, bank or shore cover, salinity, or hardness, TOC, nutrient and dissolved oxygen concentrations, and wave exposure.

6. Collect samples for physical, toxicological (if applicable), and chemical analyses as close as possible to biological sampling stations to assure correlation of findings; take such samples at the same time and from the same grab when possible. Collect substrate samples for physicochemical analyses from the upper few centimeters where most organisms live.

7. Locate sampling stations for macroinvertebrates in the best physical habitat areas that are not influenced by atypical conditions (e.g., bridges, dams, etc.).

8. Discharges in areas near a coast may be subject to variation in degree of salt water intrusion (salt water wedge). In such areas, macroinvertebrate populations may change drastically; document and/or allow for this effect.

9. When sampling in small, wadeable, first- to third-order streams, initiate sampling at the most downstream station and then proceed upstream to minimize disruptions induced by the sampling itself. This is not necessary for nonwadeable streams and rivers.

For a long-term biological monitoring program, consider collecting macroinvertebrates at each station at least once during each of the annual seasons, though this may not always be necessary and would depend on the study design.[1] More frequent sampling may be necessary if the characteristics of the effluents change or if spills occur. Make allowance for collections at night where "drift" or night feeding organisms are of special concern. In general, the most critical period for macroinvertebrates in streams is during periods of high temperature and low flow, whereas in estuarine and marine environments it is the period of maximum stratification and poor vertical mixing. If available time and funds limit sampling frequency, make at least one survey during the critical time.

2. Sampling Design

In biology, the term *population* refers to a group of individuals that are all members of the same species or taxonomic group. In statistics, *population* refers to the entire set of values for the characteristic of interest in a whole sampling universe. For example, researchers interested in determining the mean density of worms in the bottom of a lake might take ten grabs from the lake sediments. The number of worms in each grab would be an *observation*, the density of worms would be the *characteristic of interest*, and the contents within each grab would be the *experimental unit* or *sampling unit*. The entire bottom of the lake would be the *sampling universe* and enough grabs to equal the area of the entire lake bottom would be the *population* (of units).

Similarly, the term *sample* has two, often contradictory, usages. In typical studies, observations usually are not made for all the possible sampling units. Instead, observations are made that make up only a small fraction of the total possible number of observations that could be made. Statistically, this set of observations is referred to as a sample. In the example given above the ten grabs would be a sample. However, in everyday language and as used in this book and most scientific publications, the term "sample" has been used to signify a portion of the real world that has been selected for measurement, such as a water sample, plankton haul, or bottom grab. Therefore, each of the grabs in the example above would be considered a sample, i.e., "ten samples were taken."

Collecting a representative sample is difficult because of variation in successive samples. Without knowledge of sampling variability, the degree to which the data truly represent the population cannot be known. Make replicate observations of a population if definitive statistical inferences about the population are to be made.[2–11]

Standardize sampling design to consider the following requirements:

1. Approximate the set of all samples that can be selected (i.e., separate the sampling universe into all possible samples). For example, if the location (site) containing the population has an area of 1000 m^2 and the sampling device samples an area of 1 m^2, there are 1000 samples that could be collected in the sampling universe.

2. Assign each sample an equal probability of being selected. Using the situation above, divide the area to be sampled into 1000 discrete units.

3. Use a table of random numbers to select sites for sampling, i.e., sample randomly, not haphazardly.

4. The sampling design outlined above is known as simple random sampling. It is often advantageous to determine the number of samples necessary for a certain level of precision while using this type of sampling design. Use the following formula to estimate this number:

$$N = \left(\frac{t \times s}{D \times \bar{x}} \right)^2$$

where:

N = number of samples,
t = tabulated t value at 0.05 level with the degrees of freedom of preliminary survey (generally $t \approx 2.0$ at larger sampling sizes),
s = sampling standard deviation of samples, known from a

preliminary survey,
D = required level of precision expressed as a decimal (0.30 to 0.35 usually yields a statistically reliable estimate), and
$\bar{x}$ = sample mean density of preliminary survey.

Specific information (the mean and standard deviation) about the population to be sampled is necessary to estimate the number of samples. Because this information is unknown because sampling has yet to take place, estimate the population's mean and standard deviation by one of three ways: conduct a pilot study; use results from an earlier or a similar study; or make educated estimates of the population mean and standard deviation.[3,12] As an example, in determining the mean chironomid density in relatively homogeneous lake sediments during the summer, and having information that six grabs taken the previous summer produced a mean density of 4230 chironomids/m^2 and a standard deviation of 1628 chironomids/m^2, it may be satisfactory if the final estimate of mean chironomid density is correct within ± 30%, with a 5% probability of error ($\alpha = 0.05$). Using the formula given above,

$$N = \left(\frac{2.5706 \times 1628}{0.30 \times 4230}\right)^2$$

($t = 2.5706$ at a 5% probability of error and 5 degrees of freedom)

$$N = 10.88 \approx 11$$

Thus it is estimated that 11 grabs will be necessary.

5. Simple random sampling is useful in sampling relatively homogeneous areas. However, most taxa are not distributed uniformly over water bottoms. Different habitats (sand, mud, gravel, or organic material) support different densities and species of organisms. In such circumstances, use a stratified random design. In this sampling design a heterogeneous universe (different bottom substrates, current velocities, depths, temperatures, etc.) is divided into more homogeneous strata. Once the strata are defined, use random sampling, as above, within each stratum. Stratified random sampling has two important advantages: it is often valuable to have data on the various subsets of a population (e.g., density of benthic invertebrates in each of the sediment types), and stratified sampling often reduces variability because it deals with more homogeneous subpopulations, allowing for more accurate (closer to the actual value) and precise (less variation among the values) population estimates. Prior information is necessary to divide the population into the various strata. This is usually accomplished through pre-study reconnaissance (a pilot study). Systematic sampling, a third type of sampling design (in addition to simple random and stratified random), often is used in such pilot studies. In a systematic-transect design, conduct sampling at equal intervals along a number of transects within a habitat. This design can be used to identify and locate the existent strata.[3,12]

6. In descriptive studies investigators should take at least three replicate sampling units per station.[3,13] If statistical testing is planned, more replicates probably are necessary.

7. Standardize data acquisition and recording when practical. Use metric units.

3. Sampling Devices, Quantitative

Quantitative and qualitative samplers have been designed to collect organisms from the bottom of different water bodies. The most common quantitative sampling devices are the Ponar®*, Petersen, and Ekman grabs and the Surber or square-foot stream bottom sampler, all described below.

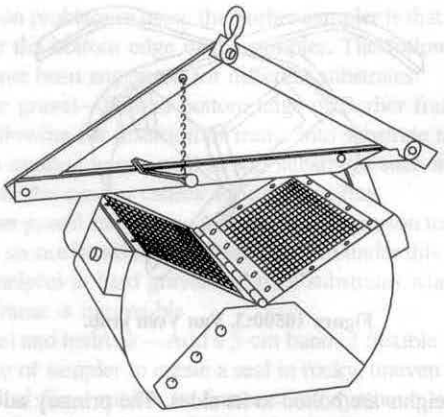

Figure 10500:1. Ponar®* grab.

a. Grab samplers:

Measure each grab-type sampler for actual surface area sampled before it is first used.

1) *The Ponar®* grab* (Figure 10500:1) is used increasingly in medium to deep rivers, lakes, reservoirs, and estuaries.[14] It is similar to the Petersen grab in size, weight, lever system, and sample compartment, but has side plates and a screen on top of the sample compartment to prevent sample loss during closure. With one set of weights, the standard 23- by 23-cm sampler weighs 20 kg. A 15- by 15-cm petite Ponar®* may be used. The large surface disturbance associated with a Ponar®* grab can be reduced by installing hinged, rather than fixed, screen tops, thereby reducing the pressure wave associated with the sampler's descent. This sampler is best used for mud, sand, gravel, or small rocks with mud, but it can be used in all substrates except bedrock.

2) *The Petersen grab* (Figure 10500:2) is used for sampling hard bottoms such as sand, gravel, marl, and clay in swift currents and deep water.[3] It is an iron, clam-type grab manufactured in various sizes that will sample an area of from 0.06 to 0.09 m^2.[2] It weighs approximately 13.7 kg, but may weigh as much as 31.8 kg when

* Registered trademark of Morris & Lee, Inc. d/b/a Wildlife Supply Co., Buffalo, NY.

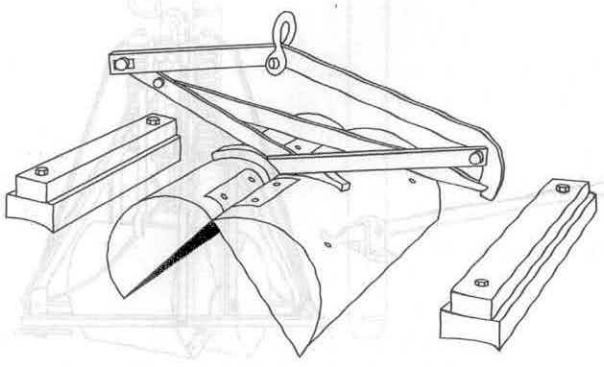

Figure 10500:2. Petersen grab.

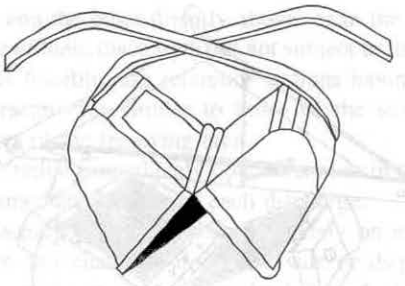

Figure 10500:3. Van Veen grab.

auxiliary weights are bolted to its sides. The primary advantage of the extra weights is to make the grab stable in swift currents and to give additional cutting force in fibrous or firm bottom materials. Modify the sampler by adding end plates, by cutting large strips out at the top of each side, and by adding a hinged 30-mesh screen as in the Ponar®* grab.[15]

To use the Petersen grab, set the hinged jaws and lower to the bottom slowly to avoid disturbing lighter bottom materials. Ease rope tension to release the catch. As the grab is raised the lever system closes the jaws.

3) *The Van Veen grab* (Figure 10500:3) is used to sample in open marine waters and in large lake environments. The long arms of the sampler tend to act as stabilizers without disturbing the water at the water-substrate interface. It is basically an improved version of the Petersen grab for mud, gravel, pebble, and sand substrates. The sampler is heavy; lower it from a boat or ship platform with mechanical or hydraulic lifts.

4) *The Smith-McIntyre grab* (Figure 10500:4) has the heavy steel construction of the Petersen, but its jaws are closed by strong coil springs.[16] Chief advantages are its stability and easier control in rough water. Its bulk and heavy weight require operation from a large boat equipped with a winch. The 45.4-kg grab can sample an area of 0.2 m^2,[17–19] but smaller models (0.1 m^2 or 0.05 to 0.06 m^2) are available.

5) *The Shipek®* grab* (Figure 10500:5) is designed to take a sample 0.04 m^2 in surface area and approximately 10 cm deep at the center in virtually any type of substrate.[3] The sample compartment

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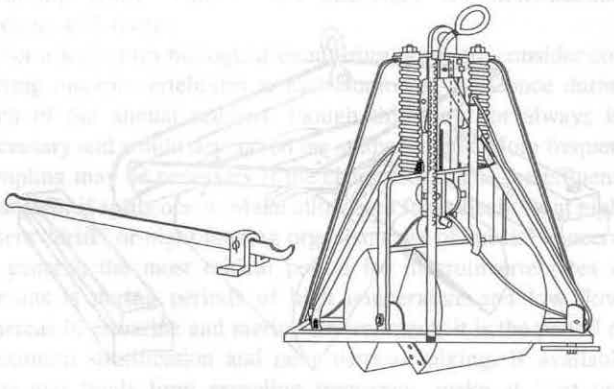

Figure 10500:4. Smith-McIntyre grab.

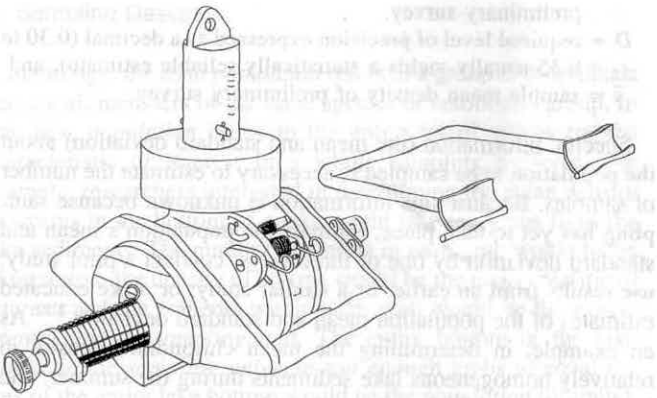

Figure 10500:5. Shipek®* grab.

is composed of two concentric half cylinders. When the grab touches bottom, inertia from a self-contained weight releases a catch and helical springs rotate the inner half cylinder by 180°. The sample bucket may be disengaged from the upper semi-cylinder by releasing two retaining latches. This grab is for special use in marine waters and large inland bodies of water, for instance, in compact substrates.

6) *The Ekman grab* (Figure 10500:6) is useful only for sampling mud, silt, muck, and sludge in water with little current.[3] It is difficult to use when rocky or sandy bottoms or moderate macrophyte growth are present because small pebbles or grit or macrophyte stems prevent proper jaw closure. The grab weighs approximately 3.2 kg. The box-like part holding the sample has spring-operated

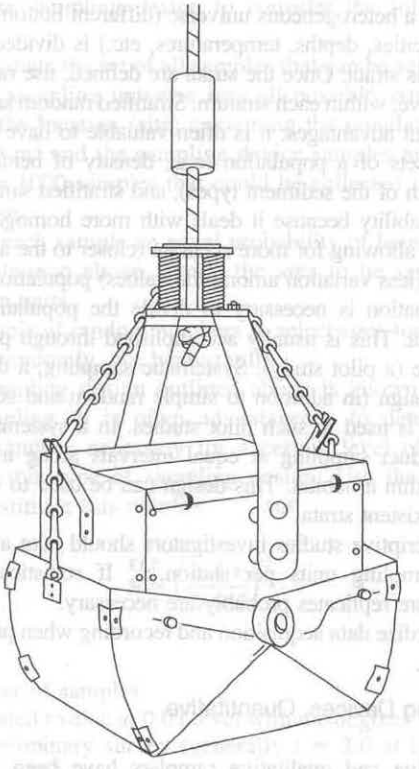

Figure 10500:6. Ekman grab.

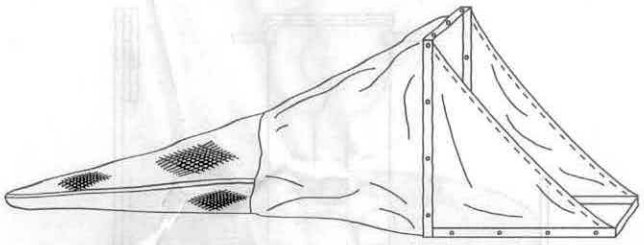

Figure 10500:7. Surber or square-foot sampler.

jaws on the bottom that must be cocked manually (exercise caution in cocking and handling the grab because of possible injuries if jaws are tripped accidentally). At the top of the grab are two hinged overlapping lids that are held open partially during descent by water passing through the sample compartment. These lids are held shut by water pressure when the sampler is being retrieved. The grab is made in three sizes: 15 × 15 cm, 23 × 23 cm, and 30 × 30 cm, but the smallest size usually is adequate. A taller model of this sampler, either 23 cm or 30.5 cm tall, is available. To prevent sample overflow and loss, place a Standard U.S. No. 30 sieve insert in the top for deep sediments.

b. Riffle/run samplers:

1) *Surber-type samplers* (Figure 10500:7)[20] consist of two brass frames, each 30.5 cm (1 ft) square, hinged together along one edge. When in use, the two frames are locked at right angles, one frame marking off the area of substrate to be sampled, and the other supporting a net to collect organisms washed into it from the sample area.

The net usually is 69 cm long with the first few centimeters and the wings constructed of heavier material (canvas, taffeta) to increase durability. Standard 30 mesh size is 595 to 600 μm. While a smaller mesh size might increase the number of smaller invertebrates and young instars collected, it also will clog more easily and exert more resistance to the current than a larger mesh. This could result in a loss of organisms due to backwashing from the sample net. This sampler is specific for macrobenthos; many microcomponents of the benthos are not collected.

Use this sampler in shallow (30 cm or less), flowing water. When it is used in deeper water some organisms may be carried over the top of the sampler. Position sampler securely on the stream bottom parallel to water flow with the net portion downstream. Take care not to disturb the substrate upstream from sampler. Leave no gaps under the edges of the frame that would allow water to wash under the net. Fill gaps that may occur along the back edge of the sampler by carefully shifting rocks and gravel along the outside edge. When the sampler is in place (it may be necessary to hold it in place with one hand in a strong current), carefully turn over and lightly hand-rub all rocks and large stones inside the frame to dislodge organisms clinging to them. Examine each stone for organisms, larval or pupal cases, etc., that may be clinging to it before discarding. Scrape attached algae, insect cases, etc., from the stones into the sampler net. Stir remaining gravel and sand with the hands or a stick to a depth of 5 to 10 cm, depending on the substrate, to dislodge bottom-dwelling organisms. It may be necessary to hand-pick some mussels and snails that are not carried into the net by the current.

Remove sample by inverting net into sample container. Carefully examine net for small organisms clinging to it. Remove these, preferably with forceps to avoid damage, and include in sample. Rinse sampler net after each use.

A common problem in using the Surber sampler is that organisms wash under the bottom edge of the sampler. The following modifications have been suggested for different substrates:

For loose gravel—Extend bottom edge of Surber frame to 5 or more cm allowing for insertion of frame into substrate to a greater depth. This method works well in soft substrates such as sand and gravel where the current causes substrate shifting.

For coarse gravel and rock—Add serrated extension to back edge of frame to secure it and reduce washing from under this edge. This method is helpful in hard gravel and rock substrates where sinking the entire frame is impossible.

For gravel and bedrock—Add a 5-cm band of flexible material to bottom edge of sampler to create a seal in rocky, uneven substrates. Make band of foam rubber or fine-textured synthetic sponge. Remove organisms that stick to foam and include in sample.

2) *Hess-type samplers* are cylindrical with enclosed sides and an open top. They function similarly to the Surber-type samplers.[3]

c. Core or cylindrical samplers: Use core or cylindrical samplers to sample sediments in depth. Efficient use as surface samplers requires dense animal populations. Core samplers vary from hand-pushed tubes to explosive-driven and automatic-surfacing models.[3,21]

1) *The Phleger corer* (Figure 10500:8) is widely used. It operates on the gravity principle.[3] Styles and weights vary among manufacturers; some use interchangeable weights that allow variations between 7.7 and 35.0 kg, while others use fixed weights weighing 41.0 kg or more. Length of core taken will vary with substrate texture.

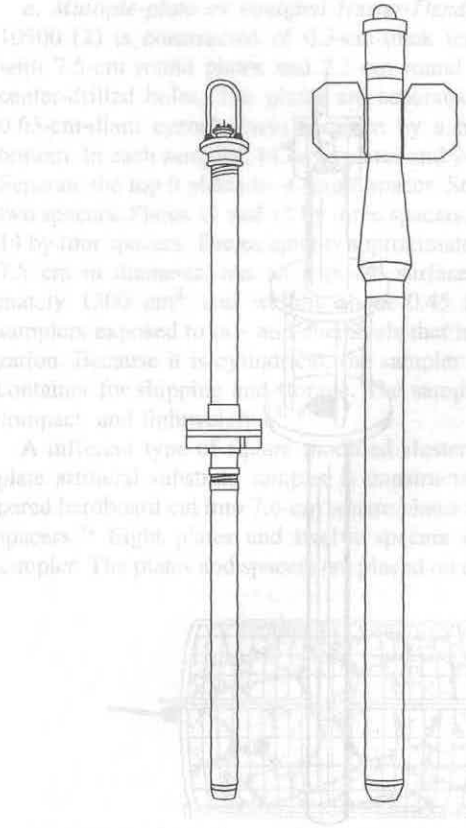

Figure 10500:8. Phleger core sampler.

2) *The KB®* core sampler* (Figure 10500:9) or a modification known as the Kajak-Brinkhurst corer, may be useful in obtaining estimates of the standing stock of benthic macroinvertebrates inhabiting soft sediments.[22]

3) *Box core samplers*[23–26] can sample a variety of sediment types. They are available in several sizes and are used in marine waters and in the Great Lakes[3,27] to collect benthic macrofauna. These devices may be deployed from ships or other platforms or they may be used by divers. Preferably use a box coring device with a rectangular corer, having a cutting arm that can seal the sample before retraction from the bottom. To sample a sufficient number of individuals and taxa, and to integrate the patchy distribution of the benthic fauna, use a sampler with a surface area of no less than 100 cm^2 and a sediment depth of at least 20 cm. A box corer capable of sampling deeper sediment may be needed to collect deep burrowing infauna. In sandy-type sediments, it may be necessary to substitute a grab sampler for the box corer to achieve adequate sediment penetration and collection. Visually inspect each sample to ensure that an undisturbed and adequate amount of sample is collected.

4) *The Wilding or stovepipe sampler* (Figure 10500:10)[28] is made in various sizes and with many modifications.[3] It is especially useful for quantitatively sampling a bottom with dense, vascular plant growth. It may be used to sample vegetation, mud-water interface sediment, or most shallow stream substrates. However, large volumes of vegetation, when sampled in this way, may require a great deal of time for laboratory processing.

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Figure 10500:9. KB®* corer.

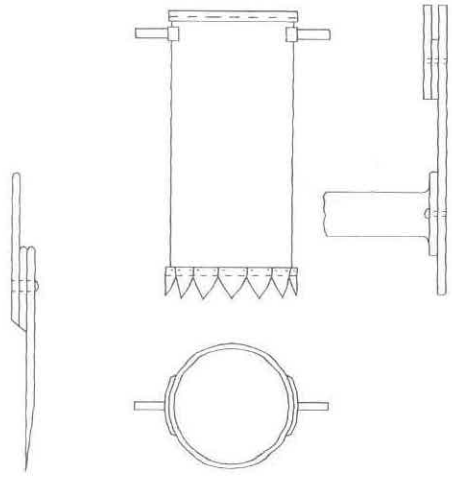

Figure 10500:10. Wilding or stovepipe sampler.

d. Drift samplers: Drift samplers, usually in the form of nets (Figure 10500:11) , are anchored in flowing water for capture of macroinvertebrates that have migrated or have been dislodged from the bottom substrates into the current. Drift organisms are important to the stream ecosystem because they are prey for stream fish and should be considered in the study of fish populations. Drift organisms respond to pollutional stresses, including spills, by increased drift from an affected area; therefore, drift is important in water-quality investigations, especially of spills of toxic materials. Drift also is a factor in recolonizing denuded areas and it contributes to recovery of disturbed streams.

Use nets having a 929-cm^2 upstream opening and mesh equivalent to U.S. Standard No. 30 screen (595-μm pore size). After placing the net in the water, frequently remove organisms and debris to prevent clogging and subsequent diversion of water at the net opening. Use replicate samples as appropriate to meet study objectives. Set drift-net samples for any specified time (usually 1 to 3 h) but use the same time for each station. Sampling between dusk and 1 AM is optimum.

The total quantity (numbers or biomass) of organisms drifting past a given station is the best measure of drift intensity. Report data in terms of numbers or biomass/m^3.[2,29–31]

4. Sampling Devices, Qualitative

When sampling qualitatively, search for organisms in as many different habitats as possible.[32] Collect samples by any method that will capture representative species.

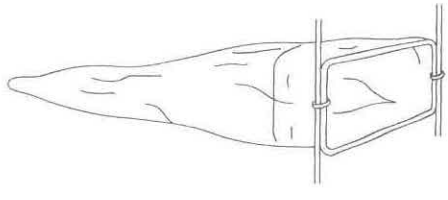

Figure 10500:11. Drift net sampler.

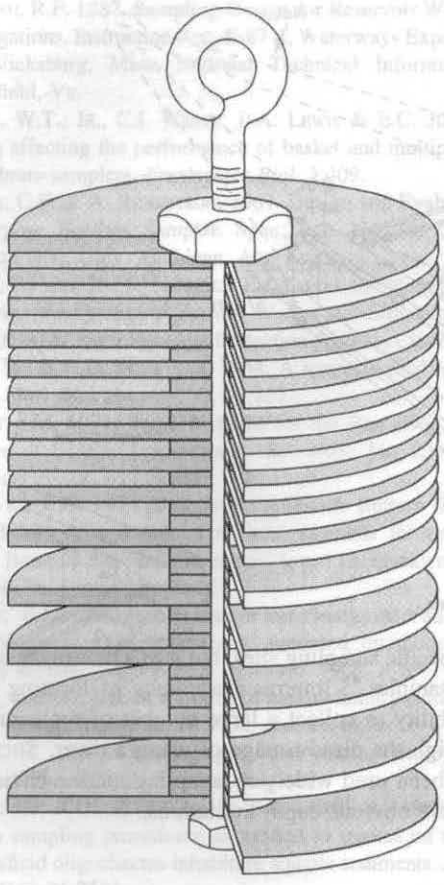

Figure 10500:12. Hester-Dendy artificial substrate unit.

a. Dip, kick nets are the most versatile collecting devices for shallow, flowing water, and are useful also for shoreline collecting in lakes. When combined with a standardized kicking technique,[33] these nets are appropriate for quantitatively sampling macroinvertebrates.[34]

b. Tow nets, dredges, or trawls range from simple sled-mounted nets to complicated devices incorporating teeth that dig into the bottom. Some models feature special apparatus to hold the net open during towing and to close it during descent and retrieval. Available styles have been discussed elsewhere.[21,35,36]

5. Sampling Devices, Artificial Substrate Samplers

Artificial substrate samplers are devices of standard composition and configuration placed in the water for a predetermined exposure period for colonization by macroinvertebrate communities. Because many physical variables encountered in bottom sampling are minimized, e.g., depth, light penetration, temperature differences, and species substrate preferences, artificial substrate sampling complements other types of sampling. Like natural submerged substrates such as logs and pilings, artificial substrates are colonized primarily by immature aquatic insects, crustaceans, coelenterates, bryozoans, and to some extent worms, gastropods, and mollusks. In lotic systems the organisms that colonize artificial substrates are primarily drift organisms, such as immature insects and eggs, carried by water currents. Placement conditions should be similar, so the numbers and kinds of organisms reflect capacity to support aquatic life.

Position artificial substrates in the euphotic zone (0.3 m) for maximum abundance and diversity of macroinvertebrates.[13] Optimum time for substrate colonization is 6 weeks for most waters. For uniformity of depth, suspend sampler from floats on a 3.2-mm steel cable. If vandalism is a problem, use subsurface floats or place sampler near the bottom. Regardless of installation technique, use uniform procedures.

At shallow water stations (less than 1.2 m deep), install samplers so that they are located midway in the water column at low flow. For samplers installed in July when the water depth is about 1.2 m and the August average low flow is 0.6 m, install 0.3 m above the bottom. Take care not to let samplers touch the bottom or they may become covered with silt, thereby increasing the sampling error. In shallow streams with sheet rock bottoms, secure artificial substrates to 0.95-cm steel rods that are driven into the substrate or secure to rods that are mounted on low, flat, rectangular blocks.

Before removing samples from the water, it may be necessary to enclose them in an oversized plastic bag (double wrapping) that is tightly sealed to prevent possible loss of organisms or to use a large dip net (openings equivalent to a U.S. Standard No. 30 sieve) when the sample is removed. Disassemble sampler and brush in a pan of water in the field or add preservative to the bag containing the intact sampler, and disassemble and brush later in the laboratory.

Although many different styles of artificial substrate samplers have been tested,[37] the Fullner[38] modification of the Hester-Dendy[39] multiplate and the basket sampler[13] are used widely.

a. Multiple-plate or modified Hester-Dendy sampler (Figure 10500:12) is constructed of 0.3-cm-thick tempered hardboard with 7.5-cm round plates and 2.5-cm round spacers that have center-drilled holes. The plates are separated by spacers on a 0.63-cm-diam eyebolt, held in place by a nut at the top and bottom. In each sampler, 14 large plates and 24 spacers are used. Separate the top 9 plates by a single spacer. Separate Plate 10 by two spacers, Plates 11 and 12 by three spacers, and Plates 13 and 14 by four spacers. The sampler is approximately 14 cm long and 7.5 cm in diameter, has an exposed surface area of approximately 1300 cm^2, and weighs about 0.45 kg. Do not reuse samplers exposed to oils and chemicals that may inhibit colonization. Because it is cylindrical, the sampler fits a wide-mouth container for shipping and storage. The sampler is inexpensive, compact, and lightweight.[13,38,39]

A different type of square modified Hester-Dendy, multiple-plate artificial substrate sampler is constructed of 0.3-cm tempered hardboard cut into 7.6-cm square plates and 2.5-cm square spacers.[32] Eight plates and twelve spacers are used for each sampler. The plates and spacers are placed on a 1/4-in. (0.64-cm)

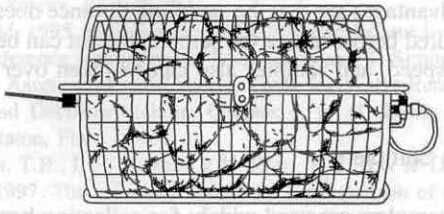

Figure 10500:13. Basket sampler.

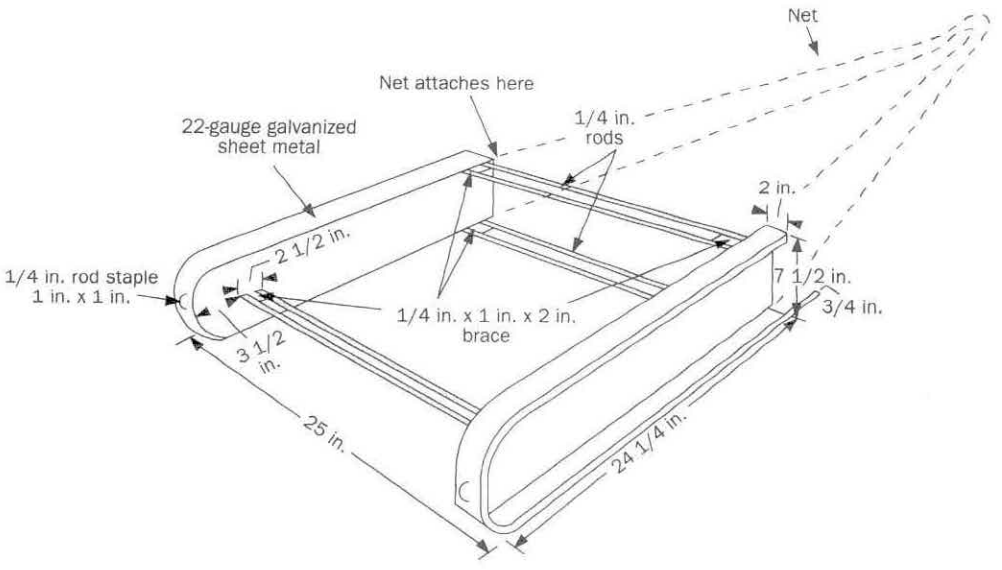

Figure 10500:14. Marsh net sampler.

eyebolt so that there are three single spaces, three double spaces, and one triple space between the plates. The total surface area of the sampler, excluding the eyebolt, is 939 cm^2 (0.9 m^2). Generally, five samplers are used and placed in streams tied to a concrete construction block as anchor. This prevents samplers from coming into contact with the natural substrates.

b. The basket sampler[13] (Figure 10500:13) is a cylindrical "barbecue" basket 28 cm long and 17.8 cm in diameter, filled with approximately 30 5.1-cm-diam rocks or rocklike material weighing 7.7 kg. A hinged side door allows access to the contents. The sampler provides an estimated 0.24 m^2 of surface area for colonization. The factors governing proper installation and collection are the same as those described for the multiplate sampler. Some investigators prefer using the basket because natural substrate materials are used for colonization.

c. Marsh net sampler (Figure 10500:14) is used for sampling macroinvertebrates in estuarine and marine environments.[40] It can be used in different habitats (e.g., marsh, beach, tidal creek, and tidal flat) of estuarine and marine intertidal zones to depths of 3 m. The metal frame is constructed of No. 22 galvanized sheet metal and 1/4-in. (6-mm) welding rods. A 0.5-m plankton net of nylon monofilament screen is laced to the posterior end of the frame. The net has a bayonet-type cod end for easy removal. The mesh size of the plankton net and cod end is about 1 mm (bar measure). The frame and net weigh 5 kg. The collecting procedures are the same in all habitats of the intertidal zone. The net is placed at one end of the sampling area and 30 m of rope is paid out in an arc to prevent the operator from disturbing the sampling site. The net is then retrieved by hand at a rate of about 0.3 m/s. Advantages are that the sampling distance does not have to be measured before taking the sample, the net can be towed at a constant speed, and samples also can be taken over soft mud bottoms.[40]

6. Suction Samplers

Suction samplers are used widely for collecting benthic macroinvertebrate samples.[41,42] These samplers can be placed directly on specific sampling sites, but a SCUBA diver is required to collect samples.[43] Improved accuracy of locating sampling sites and ability to collect a large number of replicate samples may outweigh the disadvantage of using a diver. Suction samplers have been used widely in sampling marine environments, but they have obvious depth limitations.

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10500 C. Sample Processing and Analysis

1. Sample Processing

After collecting a benthic sample, transfer it to either specially designed sieve tables (or hoppers) or a container. If a container (such as small trash can) is used, dilute with ambient water and swirl. Pour slurry gradually into a sieve bucket. Gently wash slurry over screen to prevent damaging or losing specimens. Slurries that clog the screen require removal of screened material. A series of one or two coarser screens (e.g., 1-cm and 0.5-cm mesh) will hold back larger materials such as leaves, sticks, shells, and gravel while permitting organisms and smaller materials to pass through to the bottom sieve. Carefully check rocks, sticks, shells, and other objects for attached or burrowed organisms before discarding. A soft-bristled toothbrush may be used to remove attached invertebrates from rocks, sticks, and similar objects.

Wash residual material on the screen into a container. A cheesecloth bag is very useful because it does not restrict the quantity of wash water. Label containers with a collection code but do not affix labels to lids. Similar labels can be written with pencil or indelible ink on high-rag-content paper and placed in the container. Record label code on a field sheet that describes location, date, type of sample, collector's name, and other pertinent information.

Use laboratory elutriation devices[1,2] as appropriate to reduce time required to sort benthic organisms from samples containing large amounts of silt, mud, or clay. Wash screened material into a container and fix the contents in a solution of 10% buffered formalin or 70% ethanol.[3–6] If ethanol is used, do not fill more than one-half the container with screened material. Preserve and store animals with calcareous shells or exoskeletons, i.e., mussels, snails, crayfish, and ostracods, in 70% ethanol.[6,7]

Some macroinvertebrates (soft-bodied animals) are identified more easily if they are relaxed to prevent constriction during preservation. Common relaxants include carbonated water (soda water) or carbon dioxide added to water. Other relaxants include aqueous solutions of 70% ethyl alcohol, 2% nicotine sulfate, propylene phenoxetol, or 5 to 10% solutions of either chlorotone, chloral hydrate, or magnesium sulfate added gradually to the water containing the soft-bodied animals until the degree of relaxation sought is reached. Narcotize organisms before fixing them. Ideally, fix annelid specimens (oligochaetes) in 5 to 10% buffered formalin before preserving them in 70 to 80% ethanol (note that alcohol is not a satisfactory tissue fixative). Fixation stabilizes tissue proteins to retain characteristics of the soft body (e.g., segmented worms) form.[8,9]

For qualitative samples, place rocks, sticks, and other objects in a white pan partially filled with water. Many animals will float free from these objects and can be removed with forceps.

Assign identification numbers either in the field or at the laboratory and transcribe information from the labels to a permanent ledger. The ledger provides a convenient reference in identifying number of samples collected at various places, time of sampling, and water characteristics.

Preserve and store in 70% ethanol organisms taken in the field or from artificial substrates and sieved with a U.S. Standard No. 30 sieve. For special studies and to retain anatomical form and structures, fix soft-bodied organisms first with 5 to 10% buffered formalin or 70% ethanol. NOTE: For health and safety reasons, always take care when using 5 to 10% buffered formalin, or avoid using it to fix or preserve organisms in the field or in the laboratory. Never discard fixatives or preservatives into the environment.

2. Sorting and Identification

Whether organisms are sorted in the field or the laboratory, follow consistent procedures. Before processing a sample, transfer information from the label to a data sheet that provides space for scientific names and number of individuals. Place sample directly in a shallow white tray with water for sorting. To facilitate sorting organisms from detritus, the organisms may be stained with rose bengal (200 mg/L or achieve a light pink color) in the formalin or ethanol preservative for at least 24 h.[10] NOTE: Excessive staining may prevent specific identification of some specimens. Examine entire sample and separate organisms unless they occur in very large numbers. If a subsample is sorted, take care that rare forms are not excluded. As organisms are picked from the sample, sort under a scanning lens or stereoscopic microscope, separate them into different taxonomic categories, identify to the lowest taxonomic level to meet the data quality objectives, and record on the data sheet. Place animals in separate vials according to category and fill vials with 70% ethanol. Place inside vials labels containing sample tracking number, date collected, sampling location, and names of organisms.

Identify animals in each vial using stereoscopic and compound microscopes, according to need, and available experience and resources. Identify organisms to species level if possible. Additional sources of information on laboratory techniques and identification guides and taxonomic keys of macroinvertebrates are available (see Bibliography and Section 10900).

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10500 D. Data Evaluation, Presentation, and Conclusions

There are two basic approaches used in evaluating effects of pollution on aquatic life. The first is to make a qualitative inventory of the benthic fauna, "above (upstream) and below (downstream)" or "before and after" the suspected or known areas of pollution, thereby determining species presence or absence. Then, through an understanding of the responses of various species to certain pollutants and habitat degradation, determine the significance of damage or change. The second approach is to make a quantitative analysis of the numbers of individuals, species, and structure (abundance and composition) of the aquatic community affected by pollution and then to compare with reference information. In most pollution surveys these approaches are integrated because each provides valuable interpretative information.

1. Qualitative Data Evaluation

No two aquatic organisms react identically to a pollutant because of complex relationships between genetic factors and environmental conditions. However, certain taxa are relatively sensitive to certain types of pollution such as siltation and turbidity, organic enrichment, acidity, heavy metals and other industrial toxic wastes, oil production, agricultural products, radioactive wastes, and thermal effects. For example, operculate snails, immature stages of certain mayflies, stoneflies, caddisflies, riffle beetles, hellgrammites, many marine amphipods, mysids, bivalve larvae, and echinoderms are sensitive to many pollutants. Pollution-tolerant macroinvertebrates such as certain sludge worms, midge larvae, leeches, pulmonate snails, and some polychaetes usually increase in number under organically enriched conditions. Facultative organisms, those that tolerate moderate pollution, include most snails, sowbugs, scuds, and blackfly larvae. Tolerant organisms may be found in either clean or polluted situations; thus their presence is not definitive. However, a population of tolerant organisms combined with an absence of intolerant ones is a good indication of the presence of pollution. The same species found in different geographical areas may well react differently or be present in different numbers throughout the year.

2. Quantitative Data Evaluation

Statistical methods of data evaluation and mathematical description of community structure are valuable tools in data analysis. Analysis of biological data commonly begins with the calculation of descriptive statistics (mean, standard deviation, standard error, and confidence intervals). Analysis proceeds by application of robust statistical methods of comparison (Chi-square, Student's *t*, regression, correlation, analyses of variance, or nonparametric equivalents).[1,2]

Mathematical expressions, including numerical indices of community structure, are useful in characterizing and describing aquatic communities. These expressions usually are based on the structural and functional stability of the system.[2]

Diversity indices, although limited, condense considerable biological data into single numerical values.[2–9] Unfortunately, useful information may be lost by condensing biological data.[3,10]

Select methods for analyzing multivariate benthic community data using two important criteria: the methods should test specific impact-related hypotheses suggested by the data quality objectives and study design, and the methods should objectively identify relationships among variables. Use methods that make a priori assumptions about relationships among variables only secondarily for presentations, not for primary analysis.

More powerful multivariate statistical analyses generally are less subject to criticism and may be more appropriate for some bioassessment studies.[1,10] Recommended data analyses approaches are: regression of species (or taxa) richness on abundance, analysis of variance followed by linear orthogonal contrasts,[11] various other multivariate approaches (e.g., cluster techniques and ordination, analyzing principal components, ANOVA, discriminate analyses), and macroinvertebrate community metrics[2,12] for assessing biomonitoring data and water quality.

For statistical evaluation of the data collected in pollution surveys, it always is beneficial to identify the sources of variability commonly found. Variability in macroinvertebrate data comes from the methods of sampling and the distribution of organisms. Perhaps the major source is sampling error. Organisms generally are clustered in relation to habitat distribution; therefore, random samples often show high variability among replicates. In statistical analyses of quantitative data, large numbers of samples often are required to detect statistically significant differences. Exercise care in using parametric statistical methods because the basic assumption of normal distribution is not always true. Data often have to be transformed before being tested. Do not assume that a statistically significant difference is ecologically significant.

3. Data Presentation

Data presentation may take many forms. The basic techniques include tables, bar graphs (horizontal and vertical), pie diagrams, pictorial charts (ideographs), line graphs, frequency distribution tables and graphs, histograms, frequency polygons, and cumu-

lative frequency polygons. These may be superimposed on maps. Several reports that may be useful in analyzing macroinvertebrate data have been included in the bibliography. Methods for interpreting benthic invertebrate data with measures of contamination and toxicity are available.[13]

4. Conclusions

Despite detailed data quality objectives, field methodology, and laboratory analysis and data presentation, it often requires extensive professional experience and skill and knowledge of the scientific literature to draw defensible conclusions from a data set. Even in the best circumstances, there can be more than one conclusion drawn from a study. When more than one conclusion is possible, it is appropriate to present all options.

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10600 FISHES*

10600 A. Introduction

1. Ecological Importance

Fishes are a major component of most aquatic habitats, and thousands of species occur in streams, rivers, lakes, and estuaries. These animals are the focus of economically important sport and commercial fisheries; licensing fees for both private and commercial sectors provide funds for state and federal agencies. Fishes are an important source of food and recreation and are key elements in many natural food webs. They have an impact on the physicochemical properties of the system in which they occur; they affect plankton, macrophytes, and other aquatic organisms; and they can serve as environmental indicators. Changes in the composition of a fish assemblage often indicate a variation in pH, salinity, temperature regime, solutes, flow, turbidity, dissolved oxygen, substrate composition, or pollution level. The gain or loss of certain species is a common consequence of environmental change. Because fishes are conspicuous they often are the primary indicators of the toxification of streams and lakes. In extreme cases the presence of dead or moribund fish may adversely affect potability and recreational use of waters, create foul odors, and corrupt shorelines.

Because fishes are ecologically important, there are often intense commercial and recreational interests surrounding their study. These diverse interests translate into a need for the scientist, often supported by public funds, to be aware of the sensitivity of such investigations. Fishes share many physiological properties with mammals and are used in both the laboratory and the field by the environmental manager and health specialist in biological assays.[1]

* Approved by Standard Methods Committee, 2001.
Joint Task Group: Donald M. Baltz (chair), John Homa, Donald Klemm, Lesa Meng, Thomas Minello, James Whitlock.

2. Definitions and Terminology

Fish refers to a single organism, several of the same species, or can be used as an adjective (e.g., fish market).

Fishes refers to two or more species.

A *population* is a group of individuals of any one kind of organism occupying a particular space. Its study includes definition of taxonomic position, habitat and mobility, diet, numbers of individuals by age, size, weight, sex, fecundity, and sources of mortality.

An *assemblage* (association, community) is a group of several populations sharing a common geographical area. The study of their coordinated activity is key to the understanding of the environmental system.

3. Scope of Analysis

Research often becomes necessary when existing knowledge is recognized as inadequate to answer new questions arising from real-world issues. The scope of a research project involves several critical steps that are often overlooked and may lead to findings lacking in credibility. Thus time spent in careful planning that recognizes constraints and assumptions is critical to success throughout processes that involve conceptualization, design, data collection, analysis, and interpretation.[2]

The guidelines provided here are directed to the general practitioner who may need specialists such as the commercial and sport angler, fishery biologist, taxonomist, histopathologist, population statistician, systems ecologist, and toxicologist. Adapting to the particular situation is the key element in the study of fishes in their natural habitat.[3–9]

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10600 B. Data Acquisition

1. Planning and Organization

a. Objectives: Define study goals and objectives clearly before collecting data. Refine the goals or questions into specific objectives that narrow a goal into a tractable issue. Develop testable hypotheses from the objectives, and design the sampling protocol to test these hypotheses. Fishes may be collected for many reasons including systematics, identification of species or assemblages present, age and growth, experimental laboratory studies, feeding habit studies, stocking programs, behavioral studies, or ecological studies to determine population size or patterns of habitat use. Each of these types of studies requires specific collection and analysis techniques.

b. Variables: Before initiating a study, understand the variables that affect fish populations, including life history characteristics of the species of concern. Environmental variables and habitat characteristics can affect fish abundance and the ability to capture fish. Variables to consider include the time of day, season, weather, water height, currents, tidal conditions, bottom type, presence of vegetation, and also water temperature, salinity, dissolved oxygen, pH, and turbidity. When attempting to measure fish densities, consider the effect of these variables on the catch efficiency of the gear used.

c. Regulations: A detailed understanding of licensing and permit requirements for the collection of specimens is essential. Most states have strictly enforced regulations on both the collection and disposition of fish specimens. Effective public relations usually involves the guidance of local residents and waterfront associations on the activities planned.

d. Site inspection: Study topographic maps and other relevant data before visiting the site. Make an early and thorough visual examination of the study site. Use glasses with polarized lenses to aid examination of bottom features and detection of fish. Binoculars allow field identification of fishes and help in the logging of needed behavioral information. In clear water use face plate and snorkel or SCUBA (self contained underwater breathing apparatus) to define habitat, identify fish, and observe behavior. Use wet or dry suits during colder periods and even under ice but note that special safety training is essential.

e. Data forms: Print data forms on good-quality bond paper, waterproof paper, or plastic sheets; use pencil or permanent ink to record data. Electronic media, e.g., polycorders and computer notebooks, are becoming popular. Data can be transferred directly into computer files to save time and reduce transcription errors. Back up files often and make hard copies to store at a second location. Include on the data form the following information in an order best suited for the study:

- Date and time of collection or observation;
- Exact location using the Universal Mercator System (UTM) or a local variant; township, range and section numbers; county and state; physical features such as a stream confluence, islands, bays, etc.; and station identification number or code;
- Site conditions for each sample collected, e.g., presence of ice, flood state, tidal stage, meteorological events such as air temperature, occurrence of storms and rainfall in last 48 h, water depth, discharge, vegetational cover on nearby shores, bottom type, submerged vegetation, etc.;
- Habitat information such as dissolved oxygen, turbidity, pH, substrate type or texture, aquatic vegetation, water temperature, and salinity;
- Purpose of activity or project;
- Description of collections or observations made including preservatives, photograph numbers, and gear type;
- Personnel and their functions;
- Name of person recording the data;
- Chain of custody signatures and dates;
- Information on fishes collected or observed (identity, number, mass, presence of disease, etc.).

f. Description: Collected materials may define the species represented, describe the population of a particular species, describe a species assemblage, or characterize impacts of some event such as a chemical spill. The detailed analysis may include:

- Preliminary species assignment for each specimen;
- Number of individuals of each species;
- Standard, fork, or total length for each specimen;
- Sex, if discernible;
- Maturity as indicated by gonadal condition and coloration;
- Weight of each specimen (displacement volume may be used);
- Description of unusual features such as tags, deformities, lesions, tumors, or parasites; and
- Materials taken to determine the age of fish, such as scales, otoliths, spines, or opercular bones, or stomachs for diet analysis.

g. Conduct of field workers: Obtain necessary permits for collecting, holding, transporting, and stocking of specimens. Provide adequate advance notice of activities to conservation officers, wardens, local law-enforcement agencies, and officers of lake or watershed associations. Inform them in detail of the actions planned. Understand trespass law for the study area and request access.

Be sensitive to the use of waterfront areas. Avoid, where possible, damage to amenity plantings and capture of favored specimens, e.g., a pair of large smallmouth bass holding a territory next to a dock. Deal pleasantly and in a well-informed manner with the questions of onlookers. Display the name and address of the study group through such means as name tags, arm patches, or equipment decals.

Working in, on, and near water involves potential risk. Demonstrate regard for safety. Become proficient with boat safety, proper operation of sampling equipment, and first aid including cardiopulmonary resuscitation (CPR). Wear life preservers when appropriate. Avoid wearing waders on board vessels when in deep water. Follow local restrictions on boat speed. Handle gear proficiently. Use fail-safe switches on all electrical gear—especially electro-shocking equipment. Ensure that the entire field crew is familiar with safety concerns for fisheries investigations.[1] Be especially careful when working in severe sampling conditions including extremely hot or cold temperature, ice, turbulent water, fast currents, high or gusty winds, and at facilities including diversions, dams, spillways, water intake and discharge structures, and pump stations.

Identify sampling gear with name, address, telephone number, and permit or license numbers of the using agency.

Position gear inconspicuously to minimize tampering and vandalism. Avoid navigational channels and other heavily used sites. Whenever appropriate, submerge indicator buoys and mark their location by paired range points such as navigational aids or landmarks. When transferring gear from one locality to another be careful not to translocate organisms. Maintain gear in a professional manner.

Dispose of processed specimens rationally and legally. Consider identifying and counting live specimens in the field and releasing them. Check with regional museums and/or academic institutions that regularly voucher preserved specimens. Obtain accession policies as necessary. Preserved specimens are needed in biology classes in most local school districts. Contact principals and science teachers if suitable material is available. If necessary, bury or burn specimens fixed in formalin at appropriate and legal facilities. Pay attention to local legislation regarding humane care of vertebrates including fishes. Do not overharvest. A 2-h evening set of a gill net may yield a sufficient number of specimens for a particular study while an overnight set would be wasteful. Avoid sampling nontarget species.

2. Existing Data

Published and unpublished data already exist for most larger lakes and river systems. Natural history museums and academic institutions are primary sources and often can provide preserved materials as well as the names of local specialists. Members of fishery and ecological professional associations, e.g., the American Fisheries Society, the American Society of Ichthyologists and Herpetologists, and the Ecological Society of America, also can provide information. Private engineering and environmental consulting firms often maintain detailed regional files. State agencies such as departments of health, environment, conservation, fisheries, wildlife management, and planning, and federal agencies such as the U.S. Fish and Wildlife Service, National Biological Survey, U.S. Environmental Protection Agency, Bureau of Reclamation, U.S. Army Corps of Engineers, and U.S. Geological Survey are good resources. Large data sets may be available from power utilities, refineries, food producers, and chemical companies having riparian facilities. Cooperative extension services and public colleges and universities provide expertise and information. Commercial fishermen and master sport anglers of an area are other important sources. Local libraries, newspapers, and local residents may provide useful material, including photographs, dates, and even specimens.

3. Collection and Observation Methods

All methods of collecting and observing fishes are selective and, therefore, biased. No one method of collection will completely portray the composition of a fish assemblage. The goals of an individual study dictate the appropriate method(s). Distinguish between collecting fishes for various reasons (e.g., gut

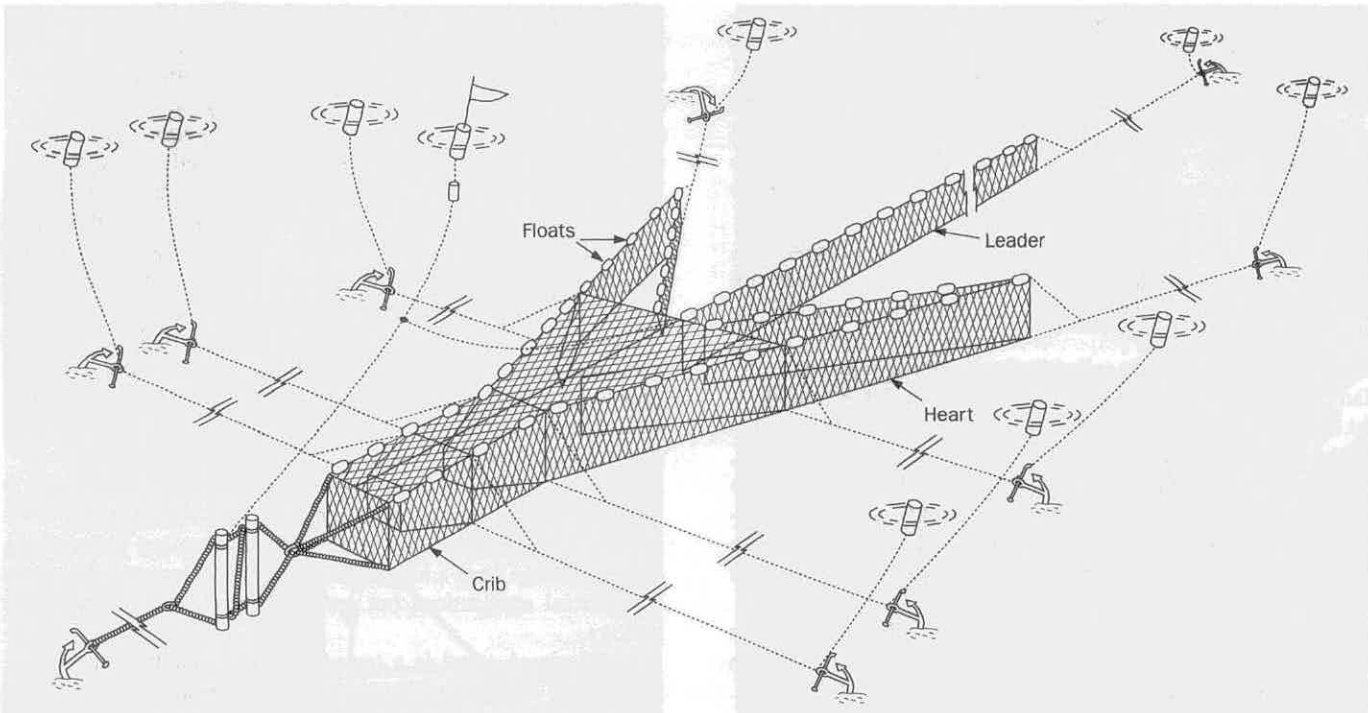

Figure 10600:1. Diagram of a sunken trap net. Source: BAGENAL, T., ed. 1978. Methods for Assessment of Fish Production in Fresh Waters. IBP Handbook No. 3. Blackwell Scientific Publ., Oxford, England.

analysis, age and growth, laboratory studies) and sampling fishes to determine absolute (fishes per m^2 or m^3) or relative (catch per unit effort, e.g., per minute, per haul, per tow) population size. Many of the gear types listed below are appropriate for collecting fishes but are not adequate for determining density or population size.[2–4] For each study, carefully and completely describe goals, objectives, testable hypotheses, and collecting methods so the adequacy of the techniques can be assessed by users of the data.

a. Angling: The use of hook and line is an ancient means and involves relatively simple gear[5] but its effective use is a matter of skill. The services of a competent angler are often valuable. The techniques depend on the resourcefulness and skill of the angler, and may be time-consuming and expensive.

b. Set line: A set line is a heavy line anchored at each end bearing regularly spaced leaders having baited hooks. It is widely used for commercial and private fishing. It usually is fished overnight on the bottom and can be used to great depths.

c. Trolling: Trolling is towing a hook and line behind a vessel. It is effective for larger fishes of open and deep waters. Use metal line equipped with metal weights or wing depressors to achieve desired depths. Specialized lures that reflect sonar often are used with acoustical electrical gear to determine depth and to locate target fish. Trolling may be the most economical means of capturing some species.

d. Spear and bow and arrow: The use of barbed and/or hooked (some automated) spears is of limited utility. It often is prohibited. Sport bow-and-arrow fishing may yield large numbers of carp, gars, or other larger fishes in shallow water. Spearing through ice often is effective, especially for sturgeon and larger esocids and percids. Efficiency varies seasonally.

e. Nets—General remarks: Netting is used in static gear such as traps and weirs and in active gear such as seines and trawls. Netting may be made of cotton, plastic, or metal. Nets of natural fiber are subject to microbial decomposition and have been supplanted by other materials but are still of value where loss of gear is likely. Plastic netting is exceedingly durable but is weakened by ultraviolet irradiation; avoid prolonged exposure to sunlight. Netting is available in colors that may hold some sampling advantage. Mesh sizes are measured in terms of "bar," i.e., along the edge of the frame, "diagonal," i.e., from opposite angles of the frame, and "stretched," i.e., from opposite angles when the net is under tension. Knotting varies; some knots are abrasive to captured fish. Knotless netting also is available.

f. Hoop, fyke, and trap nets: Elongated, tapered nets supported on hoops and variously divided into chambers with secondary funnel net sections and anchored to the bottom are common. Usually they are used at depths less than 3 m. They may be kept in place for a protracted period but usually are visited daily to remove the catch from the inner chamber or cod. Orient the cod end into the current in slowly moving water. The basic hoop or ring net may be converted into a fyke net by adding panels of netting at the open end. Those added to the sides are called wings; the single panel placed at the center of the mouth may be quite long and is called the lead. Wings and leads usually are equipped with floats and weights and are placed to deflect and trap or confuse the normal movements of fishes along shore into the net. The main body of a typical hoop or fyke net may range from 5 to 10 m in length and up to 2.5 m in diameter. A net hung on rectangular framing is called a trap net (Figure 10600:1). On commercial fishing grounds researchers usually contract for the catches of larger trap nets rather than use their own. Another net type of this general design is the pound net.

Hoop, fyke, and trap nets are effective for larger fishes that are mobile or that seek cover. They are especially useful in capturing

live fishes for spawning stock or fishes to be used in mark-and-recapture population assessment. These nets can be baited to increase their efficiency in capturing some species.

Nets of this type are available through net supply houses and commonly are built to specification. They require a boat to set and inspect. They often are conspicuous and thus attract the public. They require surveillance and the clear posting of their ownership and purpose.

g. Traps: The term "fish trap" usually is reserved for smaller, portable units commonly made of galvanized wire. They are fitted with one or more conical inserts and an opening to remove the catch.

Smaller devices usually called "minnow traps" may be useful. Many types are available, including highly durable plastic units that can be easily stacked, stored, and transported. Minnow traps may be placed in a well, a cave pool, near hydroelectric facilities, or in other awkward localities with good results. They may be made more effective through baiting. A readily degradable element (e.g., wooden lath in a plastic or metal trap) should be a part of the design of all traps used in the field so that lost traps do not continue to capture and kill fish.

Pit traps can be used to capture small fishes in marshes inundated at high tide.[6] Designs vary, but generally pit traps are plastic containers placed into a pit dug into the marsh substrate. Because fishes utilize natural depressions as refuge when the marsh drains, pit traps can be an effective collecting tool.

h. Weirs: Weirs are stationary traps usually installed along the course of a stream or river. They are of complex design and may be incorporated into a fish ladder and dam. They guide fishes into a sampling or capture sector called the "pot."

i. Gill nets: Nets constructed of thin line with mesh large enough for the target species to penetrate just beyond the operculum are called "gill nets." The fishes become entrapped while attempting to swim through the net and are harvested. Gill nets are composed of panels of netting of the same or diverse mesh size suspended between a stronger "float line" equipped with flotation devices and attached along the upper working edge and another heavier "lead line" attached along the lower working edge. Adjust weights and floats to position the net on the bottom, surface, or at an intermediate depth. The ends of the gill net are equipped with anchors, tether lines, and buoys.

The gill net may be set under ice through appropriate holes. Gill nets may be used in standing water; they are less successful in flowing water, but can be set parallel to the current.

The setting and recovery of gill nets require special attention. The sampling site must be free of ensnaring objects such as submerged trees. The boat used to set the net must be free of projections that can catch the net during the payout and the net boat must proceed at a speed in harmony with the workers discharging the net. The net must be well pleated and free of snarls to set; the anchors, tethers, and buoys must be ready for quick release as well. At the conclusion of the set pull the tether to extend the net to its full working length. The tether line must be long enough so that the buoy is not pulled out of sight. The marker buoy can be set below the surface to reduce vandalism and paired range points may be used to relocate the site. If the tether line and buoys are lost, attempt retrieval by grappling hook or SCUBA. Failure to recover lost gill netting can be environmentally disastrous because it will continue to catch and kill fish. Report lost gill netting to local fisheries authorities.

Overnight setting is common; however, a test set during dawn or dusk or a short night set may be advisable to avoid the capture of an excessive number of specimens. Use of gill nets can result in high fish mortality. To reduce mortality, set the net for a shorter time or check it at short (e.g., 2-h) intervals through the set so that all captured fishes can be removed and processed. Lifting the gill net is best during calm weather and early in the morning. Remove captured fish, if relatively few, from the net as it is lifted or retain in the net and place in a sturdy box for subsequent "picking" on shore. Record location, orientation, and mesh size of net. On completion of the "picking" remove twigs, leaves, and other matter from the net and clean and dry it. Gill nets, and all entanglement gears, are particularly effective, and selective for fishes with spines or other features that can get caught in the netting. If these fishes are abundant, time required to clear the net will increase.

j. Trammel, flag or tangle nets: Trammel nets are composed of three panels of netting hung together. The central panel is of smaller mesh and the fish is ensnared by passing through the coarse panel to form a bag in the central net. Trammel nets have commercial applications but are used infrequently in fisheries biology.

k. Trawls: A trawl is a towed net. The mouth of the net is maintained by either a frame, as in the beam trawl, or with hydraulic planes called "otter boards" or "doors" working together with weights and floats as in the "otter trawl." Trawls are specialized to work at the surface, in midwater, or on the bottom. In the surface trawl, buoying devices predominate; in the midwater trawl they are balanced against the weight; and in the bottom trawl the weights predominate. The bottom trawl usually has abrasion skirting on the lower surfaces, rollers that facilitate movement over obstructions, and special chains (ticklers) that run along the lower leading lip of the mouth. The tickler stimulates fishes to rise off the bottom and into the net. Some trawls have one or more conical inserts before the cod or terminal part. The cod is held closed with a cinch line that can be pulled to release the catch onto the sorting deck or tables. The trawl is pulled by a bridle and warp worked from a hydraulic winch. Smaller trawls may be worked strenuously by hand. The length of warp required depends on speed and depth of sampling; however, a 30° angle of warp to the water surface is typical. The speed of towing relates to the gear but is around 2 or 3 knots. Depth and the character of the bottom may be defined by echo sounder. The duration of a tow ranges from a few minutes to several hours. Night trawling may yield larger catches but is more difficult in many inland waters because of navigational aids, anchorage buoys, and other obstructions.

l. Enclosure samplers: Enclosure samplers include a wide variety of sampling gear used to rapidly enclose a known area. Active enclosure samplers include the encircling/block net, purse seine, drop net, throw trap, drop sampler (Figure 10600:2), pop net, pull-up net, and bottomless lift net. After fishes are enclosed, various methods are used to recover specimens, including dip netting, seining, pump filtration, poisoning, and pursing. Enclosure samplers generally have high catch efficiency for small fish, are adaptable for vegetated and structured habitat types that cannot be sampled with other gear, and provide quantitative estimates of fish density in different habitat types. In tidal estuaries, passive enclosure samplers such as flumes, channel nets and modified fyke nets also can be used to collect density data.

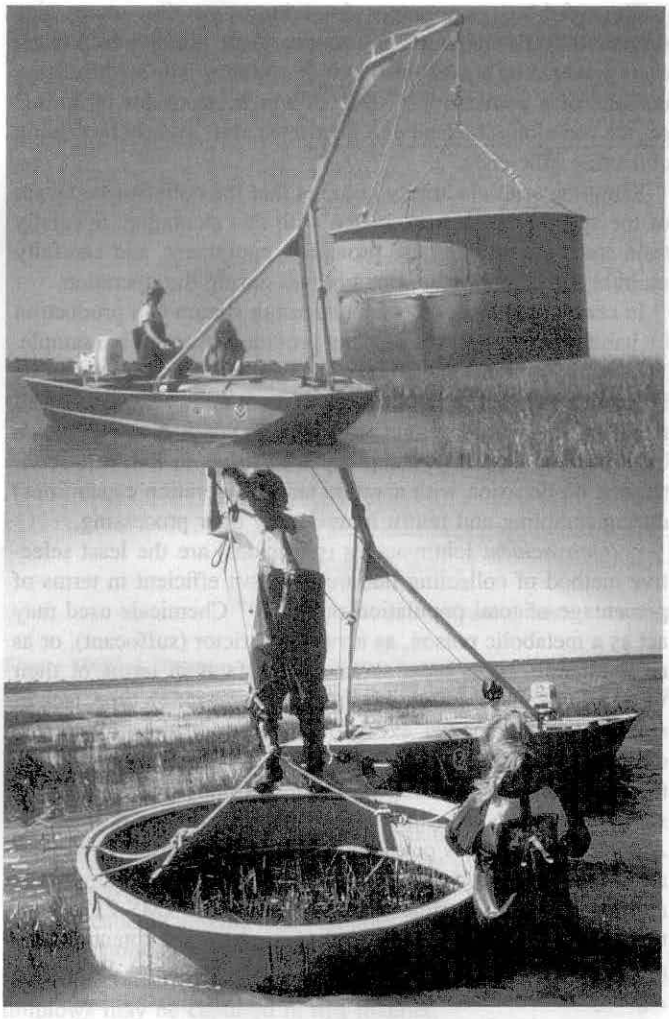

Figure 10600:2. A typical enclosure sampler, the drop sampler, in action. Photo courtesy of the National Marine Fisheries Service Galveston Laboratory.

These gear require changing water levels (generally tidal fluctuations) to sample fish distribution patterns. A review[4] compares the efficacy of these gear types and provides recommendations for gear selection in shallow water habitats.

m. Ichthyoplankton sampling: Ichthyoplankton consists of the eggs and very young stages of fish (sacfry, larvae, postlarvae).[7,8] Ichthyoplankton usually is collected either by plankton nets or bulk water sampling.[9] Nets having a mouth diameter smaller than the main body of the net may be towed faster than the usual 2 to 3 knots.[10] The towing bridle of the net affects sample collection and a number of designs such as the double net or "bongo net" have been devised to reduce this influence; comparisons of various gears have been made.[11] Measure water volume with a flow meter. Other devices may be lowered to a given depth, triggered to open, and then triggered to close again, providing a sample from a known depth. The Clarke-Bumpus, Tucker trawl, light traps, pumps, and MOCNESS are examples of quantitative plankton samplers.[12] In vertical sampling the net is lowered to the bottom or some prescribed depth and then pulled upwards, sampling the water column. Mesh size for

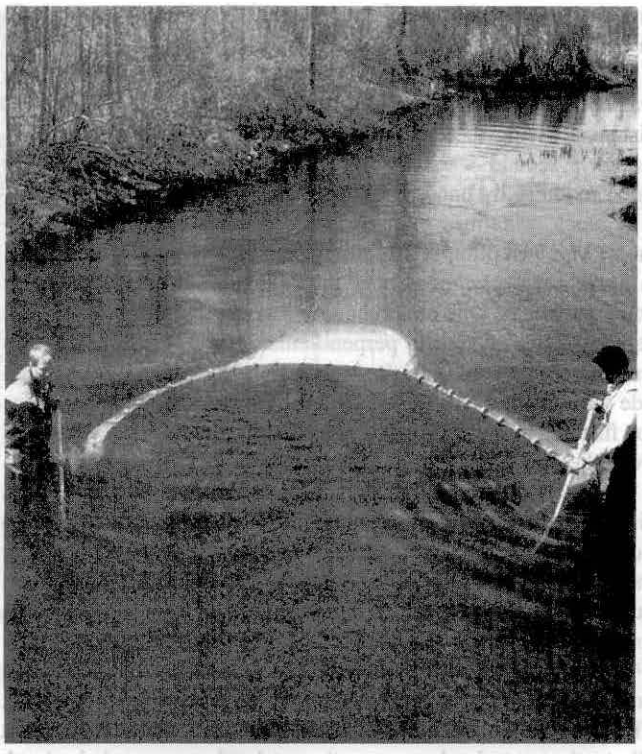

Figure 10600:3. Bag seine in operation in a stream. Photo courtesy of New York State Museum.

commercially available plankton netting ranges from 0.158 to 1.000 mm but only a few standard meshes (e.g., 0.333 and 0.500) have been specified. Because ichthyoplankton may span a wide range of developmental and sensory capabilities and they may also migrate vertically or tidally, note the time of day and/or stage of the tide (in marine and estuarine settings) at which sampling occurs. With due consideration for safety, nocturnal sampling may be warranted, if vertical migration or gear avoidance is suspected.[13]

Bulk water sampling of ichthyoplankton consists of collecting a known volume of water and separating the ichthyoplankton by filtration and/or centrifugation.

n. Seines: A seine is a simple panel of netting pulled by a bridle at each end (Figure 10600:3). In many smaller seines the bridle is attached to pulling poles or "brails." The upper line of the seine is equipped with floats and the lower with weights. Some seines are fitted with a central bag of smaller mesh that traps the fish. Seines commonly range in size from 1 to about 100 m long and from 1 to 3 m deep. Mesh size depends on the target species. The seine is an effective device for sampling smaller fishes.

Seines may be worked over shorelines relatively free of obstructions. Pulling may be either parallel, angled, or perpendicular to shore. Two samplers, wearing waders, form the net into a gentle "U" while pulling. After a suitable distance the seine, with the lead line on the bottom, is pulled to and up on the shore. A series of shorter passes may be more productive than one long one.

Small seines may be used over cobbled stream beds by placing the net poles firmly in the bottom and then rolling the cobbles upcurrent of the net; this action dislodges hidden fishes to drift

into the net. Benthic species are especially prone to capture in this manner.

Large seines (beach) usually are set from a boat. Fix a long warp on shore and pay it out by a boat working offshore to a set distance. Swing the boat parallel to shore and pay the net out. Attach a second warp and return the boat to shore. Draw the seine to shore by hand or by draft engine using the two warps.

A block seine may be used to block the mouth of an embayment. This sampling is effective when water elevations change. A flume net is a refinement of this technique for use in marshes and tidal creeks. One method of deploying a flume net is to place parallel walls of netting perpendicular to the marsh creek axis from the creek bank into the high marsh.[14] The net is set at high tide with the cod end staked out into the creek to provide refuge for fishes leaving the marsh at ebb tide. Samples are removed from the cod end when the marsh drains.

A self-contained block net is the closing seine consisting of a circular panel of netting strung on a weighted bottom ring and a floating upper ring. Drop the device onto the bottom enclosing the fishes and remove them with a dip net. The technique is quantitative to a degree but emplacement of the gear can cause fishes to leave the study area.

The commercial purse seine is a larger version of the closing net and is used in deeper water for the capture of schooling fish. The lower edge is equipped with a pursing warp or line that allows the net to be drawn together under the surrounded school. After closure the fishes are dip netted or hydraulically removed.

o. Lift and dip nets: These nets may be operated by a single worker. The lift net consists of a square panel of net held open by a pair of diagonal braces and is lifted at the crossing point by a cord. Such nets usually are baited while resting on the bottom and lifted when a sufficient number of fishes have been gathered.

Dip or pole nets are conical nets attached to a ring frame which in turn is attached to a pole. They are effective in the capture of salmonids, smelt, and various clupeids (such as blueback herring and alewife).

p. Goin dredge: The Goin dredge is a wooden box with a net bottom and one wall missing. Handles or hand holes are placed on the three remaining sides and are used to work the device through vegetation.

q. Electrofishing: Electrical devices also may be used for collecting.[15–17] They are particularly useful in areas where uneven bottoms, fast-flowing water, or obstructions make other collecting techniques difficult or impossible. Several factors affect the selectivity of electrofishing operations, including water depth and velocity, stream width, conductivity of the water, fish size and morphology, and fish behavior. An electrical field in the water is produced by passing a current between two submersed electrodes or between one electrode and the ground. Depending upon design, electrical devices produce either alternating current (AC) or direct current (DC). AC stuns fishes in its field, allowing them to be dipped from the water, whereas DC induces galvanotaxis so that the fishes move toward one of the poles, from where they are recovered. DC devices are particularly effective in turbid water or in waters with numerous obstructions or heavy vegetation. AC devices are more likely to kill fish. Effectiveness of electrofishing is affected by such environmental factors as water hardness and availability of electrolytes. Addition of salt to raise conductivity of very soft water may be necessary.

Electrofishing gear ranges from large, gasoline-motor rigs (Figure 10600:4) mounted in a boat to small, portable back-pack units powered by a gasoline motor or a battery. The electric seine consists of a conventional seine in which electrodes of an AC source run along the lead and float lines, thus greatly increasing collecting efficiency.

Sampling with electricity requires that the collector be aware of the special dangers associated with this technique. Specially train staff, provide and use protective equipment, and carefully monitor the sampling area before and during the operation.

In certain studies, e.g., when assessing stream fish production by habitat type, electrofishing can provide a quantitative sample. In streams of manageable size and flow, sampling areas can be isolated with block nets, allowing capture and processing of all fishes in the area. A second advantage is that fish mortality can be controlled carefully. Keep captured fishes in live cars (containers, on occasion with aeration and refrigeration capabilities) during sampling and return immediately after processing.

r. Ichthyocides: Ichthyocides (piscicides) are the least selective method of collecting but are the most efficient in terms of percentage of total population sampled.[18] Chemicals used may act as a metabolic poison, as a vasoconstrictor (suffocant), or as an anesthetic. Most have some serious fault in terms of their value as a fish-collecting tool, such as slow reaction time, lack of sufficient killing power except in high doses, deleterious side effects to either the environment or user, or expense. Rigorous time-consuming permitting procedures usually are required. In some places, use of ichthyocides is illegal; review ordinances. Among the chemicals or chemical preparations used in the past are cresol, sodium cyanide, sodium hypochlorite, antimycin A and B, quinaldine, tricaine methanosulfonate (MS-222), urethane, and rotenone; of these, only antimycin and rotenone now are approved for use by the U.S. Environmental Protection Agency.

Rotenone has been most widely used as a general collecting tool. It is the commercial name for a crystalline ketone ($C_{23}H_{22}O_6$) found in six genera of leguminous plants, particularly in the genus *Derris*. Rotenone functions as a vasoconstrictor and fishes affected by it literally suffocate. Rotenone is selective for gill-breathing organisms; fishes collected using rotenone can be eaten (but it is not recommended). It is rapidly detoxified by potassium permanganate, is adversely affected by light and high temperatures, and rapidly breaks down when subjected to such conditions.

Rotenone is available as powder, resin, crystals, or liquid emulsions. A 5% preparation is the usual strength employed in collecting. The powdered preparations are least stable and the resins and crystalline products the most stable. A ready-to-use liquid emulsion is most convenient, but it is relatively expensive. If weight and space problems exist, use crystals or resins. Mix resins and crystals with solvents immediately before use. For the resins, mix 100 g fragmented rotenone (broken up before mixing) with 1 L commercial-grade acetone and 100 mL emulsifier. For the crystals, mix 20 g with 3.8 L acetone. Crystals are the recommended form. The amount of rotenone to be used per collecting station varies according to volume and current of water, reaction time of the toxicant, water temperature, and other factors. A sample bioassay may be useful in determining dosage. In general, be conservative because good-quality rotenone is

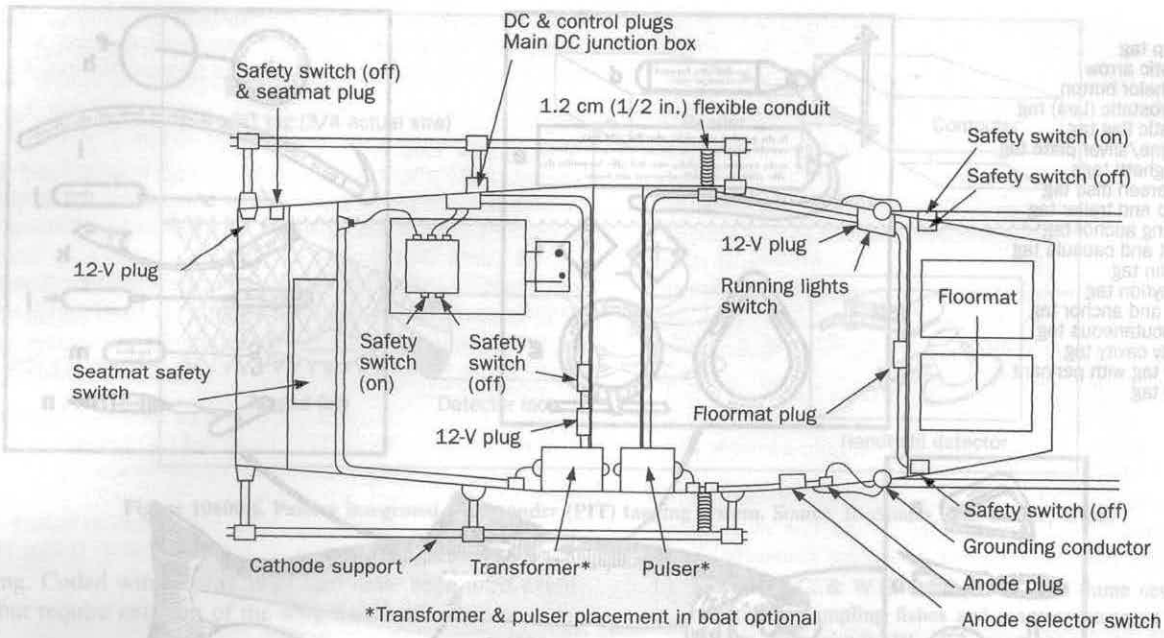

Figure 10600:4. Diagram of electrofishing boat. Source: NOVOTNY, D.W. & G.R. PRIEGEL. 1974. Electrofishing Boats, Improved Designs and Operational Guidelines to Increase the Effectiveness of Boom Shockers. Tech. Bull. No. 73, Dep. Natural Resources, Madison, Wis.

surprisingly effective. To use rotenone have properly trained personnel.

s. Concussion: Concussive methods such as explosive devices and substances have been used; however, most states and the federal government prohibit their use. A simple concussive method called "tunking" or "stoning" consists of striking an emergent boulder with another rock and then turning the boulder over for collection of the fishes underneath it. Sculpins and minnows may be captured in this manner.

t. Creel census: The systematic collection of data from sport anglers is a primary means of analysis. Field examination of fishes caught, coupled with a standard interview of the angler, is common. Allocation of fishing diaries among anglers followed by their systematic retrieval is another method. Statistically rigorous sampling methods and well-designed interview protocols are essential for the generation of reliable results.

u. Slurp gun: The slurp gun consists of a valved cylinder fitted with a plunger. It is used with SCUBA or while snorkeling and is especially effective for selective sampling of fish at nesting sites, or within otherwise inaccessible interstices.

v. Stomach and gut examination: The contents of the digestive tracts of fishes often include examples of additional fish species not otherwise sampled.

w. Serendipity: Useful specimens can be collected after various kinds of fishes mortalities. The release of toxic substances, lethal changes in water temperature, anoxic water, construction of cofferdams, dewatering of power-plant flumes, drying up of natural water bodies, and the stranding of fishes after floods exemplify events that can yield study materials.

x. Acoustic methods: Acoustic means of fish detection and quantification involve the generation of a series of sound pulses by an electroacoustic transducer mounted on a vessel, a towed body, or a fixed land feature.[19] Vertical and side or horizontal scanning are widely used. For side scanning, one or more transducers are mounted at some depth and oriented horizontally, as on the piers of a bridge crossing a river or on the walls or floor of a lock chamber. The sound impulse is reflected by suspended objects and the bottom. It is received by the same transducer, which reconverts the sound signal into an electrical signal that is amplified and displayed and/or recorded on a paper tape, VCR tape, liquid display, photographic film, etc. The usual paper tape provides a profile of the water showing the bottom and the objects suspended above it. Objects having high "acoustic impedance," such as fishes with air bladders, are especially vivid targets and produce strong signals. Gas bubbles, plankton, particulate matter, and density differences associated with a thermocline, floating leaves, and other objects may produce acoustic traces. In the more typical recording type of sounding apparatus, a single relatively stable fish will produce a chevron-shaped "echogram," with the point of the chevron pointing upwards. Several fish usually can be resolved into a corresponding number of such figures, but a school of fish may produce a cloud-like mark, complicating analysis. Under these circumstances estimation of biomass usually is undertaken.

Verify identity of species being recorded by capture or direct observation (e.g., SCUBA). Weighing captured specimens permits extrapolation to biomass estimation.

Apart from quantification, sonar methods provide high-quality data on the vertical and horizontal location of fish stocks.

y. Snorkeling and SCUBA: Snorkeling is the process of using a face plate to facilitate underwater vision and a "J"-shaped breathing tube, to allow sustained observation of underwater conditions. SCUBA consists of a compressed-air tank, a gas-flow regulator, and a hose and mouth piece. Ancillary but crucial gear includes a weight belt for adjusting buoyancy, fins, an inflatable life vest, and either a wetsuit or a dry suit.

Depths less than 15 m are accessible to those with good training. Greater depths require considerable training and experience.

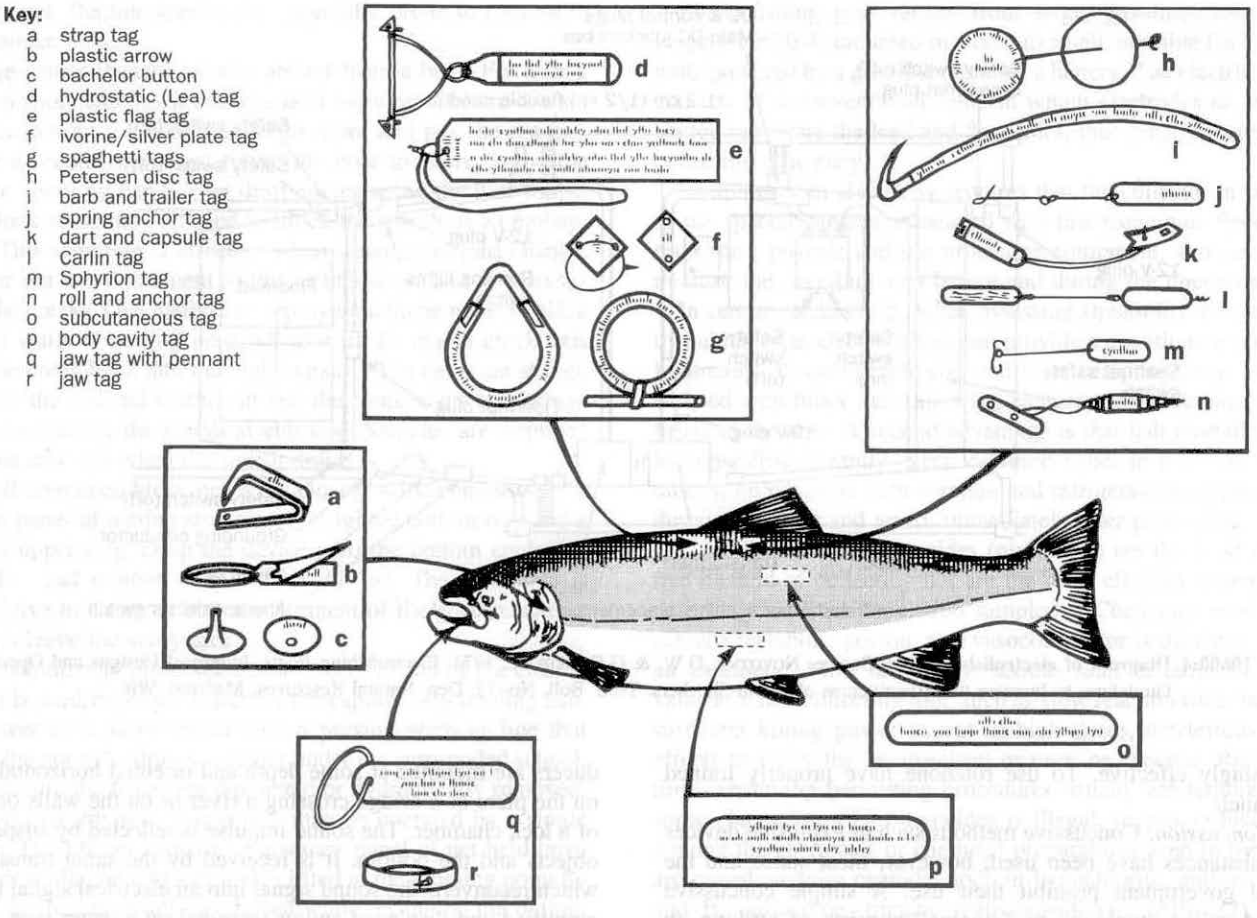

Figure 10600:5. Types of tags commonly used. Source: STOTT, B. 1971. Marking and tagging. *In* T. Bagenal, ed. 1978. Methods for Assessment of Fish Production in Fresh Waters. IBP Handbook No. 3. Blackwell Scientific Publ., Oxford, England.

Underwater study permits precise gear placement, direct observation of gear function, rapid definition of resource use by the species represented, assessment of impact of chemical spills in deeper water (when dead and moribund specimens may be resting on the bottom), the study of behavior, and estimation of population size. A team of divers can swim side-by-side along a defined course noting fishes present and making simultaneous records of observation using underwater recording materials. An underwater pad consisting of sanded vinyl plastic can be marked with the usual graphite pencil and erased with a conventional rubber eraser. Aluminum foil taped onto a plastic sheet, again marked with a pencil, is effective and has the added advantage of providing a permanent record.

4. Tags and Tagging

The marking of a fish or group of fish followed by their release and recapture provides information on movement, rates of growth, and population characteristics. The methods include: dyes and stains (including fluorescent materials), finclips, attachment of tags (Figure 10600:5), emplacement of encoded wires, sonic (transmitter) tags, the "PIT" tag (see below), branding, radioisotopes, and marker chemicals that can be detected easily or influence ageable features such as the scales, otoliths, or spines. Chemical marking may be restricted under certain circumstances. Beware the influence of the mark on the fish: for example, jaw tags can suppress growth rate through interference with feeding; brightly colored tags may increase the role of predation; large tags may impair swimming or cause entanglement with plants or other objects; infection may be induced. Marking is an important tool because it permits identification of genetic strains of hatchery stocks, observation of the behavior of individual fish submitted to particular chemicals or other stressors, observation of dominance and social rank, etc.

The passive integrated transponder (PIT) is an innovation with considerable value.[20] Small (10-mm × 2.1-mm) glass-imbedded electrical units called "PIT tags" may be injected into the abdominal cavity of a fish by using a syringe-like device (see Figure 10600:6). Fishes 5 cm and larger may be marked in this fashion. The tag is activated by a hand-held excitatory unit that elicits a distinctive signal permitting recognition of the individual fish and immediate data processing by computer. The specimen may be processed with little or no handling by using a water vessel or a flow-through cylinder (as is done with migratory salmon moving through a weir) during the tag excitation. The tags last for several years. Salmon smolt can be tagged and individually recognized years later as the adult fish return for

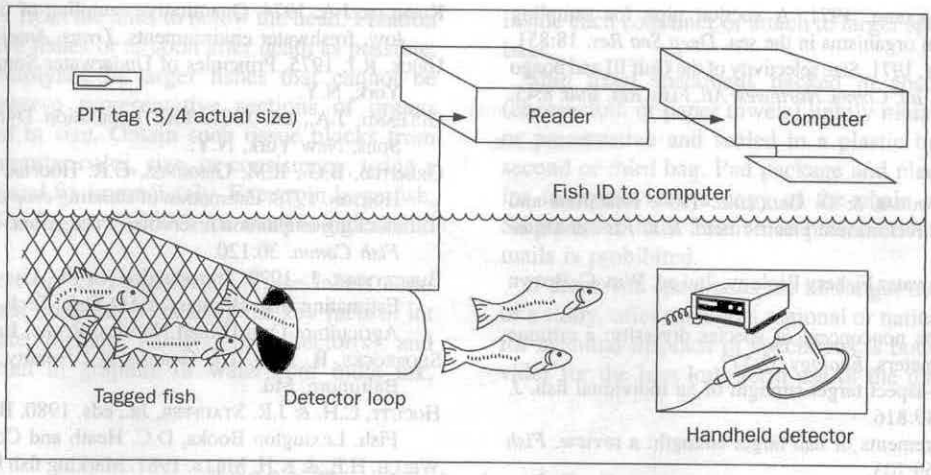

Figure 10600:6. Passive integrated transponder (PIT) tagging system. Source: BioSonics, Inc., Seattle, Wash.

spawning. Coded wire tags (CWT) also have been used extensively but require excision of the wire for identification.

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10600 C. Sample Preservation

The decision to preserve specimens depends on study objectives. Preserved material may be necessary to confirm identity of a species, to evaluate certain demographic characteristics of the population, or to estimate incidence of parasitic infection or disease. It also may be essential evidence in legal proceedings.

Do not preserve specimens unless there is clear need for them. Fixatives are toxic, dangerous if used improperly, and covered by regulations on hazardous substances. Preserved specimens require expensive and time-consuming curation.

Fix specimens in 10% formalin (a 9:1 ambient water dilution of 100% formalin). Formaldehyde gas reaches saturation in water at about 37% by weight and by convention this saturated solution is called 100% formalin. Fix fishes less than 10 cm in total length without opening the visceral cavity. Larger specimens require injection of preservative into the visceral cavity or slitting of right ventral body wall for about 25% of body length. Specimens larger than 25 cm total length (and especially oily species) usually require injection of concentrated formalin into the dorsal muscle mass.

The placement of fish in the sample container, i.e., head up or down, depends on intended use. A good ratio of specimen mass to preservative is 1:1 or with the level of preservative submerging the specimen by at least an inch.

Fix ichthyoplankton in formalin. To facilitate sorting, 1 g rose bengal stain/L fixative may be added to stain living tissue. If the sample contains a large amount of biomass (detritus, non-target organisms) split the sample into two or more sample jars rather than increase the concentration of formalin.

Ideally use wide-mouthed containers with a durable, screw-type plastic cover. If using metal caps, add about 1 g sodium borate/L preserved material.

Formaldehyde is highly allergenic and a listed carcinogen; minimize direct contact with skin and avoid breathing fumes. Formaldehyde is best transported in tightly sealed plastic containers.

After several days to two or more weeks in the fixative (depending on the size of the fish), transfer specimens to 70% ethyl or isopropyl alcohol for long-term preservation. Preserve ichthyoplankton samples in a 3 to 5% solution of formalin because alcohol tends to shrink and distort the specimens. If possible, reuse formalin.

It may be harmful and/or illegal to release even small amounts of 10% formalin into wastewater collection systems. Examine ordinances before disposal of fixatives and preservatives.

Isopropyl alcohol is a less expensive and less flammable substitute for ethyl alcohol, but weigh these advantages against the fact that isopropyl is not a good fixative and may damage the specimens for histology. Both alcohols are highly flammable when stored in bulk. Quantities stored in any building may be limited by codes. If otoliths will be used for fish age determination, do not fix specimens in formalin until after the otoliths are removed. If the otoliths cannot be removed before long-term preservation, fix the specimens in alcohol, which is adequate for small fish, or freeze them.

For pathology fix whole fishes or organs for at least 24 h in 10 times their volume of neutral buffered formalin before further processing:

37% Formaldehyde (100% formalin)	100	mL
Distilled water	900	mL
Sodium phosphate monobasic, $NaH_2PO_4 \cdot H_2O$	4	g
Sodium phosphate dibasic, Na_2HPO_4	6.5	g

Fix fishes less than 8 cm long whole. For larger specimens fix viscera either by injecting the fixative into the body cavity or by

cutting the body cavity from the anus to below the head. Fixation is most effective on live fishes or as soon after death as possible. A delay results in autolysis. In larger fishes that cannot be preserved whole, preserve representative sections of organs about 5 × 5 × 2 mm in size. Obtain such tissue blocks from organ areas having irregular color, size, or consistency, using a scalpel or razor blade and fix immediately. Except in large fish, fix the entire brain. See Figure 10600:7 for morphological and anatomical guidance.

Rigorously document preserved materials. Place labels composed of highly resistant bond paper, bearing the key facts of lot number, collection date, locality, name(s) of collector(s), and other particulars written in graphite or waterproof india ink, inside each container or attach to larger specimens held in plastic bags.

Ship fixed specimens packed in absorbent paper or cloth (cheesecloth or paper towels) lightly moistened with the fixative or preservative and sealed in a plastic bag in turn sealed in a second or third bag. Pad package and place in a box or canister for transport with a copy of the chain-of-custody documents. Shipment in bulk fixative or preservative via the United States mails is prohibited.

If preserved specimens are no longer needed after completion of a study, offer them to a regional or national museum. Planning for eventual disposal of specimens is both economical and provides for the best long-term use of the materials collected.

10600 D. Analysis of Collections

1. Identification

a. General remarks: Identification of fishes is based on diagnostic characters such as body form, color and size, shape and position of fins, meristic features such as the number of rays in a fin or the number of scales in a specific series, the presence of distinctive organs such as barbels, or the lateral line and various proportions such as the ratio of the length of the head to the total length of the body (see Figure 10600:7). Diagnostic features may vary with age, sex, reproductive condition, social status, time of year, and habitat of the fish. Diagnostic keys and other descriptive materials are available for all regions of North America and a list of selected taxonomic works appears in the bibliography.

Common names of North American fishes are listed in a special publication of The American Fisheries Society.[1]

Identification may be performed on both fresh and preserved specimens. Fresh materials are essential for color although some preserved specimens retain color if they have been preserved in a color preservative, such as BHT. Fixed specimens are suitable for determining meristic or mensural characteristics. Use a dissecting binocular microscope with illumination and dissecting tools to examine specimens less than 10 cm long.

b. Ichthyoplankton: The identification of fish eggs and larval fishes is a special discipline and selected works are cited in the bibliography. Enumeration often involves knowledge of adult populations such as sexual maturity, spawning migration, spawning groups, and presence/absence of the species in the watershed. Intensive studies may involve captive spawning, rearing, and documentation of larval development of selected species. Obtain specialized assistance for identification of ichthyoplankton and the more difficult taxa such as the Cyprinidae (minnows), Clupeidae (herrings), Catostomidae (suckers), Poeciliidae (livebearers), Cyprinodontidae (killifishes), Percidae (perches), and others.

c. Rare and endangered forms: Pay special attention to rare and endangered forms that are protected by law. If a rare or endangered form is present a special permit or memorandum of understanding may be required. Do not intentionally collect rare or endangered forms; if they are accidentally taken and fixed contact the responsible agency and transfer specimens to a designated museum.

2. Diet

The contents of the digestive tract provide information on the amount and kind of foods eaten. Stomach contents may be extracted from some living fishes by inserting a smooth and moistened glass or metal cylinder down the esophagus into the stomach. Most analyses, however, involve the sacrifice of the fish. After capture, quickly preserve either the entire fish or the viscera.

To characterize diet, use a dissecting binocular microscope with the organisms suspended in water in a shallow transparent container. Define the contents to the lowest practical taxon and express as frequency of occurrence. Each dietary item by weight, volume, or caloric content may be enumerated in special studies.

3. Structure of Populations and Assemblages

a. General remarks: The actual properties of a population such as number, average size, and weight are estimated statistics.[2] For ecological and management purposes evaluate the numbers of individuals and the biomass (total weight) along with the factors that regulate these. Four key variables define number: natality (i.e., number of individuals being added through reproduction) mortality, immigration, and emigration.

Biomass is the net result of dietary income and its conversion efficiency, respiration, defecation, and loss to other compartments of the ecosystem such as predators, parasites, and pathogens.

The ideal static characterization of a population consists of graphs that show by sex:

Age frequency and maturation
Length frequency
Weight frequency
Age versus length
Age versus weight
Length versus weight

b. Population size: One of the simplest and most practical population estimation methods is the Petersen ratio based on marked fish. Collect a sample of fish, mark, and release. Collect a second or recapture sample including both marked and unmarked fish. The method assumes random distribution and no immigration or emigration. The larger the number of marked

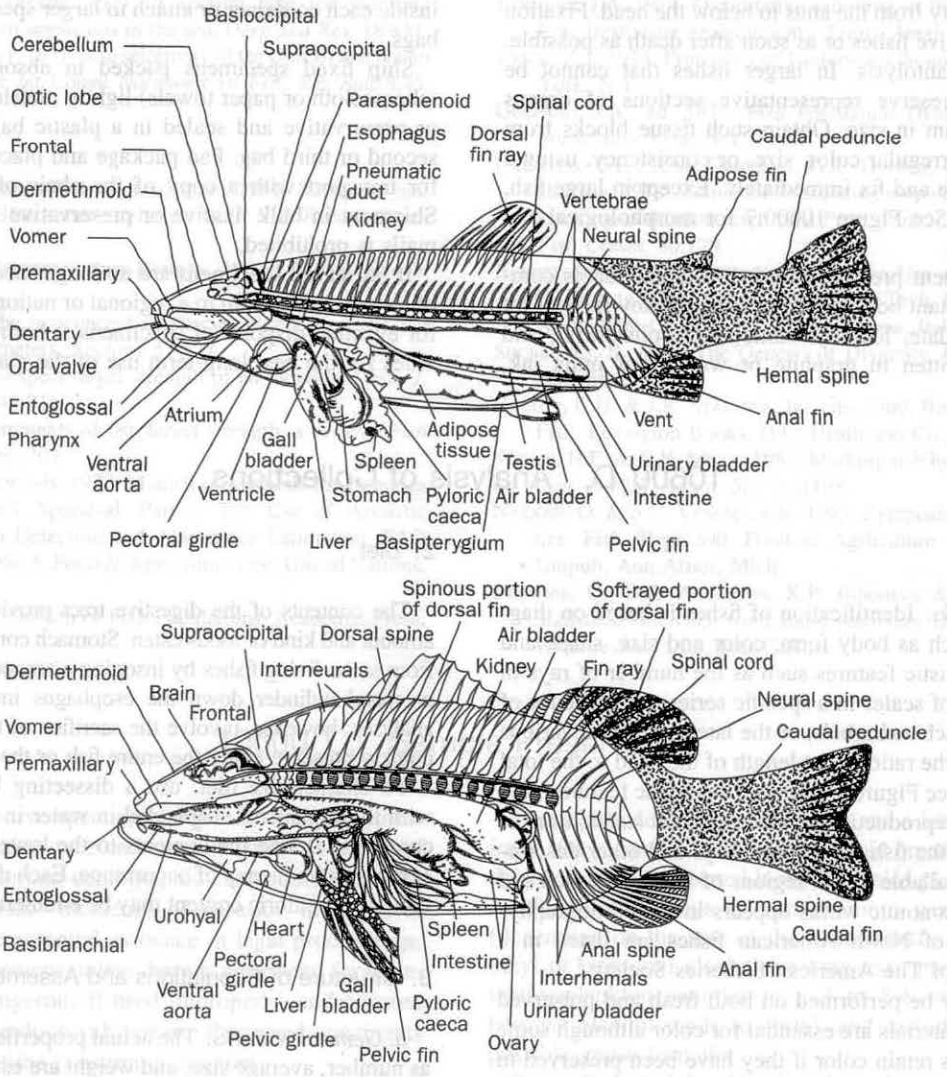

Figure 10600:7. Key organs and external body parts of a soft-rayed (upper) and spiny-rayed (lower) fish. Source: LAGLER, K.F. 1956. Freshwater Fishery Biology, 2nd ed. Wm. C. Brown Co., Dubuque, Iowa.

fish, the more reliable the estimate. It is particularly important to mark large numbers of the smaller species, if possible, because individuals of such species are less easily recovered. Estimate population size using the mark-recapture method by the formula:

$$\hat{N} = MC/R$$

where:

$\hat{N}$ = estimate of population size,
M = number of fish marked and released,
C = recapture sample size, including both marked and unmarked fish, and
R = number of marked fish recaptured.

The estimate is of the population present at the time of the first (marking and release) sample, not at the time of recapture.

c. Age and growth: Age and growth rates are useful for determining environmental effects on fish populations. The three methods used for determining age of fishes are comparison of length-frequency distribution, recovery of marked fishes of known age, and interpretation of layers laid down on hard parts of the fish. Determination of age distribution by length-frequency studies often is adequate for the first 2 to 4 years, but usually fails to separate older age groups reliably because of increasing overlap in length distribution. It also becomes less reliable as one approaches the equator because breeding seasons are more protracted in warmer areas, causing yearly age groups to be less well defined. In cold-water assemblages, growth rates often are retarded and age groups overlap, even in young fish. Another frequently used method of determining age is interpreting and counting growth zones or growth checks that appear in the hard parts of fish. Those considered to be formed once a year are called year marks, annual marks, annual rings, or annuli (Figure 10600:8). They are formed during alternate periods of faster and slower growth (or no growth at all) and reflect various environmental or internal influences. The assumption that a mark

represents an annulus requires validation.[3,4] In a temperate region, the period of little or no growth usually occurs only once annually, beginning in winter and extending into spring or early summer. Generally, the more the seasons differ with respect to temperature, the sharper the annual marks will be. The most distinct annual rings are developed in temperate climates of the northern and southern hemispheres. Scales and several bony structures also have been of value in the study of seasonal growth. Otoliths provide the most reliable record of age and growth, but require that the fish be sacrificed. Still another method of age and growth determination is by marking or tagging fish. Most tagging methods are not applicable to small fish, or if they are, they may cause mortality, so that recaptures are few. Instead of using tags, small fishes may be marked by fin clipping or fin-ray scarring or by injecting dye.

Scales are the structures most often used in age determinations of fishes because they are easily removed without serious injury to the fish. For scaleless fish, removal of other structures (otoliths, vertebrae, fin spines) may be necessary. Take scales usually from the upper mid-side of the body where they are large and symmetrical. Wherever scales are taken, remove them from the same part of the body in all individuals to be compared. Several scales may be needed for analysis, because an annulus that appears doubtful on one scale may be clear on another. In addition, some scales may have been regenerated, i.e., replaced, and may not show all annuli.

d. Index of condition: The coefficient or index of condition (also condition factor) is the length-weight relationship used to express relative plumpness or robustness of the fish. This, in turn, is related to environmental conditions. The equation usually used is:

$$K = \frac{(W \times 10^5)}{L^3}$$

where:

K = coefficient or index of condition,
W = weight, g, and
L = length, mm.

The gonadosomal coefficient (weight of gonads divided by the remaining weight of the body) and hepatosomal coefficient (weight of the liver divided by the remaining weight of the body) also are used as indicators of physiological condition.

e. Assemblage structure: Diversity indices are used to quantify the structure of the species assemblage in a particular habitat over time.[5] Usually, the number of species represented and the relative numbers of each species represented are incorporated into a single number. Margalef's index is illustrative.

$$d = \frac{(S - 1)}{\ln N}$$

where:

d = Margalef Index,
S = number of species represented in sample, and
N = total number of individuals in the sample.

The index of biotic integrity (IBI) is another method for assessing community structure. It produces a score for each sample site that allows assessment of environmental conditions at that site by comparing it to scores from other sites. It is a method for comparing changes in environmental conditions at a single site over time or at several sites within a carefully defined geographical area.

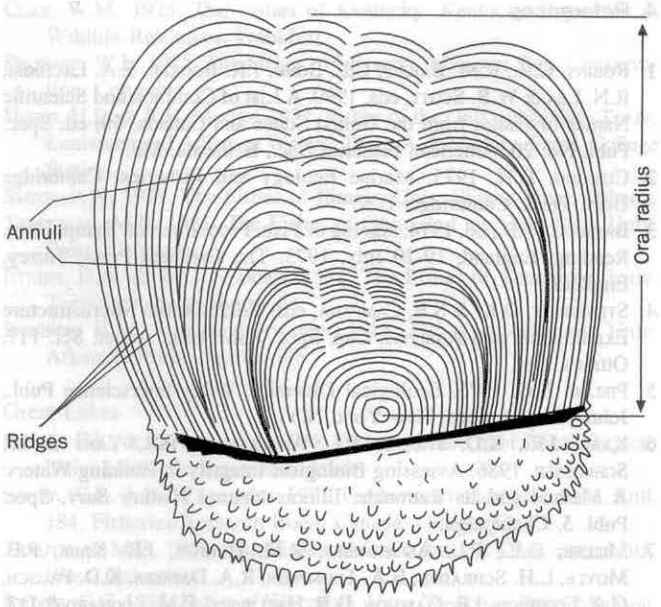

Figure 10600:8. Fish scale. Source: BAGENAL, T., ed. 1978. Methods for Assessment of Fish Production in Fresh Waters. IBP Handbook No. 3. Blackwell Scientific Publ., Oxford, England.

The IBI score is generated by using several carefully selected metrics that reflect different aspects of the aquatic system. Typically there are twelve metrics that fall into three broad categories. The first group assesses the composition of the fish assemblage. These metrics explore the relationship between the number of species and the number of individuals within each species or category. The second group examines the trophic composition of the assemblage. To score these metrics, some knowledge of the feeding ecology of the fishes is required. The third group looks at fish abundance and condition. These metrics deal with the number of individuals caught and their health.

Once the metrics are identified, develop the scoring criteria. Scores are based on the deviation from conditions at a relatively undisturbed site. Typically, there are three choices: a high score (5) is assigned if the condition is equal to that found at a similar but undisturbed site (key), a middle score (3) if the condition is worse than that found at the key site, and a low score (1) if conditions are much worse than those at the key site.

The total IBI score is the sum of the metric scores. In the example above, the key site would have an IBI score of 60 (12 metrics, maximum metric score of 5). The scores from all the sampled sites would be compared to this maximum. Sites where environmental conditions are relatively undisturbed would score near the maximum whereas degraded sites would score near the minimum of 12. The greatest advantage to the IBI is that comparisons are easy to make and the results are obvious.

The IBI metrics and scoring criteria are not universal and metrics and scoring protocols have been or must be developed for any geographic region.[6,7]

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10600 E. Investigation of Fish Kills

Fish kills vary, from the individual fish that dies of old age to the catastrophic kill, from partial to complete, and from natural occurrence to the results of human activity. No single investigative procedure can be appropriate for all situations. The following brief description may serve as an aid in investigating kills. Getting to the scene promptly before the evidence has decomposed or drifted away is vital. If surveillance of a particular body of water or area is involved, have available preset plans and equipment on a standby basis.[1–4]

1. Causes of Fish Kills

Fish kills may be caused by such natural events as acute temperature change, storms, ice and snow cover, decomposition of natural materials, salinity change, spawning mortalities, parasites, and bacterial and viral epizootics. Human-caused fish kills may be attributed to municipal or industrial wastes, agricultural activities, and water control activities.

2. Classification of Kills

One dead fish in a stream may be called a fish kill; however, in a practical sense, adopt some minimal range in number of dead fish observed, plus additional qualifications, in reporting and classifying fish kills. Any fish kill is significant if it affects fishes of sport or commercial value, results from a suspected negligent discharge or malfunctioning waste treatment facility, or causes widespread environmental damage. The following definitions, based on a stream about 60 m wide and 2 m deep, are suggested as guidelines. For other size streams, make proportional adjustments.

MINOR KILL: 1 to 100 dead or dying fishes confined to a small area or stream stretch. If recurrent, it could be significant; investigate.

MODERATE KILL: 100 to 1000 dead or dying fishes of various species in 1 to 2 km of stream or equivalent area of a lake or estuary.

MAJOR KILL: 1000 or more dead or dying fishes of many species in a reach of stream up to 16 km or greater, or equivalent area of a lake or estuary.

3. Investigation Techniques

In preparing for a field investigation, study area maps and determine the zone of fish mortality and the access to it. Identify waste dischargers. Contact participating laboratories to discuss number and size of samples that will be submitted, types of analyses required, dates of sample receipt, method of sample shipment, date by which results are needed, and to whom results are to be reported. Use two information record forms for fish kill investigations, an initial contact form and a field investigation form.

On all fish kill investigations take a thermometer, dissolved oxygen test kit, conductivity and pH meters; or a general chemical kit, biological sampling gear, sample bottles, fixatives, and other specimen containers. Take a camera to provide a photographic record of the event. Include in the investigating team at least one person who is experienced in investigating fish kills.

The field investigation consists of visual observations, sampling of fish, water, and other biota, and physical measurements of the environment. The first local observer of the kill is a useful guide to the area, which should be reconnoitered initially to establish that a fish kill actually has occurred.

If a fish kill has taken place, immediately start fish sampling because collection of dying or recently dead fishes is critical. Moribund fish usually are preferred. For purposes of comparison, if possible, collect healthy fishes from an unaffected area.

Examine moribund or recently dead animals immediately for external and internal abnormalities. Record all changes in color, size, location and consistency of organs. Record the location of lesions by organ (Figure 10600:7). Colored photographs, with indication of scale, taken at the site are an excellent way of documenting observations and greatly help the consulting pathologist. If photography is not possible describe exactly what was seen, recording the number and size of abnormalities. Provide healthy fish of the same species when possible.

Do not freeze samples for pathology. If fixatives are unavailable place samples in plastic bags on ice and rush to the pathologist.

If virology or bacteriology testing is indicated freeze additional specimens of key organs such as the liver, kidney, spleen, heart, and brain and other parts showing abnormality or lesions, label, and forward for analysis.

Bleed dying fish at collection time to obtain at least 1 g blood. Collect a blood sample in a chemically clean, solvent-washed glass bottle with a TFE-lined screw cap.

Identify and count dead fish. In a large river count dead fishes from a fixed station such as a bridge during a fixed period of time. Extrapolate to the total time involved. Alternatively, in a large river or lake, make a shore count and project to entire area of kill. In smaller water bodies traverse entire area to enumerate dead fish.

Collect water samples representative of unpolluted and polluted areas in accordance with the instruction given in Section 10200. As a minimum, measure temperature, pH, dissolved oxygen, and conductivity. Make additional tests depending on suspected causes of the fish kill. Take samples for examination of plankton, periphyton, macrophytes, and macroinvertebrates.

Record observations on water appearance, streamflow, and weather conditions. Color photographs are valuable in recording conditions.

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10700 BENTHIC MEIOFAUNA

At least 20 of the currently recognized 34 metazoan phyla are represented in the benthic meiofauna: Porifera, Placozoa, Cnidaria, Ctenophora, Platyhelminthes, Orthonectida, Rhombozoa, Cycliophora, Acanthocephala, Nemertea, Nematomorpha, Gnathostomulida, Kinorhyncha, Loricifera, Nematoda, Rotifera, Gastrotricha, Entoprocta, Priapulida, Pogonophora, Echiura, Sipuncula, Annelida, Arthropoda, Tardigrada, Onychophora,

Joint Task Group: Byron J. Adams (chair).

Mollusca, Phoronida, Bryozoa, Brachiopoda, Echinodermata, Chaetognatha, Hemichordata, and Chordata. Most are marine, but 14 of these phyla have members found in freshwater systems: Porifera, Cnidaria, Platyhelminthes, Nemertea, Nematoda, Rotifera, Gastrotricha, Entoprocta, Annelida, Arthropoda (Copepoda, Halacaroidea, Ostracoda, Mystacocarida, Tantulocardia), Tardigrada, Mollusca, Bryozoa, Chordata. The contribution of these metazoans to nutrient cycling and other ecological processes is probably substantial, but the details of such interactions are still poorly understood. Benthic meiofauna have an extreme range of morphological and life history diversity and have free-living, parasitic, and/or symbiotic trophic habits. Comprehensive information about these taxa is available.[1,2]

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10750 NEMATOLOGICAL EXAMINATION*

10750 A. Introduction

1. Occurrence and Impact

Nematodes are aquatic animals present in all parts of the world in fresh, brackish, and salt waters and in soil. A freshwater nematode has been defined as "any nematode species inhabiting either fresh water or non-brackish swampy soil below the water table; hence a species that will not drown in fresh water; a species fitted to utilize oxygen dissolved in fresh water."[1] Nematodes are a food source for other invertebrates, small fish, and fungi, and play a fundamental role in cycling carbon and nitrogen through the benthic ecosystem. This role is filled primarily by bactivorous nematodes. Predaceous nematodes from families Aporcelaimidae, Diplogasteridae, Dorylaimidae, and Mononchidae abound in fresh water, devouring other nematodes, oligochaetes, and other small invertebrates. Their role in ingestion of algae and diatoms is less clear; however, dorylaimids occasionally are seen with bright amber, yellow, or green coloration as a consequence of algae in the gut.

Bactivorous freshwater nematodes can ingest human enteric pathogens, and these bacteria and enteric viruses can survive chlorination inside nematode bodies. These nematodes often appear in large numbers in secondary wastewater effluents and have been used as bioindicators of water quality.[2]

2. Nematode Characterization

Aquatic nematodes are well adapted to their habitat. Their long, slender bodies (see Figure 10750:1) are encased by a strong, protective, usually smooth cuticle (skin) inflated by a high-turgor pressure system. Some aquatic species have a long, filamentous, nonmuscular tail. Whipping of the tail, together with the undulating sinuous body movement, propels the nematode through the water at a very rapid rate. Many aquatic nematodes possess glands in the tail that produce a sticky secretion controlled by a spinneret at the tail tip. These secretions temporarily fasten the nematode to a substrate so that anchored nematodes can function without interference by water currents. Oxygen requirements of most freshwater nematodes are low and the metabolism of some species may be nearly anaerobic.

The body is pierced by six to eight distinct openings and few to many minute apertures. First is the oral aperture at the apex of the anterior end, followed by two amphids on the head or neck region, the excretory pore (usually near the esophagus base), the vulva and anus in females, and the cloaca in males, and, on some nematodes, two small pores on the tail called phasmids. Cuticular ornamentation such as engravings, pores, spines, alae, or inflations also may be present.

Internally a stoma may be present, armed with teeth, or unarmed, uncollapsed or collapsed, or sometimes modified to form a hollow spear. An esophagus follows the stoma and terminates in esophageal glands. The intestine extends from the base of the esophageal glands to the rectum, which leads to the anus in females or cloaca in males.

The female gonad is single or paired and consists of an ovary, uterus, and vagina. It exits at the vulva. The male gonad consists of one or two testes, vas efferans, and vas deferens, and exits in the cloaca. Males possess spicules, which are the male copulatory organ, and their guide, the gubernaculum.

The nervous system comprises a nerve ring encircling the esophagus and connected ganglia and nerve cells. Appropriate muscle cell groups are present.

Although nematodes do not possess respiratory and circulatory organs, they tolerate large variations in the levels of salts and other environmental chemicals. Aerobic metabolism is dependent on the diffusion of oxygen into their tissues. Lacking a circulatory system, nematodes rely on diffusion through the tissues for translocation of nutrients, respiratory gases, and waste products.

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* Approved by Standard Methods Committee, 1997. Formerly numbered 10550. Joint Task Group: Byron J. Adams (chair).

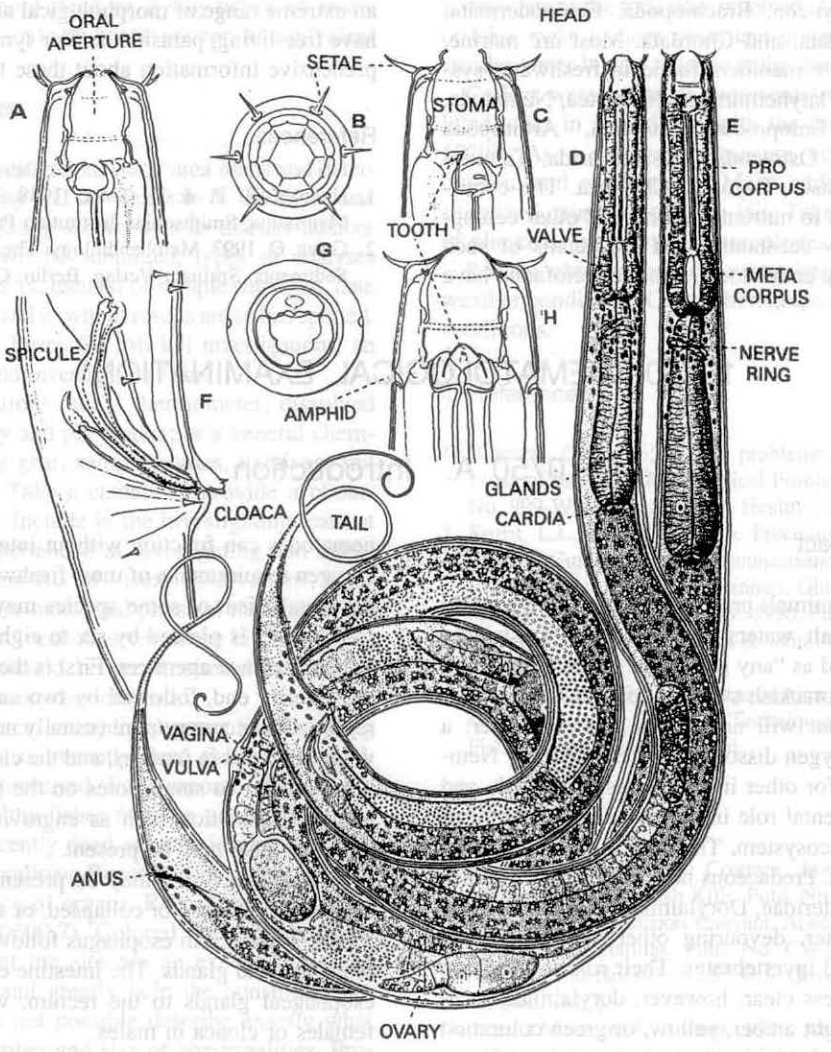

Figure 10750:1. ***Butlerius*** **sp., a freshwater nematode.** A. head; B. *en face* view of head showing six setae and a central oral aperture; C. head lateral view showing stoma and tooth; D. male; E. female; F. male tail portion showing spicules, gubernaculum, and cloaca; G. head section showing aphids; H. ventral view of head showing aphids and tooth. Russell, C.C., Department of Plant Pathology, Oklahoma State Univ., Stillwater.

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10750 B. Collection and Processing Techniques for Nematodes

1. Samples

Principal samples are: tap or well water, free-flowing or standing water without bottom sediment, bottom sediment, and aquatic plants and coarse detritus such as stones, twigs, or leaves.

a. Sample collection:

1) Tap water—Place a 20-cm, 325 mesh (45-μm pore size) sieve at a 45° angle under the discharge. Adjust water flow to a moderately slow rate, with no splashing, striking upper one-third of sieve. Run for 4 h.

2) Free-flowing or standing water—Take samples from sites where bottom sediments are absent or too deep to be collected. Collect five subsamples as follows: Hold 20-cm, 325 mesh sieve firmly at a 45° angle. Dip 3- to 4-L stainless steel pitcher in water and fill to 1-L mark. Pour contents slowly through top one-third of sieve. Repeat three more times. Collect additional 1 L to wash and concentrate detritus on sieve surface from top to bottom of sieve.

3) Bottom sediment sample—For bottom-to-surface depth less than 20 cm, stir bottom with hand garden rake. Scoop up stirred sediments in stainless steel pitcher. Add water to pitcher to within 5 cm of top. Stir, then wait 30 s. Pour contents of pitcher on to a 20 mesh (1-mm pore size) sieve nested on a 325 mesh sieve with the surface held at a 45° angle until dense detritus reaches pitcher lip. (Usually about 9/10 of the pitcher is poured off.)

For bottom-to-surface depth of 20 to 30 cm, collect duplicate samples by holding a 325 mesh sieve at a 90° angle near the bottom. Using hand rake, stir bottom sediments so that they roil up in a dense cloud in front of sieve. Let cloud settle about 10 s, then move sieve into cloud about 2.5 to 5 cm above the bottom. Bring sieve out of water while holding it at a 45° angle.

4) Aquatic plants, plant or inorganic debris—Randomly collect live floating or submerged plants of one species from target site and place in 1-L jars filled with collection site water. Do not fill more than half of jar with plant material. If several plant species are present, take two or more samples. Place plant and inorganic debris (sticks, leaves, pebbles, etc.) in 1-L jar to about half its volume.

b. Sample concentration: Concentrate detritus present on sieve face by washing tap water across sieve face from top to bottom. Place sieve on lip of a clean, empty 250-mL beaker, bring beaker forward until bottom side is up at a 45° angle. Wash detritus into beaker by flushing tap water from another beaker through the bottom one-third of the bottom side. Pour sample into jar.

c. Sample transport and storage: Regardless of collection mode keep sample jars cool. On very hot days use ice to cool them. Accurate taxonomic determination is most effective when nematodes are live and healthy. Because nematode mortality, deterioration, and obfuscation of diagnostic characters begins at time of collection, process samples for diagnosis within 24 h and complete diagnostic processing within 48 h.

Cold storage retards, but does not entirely halt, deterioration and rot. Plan survey so that samples can be processed on the same day they are taken. In an emergency, preserve entire sample indefinitely in 4% formalin (*never* use alcohol). Add equal volume of 8% formalin solution to sample. If sample jar is more than half full, decant excess water after a minimum 40-min settling period. Preserved specimens will shrink to some degree and body pores and lumens may be made obscure.

2. Sample Processing

a. Specialized apparatus:

1) *Custom pipet*, for clean-water samples—Take a 29-cm-long disposable pipet and place a piece of 12-cm-long rubber tube snugly over about 3 cm of the conical pickup end. Add a wire buret clamp on the rubber tube. (Clean by removing clamp and flushing with a syringe.)

2) *Baermann funnel*, for samples containing debris—Use a glass funnel with a 15.5-cm top opening and 1.5-cm tube. Fit a rubber tube to the exit tube and close with a buret wire clamp. Place an 8- to 10-cm-diam coarse screen wire disk (3-mm pores) in the funnel opening. Add tap water until it lies just above the wire disk. Insert a facial tissue over the disk.

b. Procedure:

1) Clear or relatively clear water—Shake to obtain homogenous mix, then pour slowly onto the surface of a 7.6-cm, 325 mesh sieve. Concentrate as indicated in ¶ 1*b*, above. Pour concentrated residues into 50-mL conical-bottom centrifuge tube or tubes. Let nematodes settle for 40 min. Insert a custom pipet, ¶ *a*1), above, with rubber tube closed by finger pressure, to tube bottom. Depress rubber tube to take up the ball of nematodes on bottom of cone. Discharge about 0.05 mL (small drop) of pipet contents on to a microscope slide. Cover drop with a 22-mm cover slip. Diagnose nematodes using a *compound* microscope.

2) Samples with much debris—Pour concentrated samples very slowly onto the facial tissue in a Baermann funnel. After 24 h flush funnel into a 250-mL beaker. Process as directed in ¶ 2*b*1), above.

3) Samples containing live plants, plant debris, or inorganic material—Process samples immediately on return to laboratory. Shake vigorously and pour contents into beaker. Concentrate samples as directed in ¶ 1*b*, above, and, depending on clarity of sample, proceed according to ¶ 2*b*1) or 2), above. If the laboratory is equipped to process samples with an excess of debris using the centrifugal flotation technique,[1] preferably use this technique.

3. Reference

1. Caveness, F.E. & H.J. Jensen. 1955. Modification of the centrifugal technique for the concentration of nematodes and their eggs from soil and plant tissue. *Proc. Helminth. Soc. Wash.* 22:87.

10750 C. Illustrated Key to Freshwater Nematodes

1. General Discussion

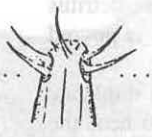
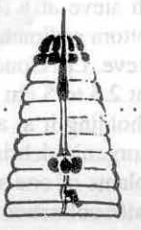
The following key was devised so that persons trained in biology, but not necessarily in nematology, could use it. The illustrations include original drawings, photocopies of published drawings, or photocopies on which figures were redrawn. The two most important references were Goodey[1] and Chitwood & Chitwood.[2] Other publications used as references and for illustrative material are listed in the bibliography.

Published literature indicates that several genera in this key contain species predominantly associated with terrestrial habitats. Presence of such nematodes suggests runoff from banks or higher ground in which various plant species (often food sources for these nematodes) are growing. These genera are indicated by an asterisk (*).

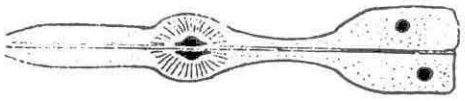
2. Key

		Refer to Couplet No.
1.	Cephalic setae indistinct or absent	2
	Cephalic setae absent but setae-like head appendages present	64
	Cephalic setae present	69
2.(1)	Stylet present	3
	Stylet absent	38
3.(2)	Base of stylet knobbed or flanged	4
	Stylet knobs or flanges absent	29

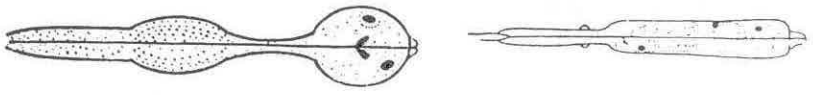


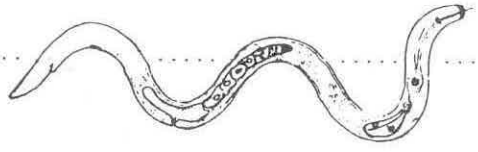



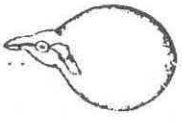

Refer to Couplet No.

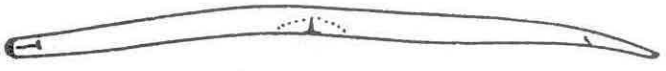
4.(3) Valvate median esophageal bulb present .....  5

Valvate median esophageal bulb absent 22

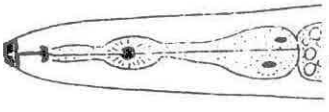


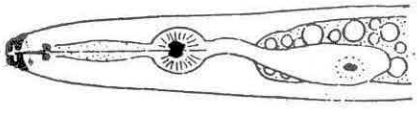
5.(4) Females eel-like .....  6

Females swollen .....  21

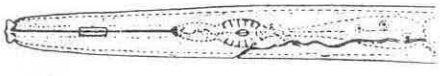
6.(5) Vulva at mid-body .....  ... 7

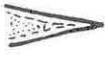
Vulva on lower third of body ....  14

7.(6) Esophagus not overlapping intestine .....  8

Esophagus overlapping intestine ....  11

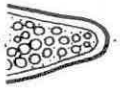
8.(7) Stylet length less than 50 μm 9

Stylet length
greater than 80 μm .....  ..... *Dolichodorus*

9.(8) Tail terminus pointed .....  ..... *Tetylenchus**

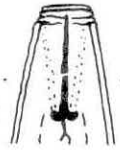
Tail terminus not pointed 10

10.(9) Tail terminus knobbed .....  ..... *Psilenchus**

Tail terminus never knobbed or
pointed .....  ..... *Tylenchorhynchus**

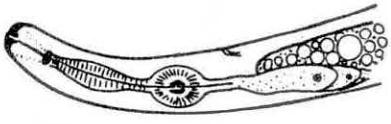
Refer to Couplet No.

11.(7) Labium offset .....  12

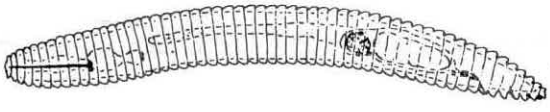
Labium flattened, amalgamated or nearly so .....  13

12.(11) Stylet massive, 40–50 μm long .....  ..... *Hoploaimus**

Stylet long and thin, longer than 90 μm .....  ..... *Belonolaimus**

13.(11) Body 0.5–1.0 mm long, tail tip not mucronate .....  ..... *Radopholus**

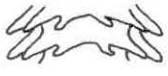
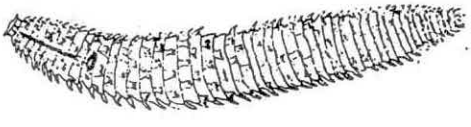
Body 2–3 mm long, tail tip usually mucronate ..... *Hirschmanniella*

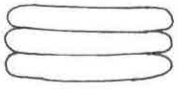
14.(6) Cuticle heavily annulated, stylet elongate .....  15

Cuticle not heavily annulated, stylet short .....  17

15.(14) Cuticular sheath absent .....  16

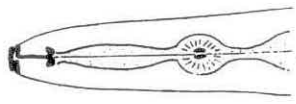
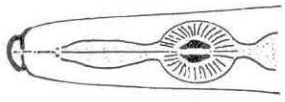
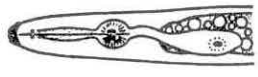
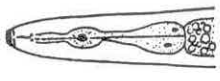
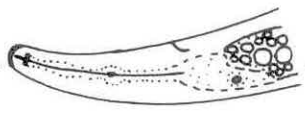
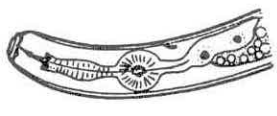
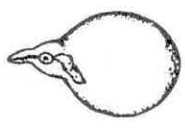
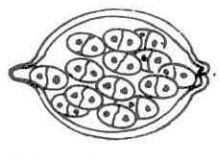
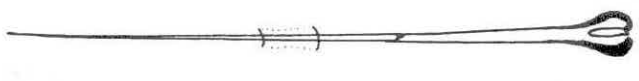
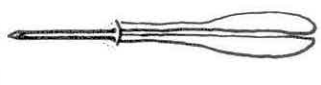
Cuticular sheath present .....  ..... *Hemicycliophora*

16.(15) Annules with cuticular spines or scales ..   .. *Criconema**

Annules plain without spines or scales .....   .... *Criconemoides**

17.(14) Body death position straight .....  ...18

Body death position spiral ....  ....  ..... *Helicotylenchus**

		Refer to Couplet No.
18.(17)	Median esophageal bulb distinct but not pronounced 	19
	Median esophageal bulb well developed 	*Aphelenchoides*
19.(18)	Esophagus overlapping intestine 	20
	Esophagus not overlapping intestine 	*Tetylenchus**
20.(19)	Median bulb and valves small, stylet usually weak 	*Ditylenchus**
	Median bulb, valves and stylet well developed, labium flattened 	*Pratylenchus**
21.(5)	Female body soft, white, with few or no internal eggs 	*Meloidogyne**
	Female body a rigid brown cyst usually with many internal eggs 	*Heterodera**
22.(4)	Stylet short, less than 100 μm 	23
	Stylet long, greater than 100 μm 	*Xiphinema**
23.(22)	Stylet complex 	24
	Stylet simple 	25
24.(23)	Stylet with anterior arch-like portion 	*Diphtherophora**
	Stylet with dorsal thickening piece 	*Tylencholaimellus**
25.(23)	Stylet knobs elongate, flange-like 	26
	Stylet knobs round 	27

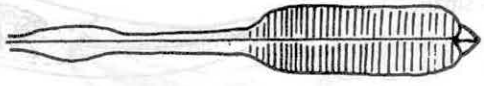
Refer to Couplet No.

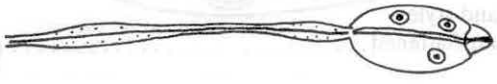
26.(25) Filiform tail .....  ..... *Aulolaimoides*

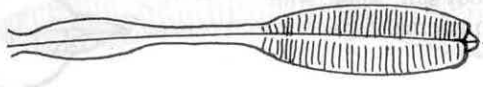
Round tail .....  ..... *Enchodelus*

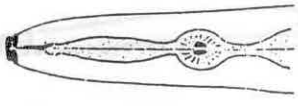
27.(25) Tail rounded .....  28

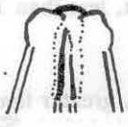
Tail pointed .....  ..... *Nothotylenchus*

28.(27) Esophagus base elongate .....  ..... *Tylencholoaimus**

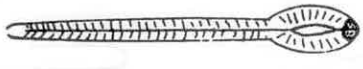
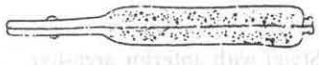
Esophagus base oval .....  ..... *Doryllium*

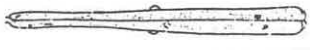
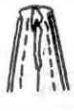
29.(3) Valvate median esophageal bulb absent .....  30

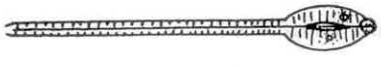
Valvate median esophageal bulb present .....  37

30.(29) Stomal walls not cuticularized .....  31

Stomal walls cuticularized (*Actinolaimus, Metactinolaimus, Paractinolaimus*) .....  ..... *Actinolaiminae*

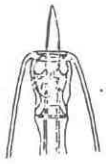
31.(30) Esophagus with basal expansions .....   32

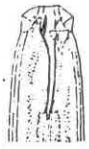
Esophagus expanding uniformly .....   ..... *Oionchus*

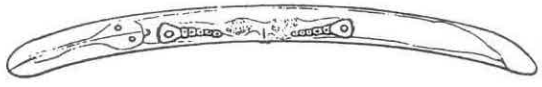
32.(31) Terminal fifth or sixth of esophagus an ovoid bulb .....  33

Posterior third of esophagus swollen .....  36

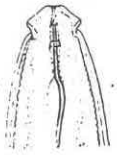
Refer to Couplet No.

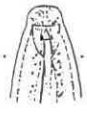
33.(32) Stylet axial, positioned centrally  34

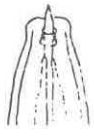
Stylet not axial, originating from tooth in stoma wall  ..... *Campydora**

34.(33) Gonads paired; vulva usually near mid-body  35

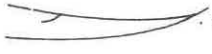
Gonad single, posterior to vulva; vulva anterior to mid-body ..... *Tyleptus**

35.(34) Stylet slender  ..... *Leptonchus**

Stylet not slender  ..... *Dorylaimoides**

36.(32) Stylet axial, positioned centrally (*Dorylaimus, Eudorylaimus, Labronema, Mesodorylaimus, Thornia, Laimydorus, Prodorylaimus*)  ..... Dorylaiminae

Stylet not axial, originating from tooth in stoma wall  ..... *Nygolaimus*

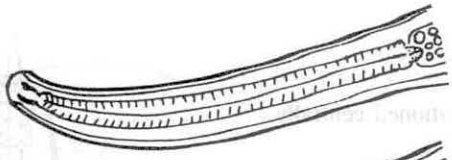
37.(29) Tail pointed  ..... *Seinura**

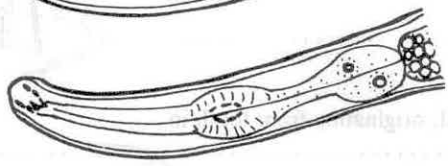
Tail rounded  ..... *Aphelenchus**

38.(2) Teeth present, prominent  39

Teeth absent, minute, or indistinct  50

Refer to Couplet No.

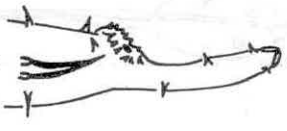
39.(38) Esophagus without mid-region expansion .....  40

Esophagus expanded at mid-region .....  49

40.(39) Tail pointed or tapering.....  41

Tail rounded .....  47

41.(40) Male tail without setae .....  42

Male tail with setae .....  .....*Oncholaimus*

42.(41) Stoma with denticles .....  43

Stoma without denticles.....  45

43.(42) Denticles scattered or in longitudinal rows .....  44

Denticles in transverse rows .....  .....*Mylonchulus*

44.(43) Denticles situated on longitudinal rib of stoma.....  .....*Prionchulus*

Denticles scattered on stoma wall .....  .....*Sporonchulus*

45.(42) Tooth anteriorly directed.....  46

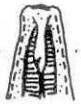
Tooth retrorse .....  .....*Anatonchus*

Refer to Couplet No.

46.(45) Tooth in basal part of stoma .....  ..... *Iotonchus*

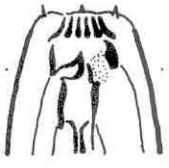
Tooth in anterior part of stoma .....  ..... *Mononchus*

47.(40) Stoma with prominent medial or apical tooth .....  48

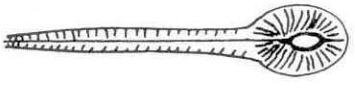
Stoma with small basal tooth .....  ..... *Bathyodontus*

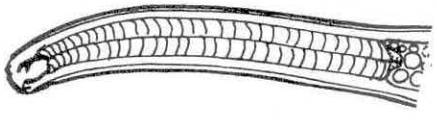
48.(47) Stoma with 3 teeth, without small basal tooth, caudal glands opening terminally .....  ..... *Enoplocheilus*

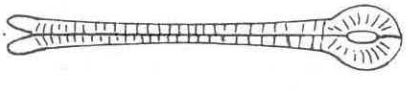
Stoma with large anterior and small basal tooth, caudal glands opening ventrally .....  ..... *Mononchulus*

49.(39) Lip region with rib-like armature .....  ..... *Mononchoides*

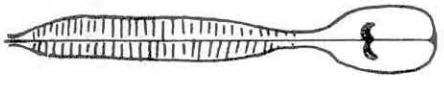
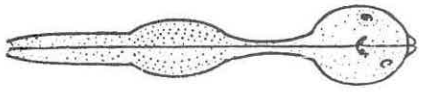
Lip region without rib-like armature .....  ..... *Diplogaster*

50.(38) Esophagus with basal expansions .....  51

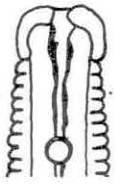
Esophagus uniformly cylindrical .....  60

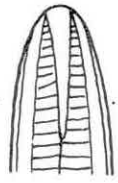
51.(50) Esophagus without mid-region expansion .....  52

Esophagus expanded at mid-region 55

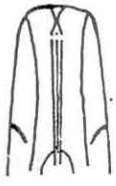
Refer to Couplet No.

52.(51) Amphids distinct  53

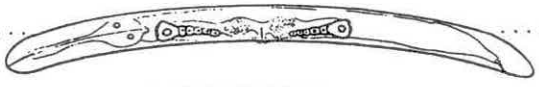
Amphids indistinct  54

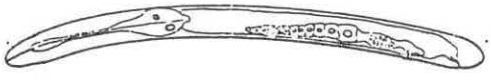
53.(52) Stoma walls anteriorly inflated with minute tooth  ..... *Microlaimus*

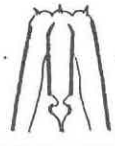
Stoma walls without tooth and with straight, tapering sides  ..... *Leptolaimus*

54.(52) Stoma with 3 rod-like thickenings  ..... *Rhabdolaimus*

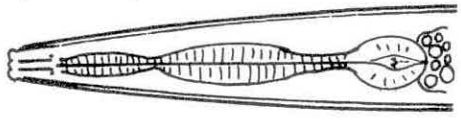
Stoma without rod-like thickenings  ..... *Monochromadora*

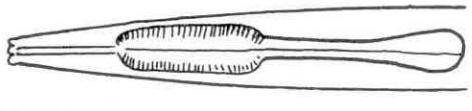
55.(51) Gonads paired  56

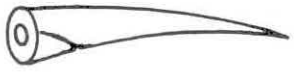
Gonads single  58

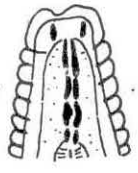
56.(55) Stomal walls straight, amalgamated  57

Stomal walls separated, not straight  ..... *Alloionema*

57.(56) Moderately swollen metacorpus, stoma not excessively elongate  ..... *Rhabditis*

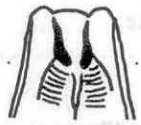
Elongate, cylindrical metacorpus, stoma elongate  ..... *Cylindrocorpus*

58.(55) Tail with sharp terminus  59

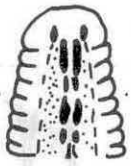
Tail bluntly conical   ..... *Cephalobus*

Refer to Couplet No.

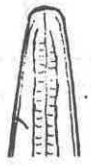
59.(58) Anterior part of stoma a broad, open chamber ..... *Panagrolaimus*



Stoma narrow, collapsed ..... *Eucephalobus*

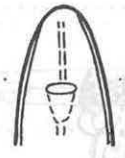


60.(50) Stoma absent or indistinct 61

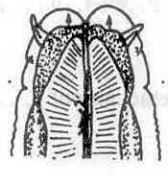


Stoma distinct 63

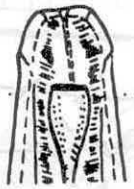
61.(60) Lip region narrow, tooth absent 62



Lip region broad, small denticle apparent in stomal area ..... *Tripyla*



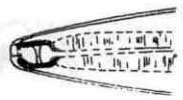
62.(61) Amphid aperture appearing as large slit ..... *Amphidelus*



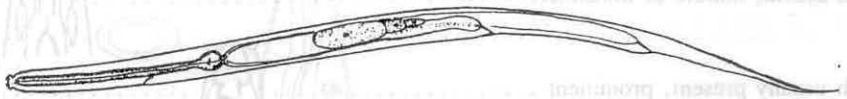
Amphid aperture appearing as minute pores ..... *Alaimus*

63.(60) Stoma narrow and long ..... *Cryptonchus*

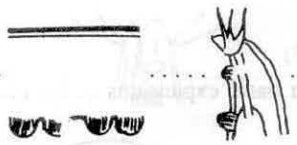
Stoma wide and shallow ..... *Bathyonchus*



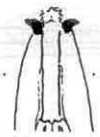
64.(1) Body symmetrical 65



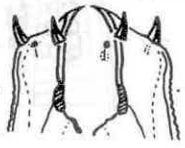
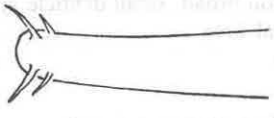
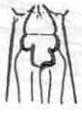
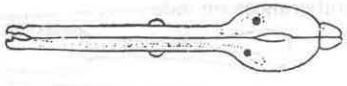
Body asymmetrical, bearing series of protuberances on side ..... *Bunonema**



65.(64) Lip appendages not elaborate 66



Lip appendages elaborate 68

		Refer to Couplet No.
66.(65)	Lateral lip appendages thorn-like, directed laterally 	*Diploscapter*
	Lateral lip appendages not thorn-like or directed laterally	67
67.(66)	Papillae or setae horn-like 	*Macrolaimus*
	Lips flap-like and pointed anteriorly 	*Teratocephalus*
68.(65)	Lip appendages forked and elaborately fringed 	*Acrobeles**
	Lip appendages membranous and wing-like 	*Wilsonema**
69.(1)	Post-cephalic setae absent 	70
	Post-cephalic setae present (may be very faint ex. *Tobrilus*) 	92
70.(69)	Stylet absent 	71
	Stylet present 	91
71.(70)	Teeth absent, minute or indistinct 	72
	Teeth usually present, prominent 	85
72.(71)	Esophagus with basal expansions 	73
	Esophagus uniformly cylindrical 	82
73.(72)	Amphids oval, spiral, or stirrup-shaped 	74
	Amphids circular	80

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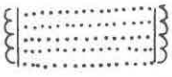
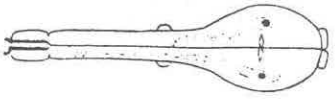
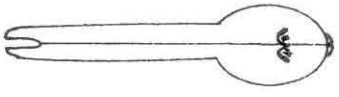
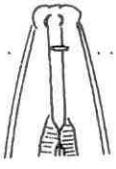
74.(73) Amphids spiral  75

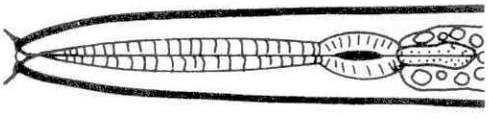
Amphids not spiral 79

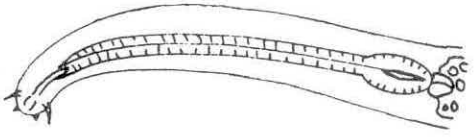
75.(74) Cuticular punctations absent  76

Cuticular punctations present  78

76.(75) Esophageal bulb without valves  77

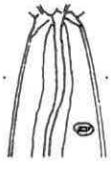
Esophageal bulb valvate   ..... *Plectus & Anaplectus*

77.(76) Esophageal-intestinal valve elongate  ..... *Paraplectonema*

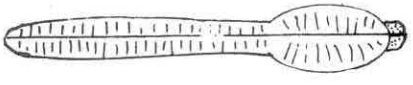
Esophageal-intestinal valve shortened  ..... *Paraphanolaimus*

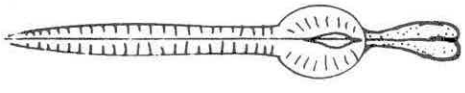
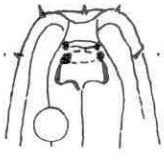
78.(75) Labial region characteristically flap-like  ..... *Euteratocephalus*

Labial region not flap-like, lips bluntly rounded  ..... *Ethmolaimus*

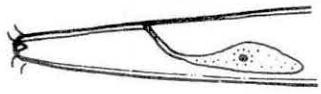
79.(74) Amphids oval   ..... *Greenenema*

Amphids stirrup-shaped   ..... *Chronogaster*

80.(73) Esophageal-intestinal valve shortened  81

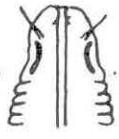
Esophageal-intestinal valve elongate   ..... *Desmolaimus*

Refer to Couplet No.

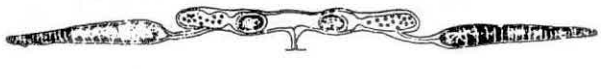
81.(80) Excretory pore and large excretory gland present .....  ..... *Domorganus*

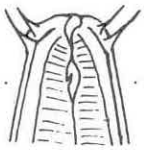
Excretory pore and gland indistinct or absent .....  ..... *Monhystera*

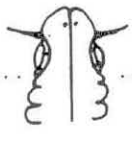
82.(72) Stoma wide and shallow, conspicuous, tail filiform .....  ..... *Prismatolaimus*

Stoma narrow, elongate, collapsed or inconspicuous .....  83

83.(82) Gonad single .....  ..... *Cylindrolaimus*

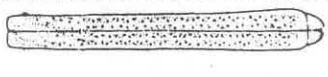
Gonads paired .....  84

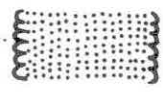
84.(83) Amphids inconspicuous .....  ..... *Tripyla*

Amphids conspicuous .....  ..... *Aphanolaimus*

85.(71) Terminal fifth or sixth of esophagus an ovoid bulb .....  86

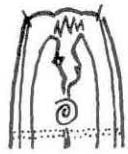
Esophagus uniformly cylindrical, stoma with massive teeth .....  ..... *Ironus*



86.(85) Cuticular punctations present .....  87

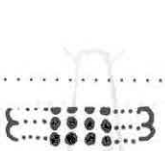
Cuticular punctations absent .....  89

87.(86) Amphids not spiral 88

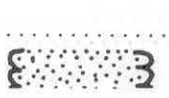
Amphids spiral .....  .....  ..... *Achromadora*

Refer to Couplet No.

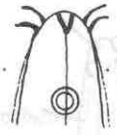
88.(87) Four longitudinal rows of cuticular markings present ..... *Chromadora*



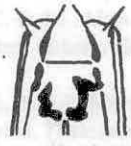
No longitudinal rows of cuticular markings present ..... *Prochromadorella*



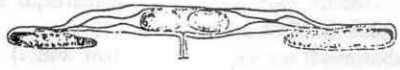
89.(86) Amphids distinct 90



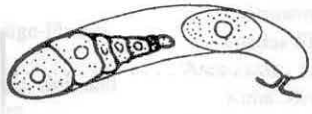
Amphids indistinct ..... *Butlerius*



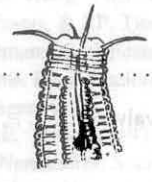
90.(89) Female gonad double, amphids hook-shaped ..... *Anonchus*



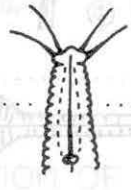
Female gonad single, amphid circular ..... *Monhystrella*



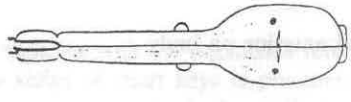
91.(70) Lip region annulated, not set off ..... *Atylenchus*



Lip region smooth, set off ..... *Eutylenchus*

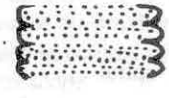


92.(69) Esophagus with basal expansion 93



Esophagus uniformly cylindrical 98

93.(92) Cuticular punctation present, amphids not circular 94

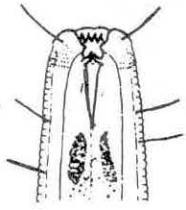


Cuticular punctation present, amphids circular 97

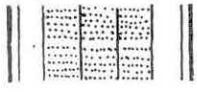
Refer to Couplet No.

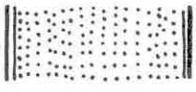
94.(93) Ocelli (eye spots) present  95

Ocelli absent  96

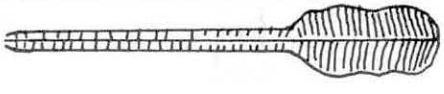
95.(94) Stoma with three equal-sized teeth  ..... *Chromadorina*

Stoma with at least one large tooth  ..... *Punctodora*

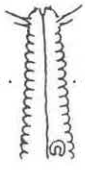
96.(94) Cuticle with lateral longitudinal rows of punctation  ..... *Hypodontolaimus*

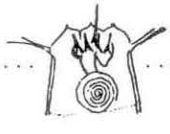
Cuticle without lateral differentiations  ..... *Chromadorita*

97.(93) Esophageal bulb valvate  ..... *Prodesmodora*

Esophageal bulb without valves   ..... *Odontolaimus*

98.(92) Amphid anterior on body  99

Amphid posteriorly located  ..... *Bastiania*

99.(98) Amphid spiral  ..... *Paracyatholaimus*

Amphid cup-shaped or obscure 100

100.(99) Stomal teeth massive ..... *Oncholaimus*

Stomal teeth small ..... *Tobrilus*

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10900 IDENTIFICATION OF AQUATIC ORGANISMS*

Experienced aquatic biologists will be familiar with most organisms illustrated in Plates 1 through 35, and seldom will need the assistance of keys to identify organisms to the level illustrated. Because these plates are not intended for critical identification, specific (species) names are not cited. Organisms most likely to be observed are illustrated. For the convenience of those less familiar with the organisms referred to in preceding sections, a series of short keys is presented to enable them to identify most organisms to the level illustrated by the plates.

In conformity with preceding sections, organisms are arbitrarily divided into microscopic and macroscopic, depending on whether or not they pass through a U.S. Standard No. 35 sieve (0.5 mm). For the study of microscopic forms, use a compound microscope. For examination of the smaller macroscopic organisms and to resolve the finer structures of larger forms, use a wide-field stereoscopic microscope.

* Approved by Standard Methods Committee, 1997.
Joint Task Group: Donald J. Reish (chair), E.F. Benfield, Donald B. Cadien, Donald G. Huggins, Donald J. Klemm, Vincent H. Resh, Ronald G. Velarde.