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Techniques of Water-Resources Investigations
of the United States Geological Survey

Chapter A4

METHODS FOR COLLECTION AND ANALYSIS OF AQUATIC BIOLOGICAL AND MICROBIOLOGICAL SAMPLES

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Revised 1987
Book 5

LABORATORY ANALYSIS

AQUATIC VERTEBRATES

Introduction

In most aquatic ecosystems, fish are the most common vertebrates. Because they are dependent on lesser life forms for food, the health of a local fish population commonly is used as an index for water quality and for the health of other aquatic organisms. Fish, however, are mobile animals and may avoid undesirable water quality (Whitmore and others, 1960). Moreover, they may exist for relatively long periods of time without food.

Although the investigation of fish populations is not a major interest of the U.S. Geological Survey, such investigations may at times provide valuable information about the aquatic environment. For example, length-weight relations can be used to compare fish from several streams, and changes in species composition with time may reveal water-quality trends, such as increased enrichment or a temperature increase of a particular aquatic environment. Stomach analyses reveal the organisms on which the fish feed; this information is essential to understanding the aquatic ecosystem.

The presence of dead or dying fish is indicative of lethal environmental conditions, unless it is a postspawning mortality or a delayed mortality resulting from cellular buildup of toxic materials. Onsite personnel can acquire valuable information by observing and collecting distressed fish. Pathological and histological examination of such fish may disclose the cause of death; however, on-the-spot observations of existing conditions, such as color of the water, floating material, effluent discharge, and the immediate collection of a water sample, are vital for a true explanation of the mortality (American Public Health Association and others, 1985).

In all States, some fish species and other aquatic vertebrates are protected by law, and the collection of others is regulated. Onsite personnel should ensure that they have complied with State laws before collecting samples of fish and other aquatic vertebrates. Hocutt (1978, p. 88) has prepared a listing, by specific year, for those States that require a permit or a license, or both, to collect fish. Czajka and Nickerson (1977) have prepared a similar list for the collection of reptiles and amphibians.

Although the methods described in this section are applicable to fish and other aquatic vertebrates, the emphasis generally will be on fish.

Collection

Collecting specimens for study requires a knowledge of the selectivity, limitations, and efficiency of the different types of sampling gear. Sampling gear and its use are discussed in Lagler (1956), Ramsey (1968), Weber (1973), Everhart and others (1975), Hocutt (1978), and American Public Health Association and others (1985).

Because of the nonrandom distribution of fish populations, the choice of sampling method, time of sampling, and frequency will depend on the objective of the particular investigation.

Active sampling gear

Active sampling gear, such as seines, trawls, electrofishing, chemical fishing, and hook and line, generally are less selective and commonly are preferred to passive techniques, such as gill, trap, hoop, and fyke nets.

If the data are to be used statistically (quantitatively), the method(s) of collection must be comparable numerically. Many fishery studies, for example, are concerned with determining yield biomass per unit area or estimating population densities in number per unit area based on a sample of the total population.

Ichthyocides (fish toxicants) provide the best method for collecting quantitative data; however, electrofishing often is the method of choice where chemicals cannot be used. While seines and other types of nets are basically qualitative gear, quantification of data is possible when the same experienced personnel do the collecting and all other factors are equal.

Seines

Seines consist of a length of strong netting material attached to a float line at the top and a heavily weighted lead line at the bottom. The ends of the seine are attached to a short stout pole or brail. If the net is large, hauling lines are attached to the top and bottom of the brail by a short bridle (fig. 50).

The sides, or wings, of the seine generally are of larger mesh than the middle, or bunt, part. The bunt may be in the form of a bag to confine the fish. Bag seines are most useful in ponds and lakes, and straight seines usually are used in streams and rivers. Small seines (50 ft or less) are adequate for capturing small fish. For capturing larger fish, especially in clear water, seines of 100 ft or more are necessary.

Bag seine

The bag seine is most useful in small ponds or lakes but may be used in slow-flowing rivers. Select a shoreline section that is free of stumps and other obstructions. Secure or hold one end of the seine to the bank, and extend the seine into the water at right angles. Pull the extended end of the seine toward the bank so the seine forms the radius of a circle (Lagler, 1956, p. 8, fig. 2). With both ends of the seine

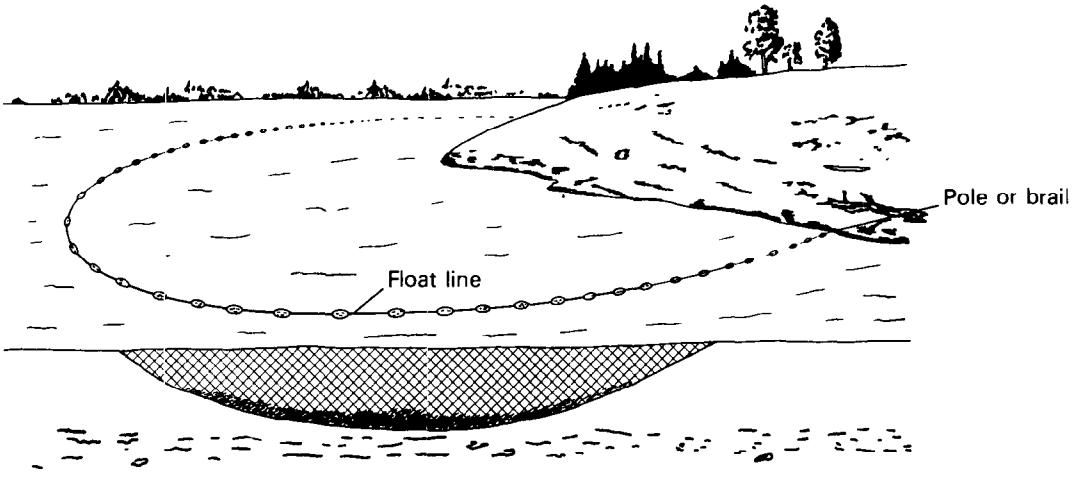

Figure 50.—Common haul seine (modified from Dumont and Sundstrom, 1961).

beached, pull the remainder of the seine slowly into shore, keeping the lead line in contact with the bottom. Continue pulling until the opening of the bag reaches the shoreline. Remove the specimens, and process using the method selected based on the objectives of the study.

Straight seine

Select a suitable area, usually a stream section having a smooth or relatively smooth bottom. Beginning at the downstream boundary of the area, pull the seine upstream into the current as rapidly as possible. Ensure that the bottom edge of the seine (lead line) is in contact with the stream bottom at all times. At the upstream boundary of the area, beach or bring the seine to the bank and quickly lift it from the water, forming a pocket in its center.

When using the larger seines in rivers and lakes, the usual method is to leave one end of the net, or hauling line, on shore while the net is played out by hand or boat perpendicular to the shore until the net is nearly extended. Direction then is changed (usually downstream) to lay out the remaining net parallel to the shore. When the net is fully extended, the end of the second haul line, or brail, is brought to the shore.

When fishing for pelagic or schooling species, one end of the net may be hauled first to form a hook against the shore. As soon as a school of fish enter the area, the second line is hauled. When fishing for nonschooling species, both ends of the net usually are hauled in at once.

With either type of net, be certain the lead line remains in contact with the bottom at all times. Continue pulling until the pocket, or bag, reaches the shoreline. Remove specimen(s) and process using the method selected based on the objective of the study.

Trawls

Trawls are specialized seines used in large, open-water areas where they are towed behind boats at sufficient speeds to overtake and enclose fish on the bottom or to collect schooling fish at various depths (figs. 51, 52). Because of the size and weight of the equipment, trawls have limited usefulness in lakes and reservoirs. For more information, refer to Massman and others (1952), Rounsefell and Everhart (1953), and Dumont and Sundstrom (1961).

Electrofishing

Applying alternating or direct electrical current [at the specified (110 V ac or 220 V dc) output amperage] to water to induce subnarcosis or the temporary immobilization of fish is an efficient method of capturing fish. A pulsed direct current of 50 to 100 pulses per second, at the specified output amperage, includes electrotaxis of the fish and attracts it to the positive electrode, or anode, where it is netted (Sharpe and Burkhard, 1969). Alternating current is most useful in streams of very weak resistance.

Electrofishing can be hazardous and must be used with caution. All personnel engaged in electrofishing must wear protective rubber waders and low-voltage Trapper's gloves, and adhere strictly to safety precautions. Training of all crew members in first-aid for electrical shock and drowning is advisable. The method is best suited for small streams but is adaptable to lakes and slow-flowing rivers as described by Frankenberger (1960) and Sharpe (1964).

After selecting a suitable site, position the electrodes according to the manufacturer's instructions for the type of water being sampled. Electrofishing generally is done upstream from a natural barrier or block seine placed across the stream. Shock all areas that may have fish, such as brush, fallen trees, boulders, and undercut banks. When making population estimates, shock the same reach three or more times (Zippin, 1956). Capture efficiency varies with the species of fish, current velocity, turbidity, water conductivity, experience of personnel, and other variables (Cross and Stott, 1975). Friedman (1974) prepared a selected bibliography about the use of electrofishing that included the state of the art during 1974.

Captured fish should be placed in live cages for processing. When possible, identify specimens onsite and release

after processing. If onsite identification is not possible or only tentative, count the number of individuals in each taxa, and preserve about 20 representative specimens for laboratory examination. Processing of specimens will depend on study objectives but generally includes length, weight, sex, and scale samples for age-growth analysis. Lagler (1956) and Everhart and others (1975) are excellent sources for additional information about fishery science.

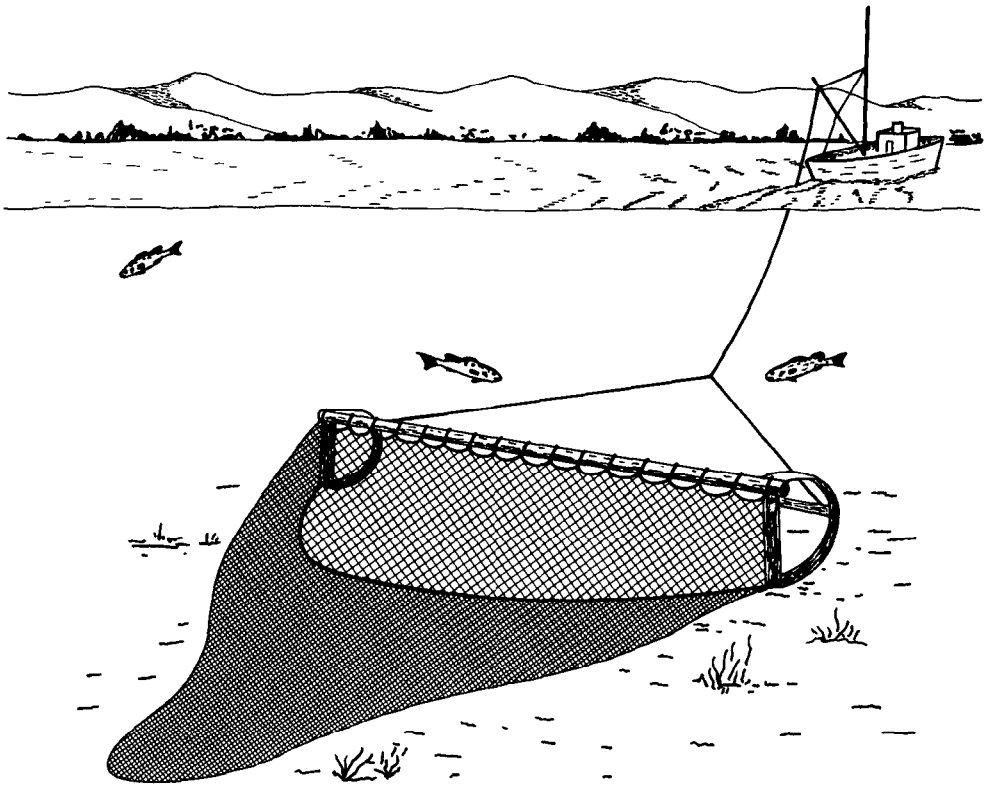

Figure 51.—Beam trawl (modified from Dumont and Sundstrom, 1961).

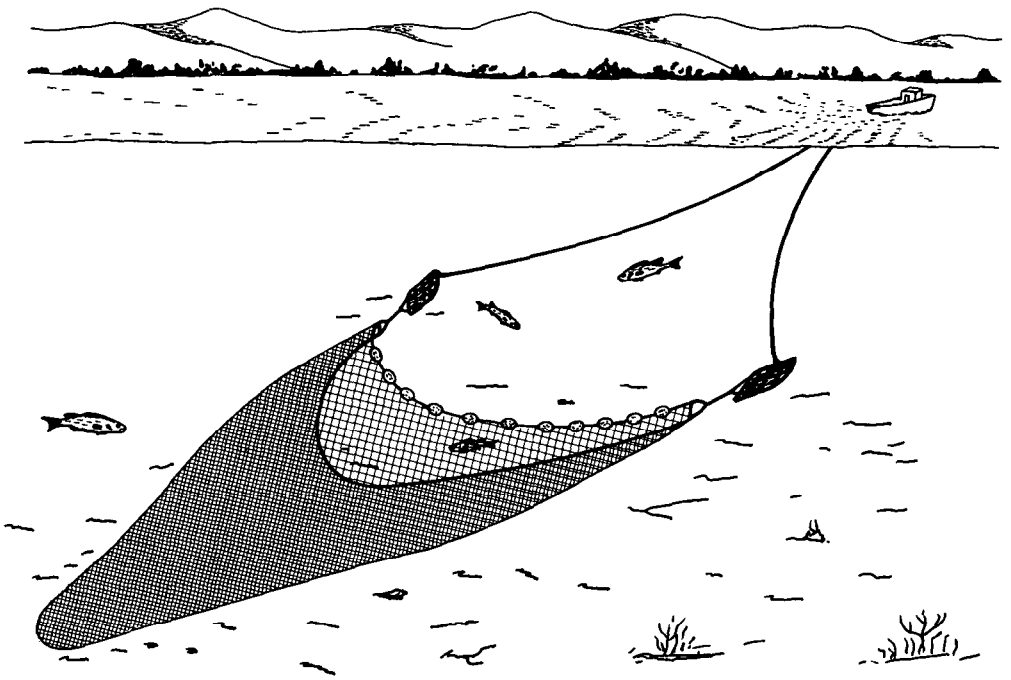

Figure 52.—Otter trawl (modified from Dumont and Sundstrom, 1961).

Ichthyocides

Ichthyocides, or fish toxicants, provide a good sampling method for making qualitative and quantitative studies of fish populations. Relative abundance, diversity, and biomass can be estimated more precisely using ichthyocides than using any other means. However, their use requires careful planning, and special permits from State conservation agencies usually are required.

Rotenone obtained as an emulsion, containing 5-percent active ingredient, is the most popular chemical because it is relatively safe to use, is not persistent in the environment, and is fairly easy to detoxify. A general review of the literature about ichthyocides was prepared by Lennon and others (1971) and about rotenone specifically by Schnick (1974).

Fish toxicants generally are used in areas such as small embayments of lakes and reservoirs or short reaches of streams or rivers. The concentration of active ingredient necessary to effect a good recovery of most fish is dependent on the species present and the alkalinity of the water. Alkaline water requires a larger concentration as do species of bullheads, carp, and eels. The successful use of rotenone is dependent on exposing the desired fish population to a lethal dose (generally 0.25 to 1 mg/L) for at least 15 minutes.

The use of rotenone in small streams is discussed by Lennon and Parker (1959) and Boccardy and Cooper (1963), in large rivers by Hocutt and others (1973), and in impoundment surveys by Eschmeyer (1939), Lambou (1959), and Bone (1970). Weber (1973) describes several methods of application.

To determine the quantity of rotenone to use, calculate the volume (acre-feet) of water to be treated. For lakes, the volume is simply the area times the mean depth, divided by 43,560 to obtain acre-feet. Because 1 acre-ft of water weighs 2,718,144 lb, an investigator would need approximately 2.7 lb of rotenone for a concentration of 1 (mg/L)/acre-ft. For streams, the quantity of rotenone is based on the cubic feet of water passing a point in the stream for the 15 minutes necessary for the exposure period. To calculate, multiply width times mean depth times velocity, which equals cubic feet of water per second. Cubic feet per second times 900 seconds (15 minutes) equals total cubic feet of water to treat. Total cubic feet divided by 43,560 equals acre-feet of water.

Potassium permanganate ($KMnO_4$) is used to detoxify the rotenone. To calculate the quantity of $KMnO_4$ necessary to detoxify the rotenone, calculate the weight of water treated and apply $KMnO_4$ at the same concentration that the rotenone was applied.

Hook and line

Although the method is too selective to be used for population studies, it is a useful technique for capturing small numbers of adult fish for metal or pesticide analyses when other methods are impractical.

Passive sampling gear

Gill nets and other entanglement and entrapment devices are used to passively sample fish communities in lakes, reservoirs, estuaries, and large slow-moving rivers. Gill nets hang vertically in the water and may be fished at the surface or at any depth. Because fish caught in the net die within a short period of time, the nets need to be checked at least once every 12 hours. Gill nets are set most successfully in the evening and recovered early the next morning. Gill nets generally are set perpendicular to the shoreline. Lackey (1968) and Jester (1977) describe the effective use of gill nets (fig. 53).

Drifting gill nets are set and fished the same way as stationary gill nets except they are allowed to drift with the current. Gill nets are selective in what they capture because of the size of the mesh of the net and because some species are more susceptible to nets than others (Berst, 1961).

Entrapment devices include a variety of nets and traps designed to lure and guide the fish through a series of funnels from which it cannot escape (Beamish, 1973; Yeh, 1977). The two most common devices are the hoop net (figs. 54, 55) and the trap net. These devices are easily set from a small boat. The nets are held in place by anchors or poles and are used in water less than 4 m deep. Fyke nets are a type of hoop net that has wings, or a lead, or both. They are used in lakes and reservoirs where fish movement is more random. Trap nets are similar to hoop nets except floats and weights instead of hoops are used to give the net shape. An adequate sample of fish often can be captured by using a combination of hoop and trap nets of various mesh sizes in the available habitat.

Investigation of fish kills

For investigation of fish kills, collect live or distressed specimens, if possible, because they are more suitable for pathological and histological examination. Specimens generally can be collected using a dip net. Specimens that have died recently are a second choice, but the fact that they were dead when collected should be noted clearly on the sample label. Collect about 0.5 kg of fish or other vertebrates and, if possible, about five individuals if the whole animal is to be ground for analysis. Collect a proportionally larger sample when individual tissues are to be analyzed. Generally, a sample of 5 kg will be adequate.

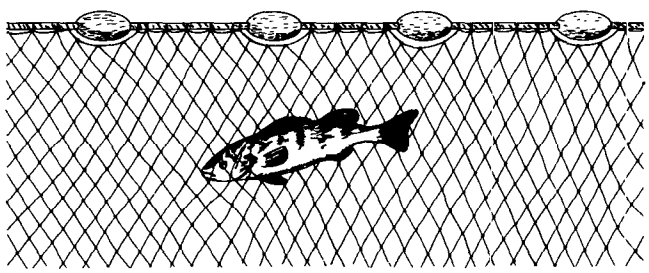

Figure 53.—Gill net (modified from Dumont and Sundstrom, 1961).

Collect specimens of the same type of organism as those affected from an area within the same body of water that had not been contaminated by the causative agent. These specimens should be handled separately. Collect 20 or more drops of blood from these specimens in a solvent-rinsed vial, seal with teflon or aluminum foil, cap, and freeze. Collection method will depend on the type of habitat to be sampled (Lagler, 1956).

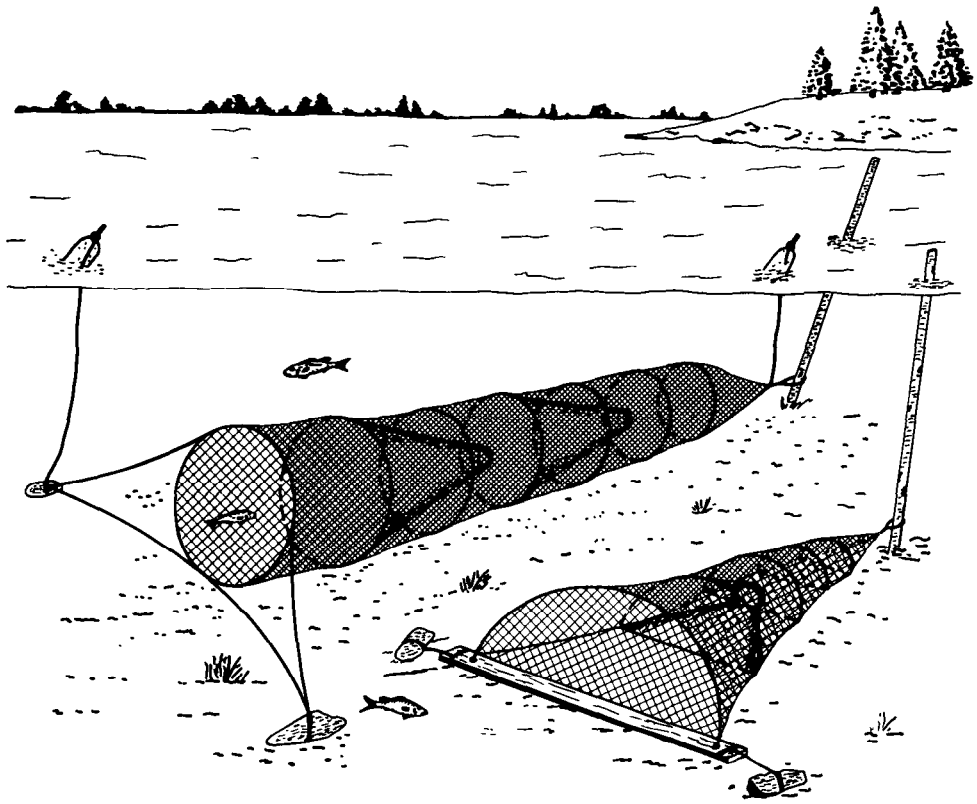

Figure 54.—Hoop net (modified from Dumont and Sundstrom, 1961).

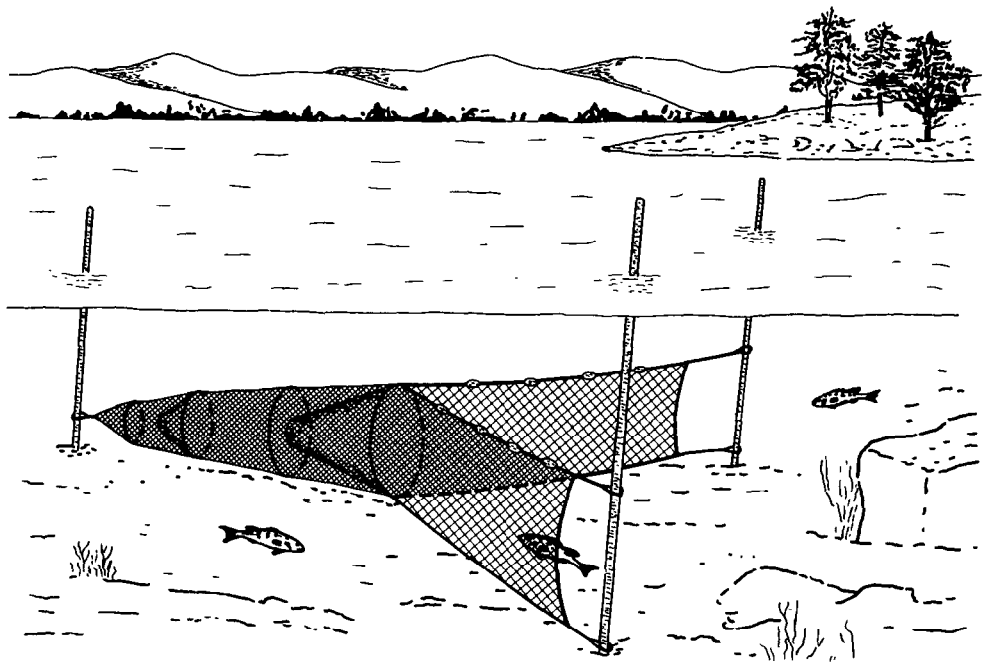

Figure 55.—Fyke net (modified from Dumont and Sundstrom, 1961).

Identify preserved specimens using the best available taxonomic keys or other appropriate means. Proper identification of species involved is necessary to assess the monetary loss due to the destruction of valuable fish and other animal life.

Preparation and storage

Package the fish in labeled polyethylene bags and freeze (Note 1). Samples may be packed in insulated cartons or chests and refrigerated using about 5 kg of dry ice per 5 to 8 kg of fish.

Note 1: Samples collected for polychlorinated biphenyl (PCB) or other organic-compound analysis should be stored in glass containers or wrapped in aluminum foil. If freezing facilities are not available, preserve the fish in ethyl alcohol (Cope, 1960; Wood, 1960).

Before placing in the preservative, slit each fish from the anus to the gills. Use at least five volumes of preservative for each volume of fish. To avoid contamination, package the fish collected dead separately from those that were collected alive. Labels placed in the same bag with wet fish may become illegible. Tie labels to the outside of the bag.

Estimate the intensity or degree of kill by counting the number of distressed or dead fish per unit length of shoreline, water-surface area, or number of fish passing a point per unit time. Record any factors at the site of the kill that will be useful in identifying the source of the kill. At a minimum, record the name and location of water, time, date, general characteristics of water (color, odor, and other characteristics), and present and previous weather conditions. Also, record name and telephone number of agency or individual reporting the kill, suspected causative agent(s), and suspected source(s).

Whenever possible, measure dissolved oxygen, temperature, pH, and specific conductance upstream and downstream from suspected source(s) of pollutant(s). Also, collect an adequate number of water samples (at least 1 L) upstream from and at the source(s) of suspected pollutant(s). The samples should be chilled to 4 °C.

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Faunal survey (qualitative method)

(B-6001-85)

Parameter and Code: Not applicable

1. Applications

The methods are applicable to all water.

2. Summary of method

Fish and other aquatic vertebrates are collected, preserved, and identified using appropriate taxonomic keys.

3. Interferences

Physical factors, such as stream velocity, depth of water, and turbidity, may make collection difficult. Filamentous algae and macrophytes may interfere with the operation of nets and seines.

4. Apparatus

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

Methods and equipment for the collection of fish are described by Lagler (1956), Needham and Needham (1962), Calhoun (1966), Weber (1973), Everhart and others (1975), Hocutt (1978), and American Public Health Association and others (1985). Hocutt (1978) also discussed methods and equipment for the collection of amphibians and reptiles. State conservation agencies, the U.S. Fish and Wildlife Service, and commercial fishermen are other sources of information for obtaining the proper collecting equipment. Weber (1973, p. 171) lists publications containing information about fishery sampling equipment.

4.1 *Bag seine*, about 25 to 50 ft × 6 or 8 ft. The mesh size should be ½ in. square for the wings and ¼ in. square for the bag.

4.2 *Dip net*, about 15-in. bow, 45-in. handle, 18-in. depth knotless nylon net, and ⅜-in. square mesh.

4.3 *Dissecting kit*. Routine dissecting tools. Dissection of the fish for internal examination frequently is required.

4.4 *Dissecting microscope*, low power of about 7× and stronger, either rotary or stereozoom type of binocular microscope. A substage mirror is essential.

4.5 *Divider*, fine-pointed, or dial caliper, for measuring body proportions.

4.6 *Electrofishing gear*. The basic unit consists of a generator (110 V ac or 220 V dc), sufficient insulated electrical wire, and two or three electrodes.

4.7 *Forceps*, long, for removing specimens from jars, and fine-pointed forceps that meet at the tip, for proper grasping of fins of small fishes and for removal of pharyngeal teeth of small cyprinids.

4.8 *Gill net*, experimental, about 6×125 ft. Most nets are made in 25-ft panels joined into continuous lengths that have four to five panels of different mesh size. The mesh size should range from about ½ in. at one end to about 2 in. at the other end. When equipped with poly-foam float line and lead-core leadline, the nets are virtually tanglefree. Mesh combinations and hanging sequence may be varied to suit individual requirements.

4.9 *Gloves, waterproof*, low-voltage rubber, Trapper's, shoulder length, for use with electrofishing gear.

4.10 *Light source*, that has very intense illumination. Many investigators favor a gooseneck lamp and a 100-W lightbulb; others favor the smaller lamps that project a concentrated beam of light. The important goal is to bring the light as close to the subject as possible.

4.11 *Nylon-mesh cage*, about 4×4×4 ft, and ¼-in. mesh to hold fish after capture.

4.12 *Rule*, stainless steel, metric, and a divider for obtaining actual measurements.

4.13 *Sample containers*, plastic, wide-mouth jars, about 0.5-, 1-, and 2-L capacity. Lids should be of plastic if used for prolonged storage of preserved specimens.

4.14 *Straight seine*, 10×5 ft × ⅛-in. mesh, minnow type, and 25×6 ft × ¼-in. square mesh.

4.15 *Trawls, traps, and hoop nets*, available through commercial fishing supply outlets.

4.16 *Waders*, chest-type, for use with electrofishing gear.

4.17 *Waterproof ink*.

4.18 *Waterproof labels*, or labels may be cut from sheets of plastic paper.

5. Reagents

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

5.1 *Alcohol, isopropyl*, 40-percent solution. Dilute 40 mL concentrated isopropyl alcohol to 100 mL using distilled water.

5.2 *Distilled or deionized water*.

5.3 *Formaldehyde solution*, 4 percent. Dilute 10 mL 37- to 40-percent aqueous formaldehyde solution (formalin) to 100 mL using distilled water.

5.4 *Household borax*. Add about 3 g borax to 1 L 4-percent formaldehyde solution to prevent shrinkage of biological specimens.

6. Analysis

6.1 Preserve specimens in 4-percent formaldehyde solution (10-percent formalin) containing about 3 g borax per liter. Specimens more than 8 cm in length should be slit on the right side to ensure penetration of the preservative into the body cavity. After about a week in the formaldehyde solution, remove the specimens, wash thoroughly by several changes of tap water for at least 24 hours, and transfer the specimens to a 40-percent isopropyl alcohol solution. One change of alcohol is necessary to remove traces of formaldehyde before permanent preservation in 40-percent isopropyl alcohol solution (Needham and Needham, 1962).

6.2 Identify specimens using the best available taxonomic keys, such as Jordan and Everman (1890-1900) and Eddy (1978). Lagler (1956, p. 19-64) described the families of North American freshwater fish and listed local and regional publications about fish taxonomy. Weber (1973) also lists taxonomic references by region. Widely used regional fish keys include, for example, Schultz (1936), Hubbs and Lagler (1958), and Clemens and Wilby (1961). Examples of local keys are Simon (1946), Trautman (1957), and Cook (1959). The recognized common and scientific names of North American fish are reported in Bailey and others (1970). For the identification of other aquatic vertebrates, refer to Bishop (1947), Carr (1952), and Conant (1975).

6.3 When a tentative species identification has been made using a key, confirmation or rejection of the determination is based on: (1) A comparison with species characteristics listed in the key, (2) determination of correct geographic range, (3) comparison with photographs and drawings in various keys, and (4) identification by a specialist of individuals of questionable species.

7. Calculations

No calculations are necessary.

8. Reporting of results

Report the number of taxa and individuals of each taxon and the type of collection method used.

9. Precision

No numerical precision data are available.

10. Sources of information

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Life history (quantitative method)

(B-6020-85)

Parameter and Code: Not applicable

1. Applications

The method is applicable to all water.

2. Summary of method

Fish and other aquatic vertebrates are collected and identified. Fish studies commonly include the number of specimens captured per unit area or unit time. The fish also may be measured, weighed, sexed, and aged to provide comparative information between populations in the same environment or between populations in different environments. Methods used in the study of fish and fish populations are described by Lagler (1956), Ricker (1971), and Everhart and others (1975). Methods for the direct and indirect enumeration of populations are described in this section.

3. Interferences

Physical factors, such as stream velocity, depth of water, and turbidity, may make collection difficult. Filamentous algae and macrophytes may interfere with the operation of nets and seines.

4. Apparatus

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

Methods and equipment for the collection of fish are described by Lagler (1956), Needham and Needham (1962), Calhoun (1966), Weber (1973), Everhart and others (1975), Hocutt (1978), and American Public Health Association and others (1985). Hocutt (1978) also discussed methods and equipment for the collection of amphibians and reptiles. State conservation agencies, the U.S. Fish and Wildlife Service, and commercial fishermen are other sources of information for obtaining the proper collecting equipment. Weber (1973, p. 171) lists publications containing information about fishery sampling equipment.

4.1 *Bag seine*, about 25 to 50 ft × 6 or 8 ft. The mesh size should be ½-in. square for the wings and ¼-in. square for the bag.

4.2 *Balance*, capable of weighing to at least 1 g.

4.3 *Container*, for holding anesthesia.

4.4 *Dip net*, about 15-in. bow, 45-in. handle, 18-in. depth knotless nylon net, and ⅜-in. square mesh.

4.5 *Dissecting kit*. Routine dissecting tools. Dissections of the fish for internal examination frequently is required.

4.6 *Dissecting microscope*, low power of about 7× and stronger, either rotary or stereozoom type of binocular microscope. A substage mirror is essential.

4.7 *Divider*, fine-pointed, or dial caliper, for measuring body proportions.

4.8 *Electrofishing gear*. The basic unit consists of a generator (110 V ac or 220 V dc), sufficient insulated electrical wire, and two or three electrodes.

4.9 *Forceps*, long, for removing specimens from jars, and fine-pointed forceps that meet at the tip, for proper grasping of fins of small fishes and for removal of pharyngeal teeth of small cyprinids.

4.10 *Gill net*, experimental, about 6×125 ft. Most nets are made in 25-ft panels joined into continuous lengths that have four to five panels of different mesh size. The mesh size should range from about ½ in. at one end to about 2 in. at the other end. When equipped with poly-foam float line and lead-core leadline, the nets are virtually tanglefree. Mesh combinations and hanging sequence may be varied to suit individual requirements.

4.11 *Gloves, waterproof*, low-voltage rubber, Trapper's, shoulder length, for use with electrofishing gear.

4.12 *Light source*, that has very intense illumination. Many investigators favor a gooseneck lamp and a 100-W lightbulb; others favor smaller lamps that project a concentrated beam of light. The important goal is to bring the light as close to the subject as possible.

4.13 *Measuring board*, or similar apparatus. A metric ruler that has a piece of wood at a right angle to the zero end is an adequate measuring device.

4.14 *Nylon-mesh cage*, about 4×4×4 ft, and ¼-in. mesh to hold fish after capture.

4.15 *Rule*, stainless steel, metric, and a divider for obtaining actual measurements.

4.16 *Sample containers*, plastic, wide-mouth jars, about 0.5-, 1-, and 2-L capacity. Lids should be of plastic if used for prolonged storage of preserved specimens.

4.17 *Scalpel or knife*, that has small sharp blade.

4.18 *Small envelopes*, 2¼×3¼ in., and bond typing-paper inserts for scale samples.

4.19 *Straight seine*, 10×5 ft × ⅛-in. mesh, minnow-type, and 25×6 ft × ¼-in. square mesh.

4.20 *Trawls, traps, and hoop nets*, available through commercial fishing supply outlets.

4.21 *Vials or small bottles*, for stomach-content samples.

4.22 *Waders*, chest-type, for use with electrofishing gear.

4.23 *Waterproof ink*.

4.24 *Waterproof labels*, or labels may be cut from sheets of plastic paper.

5. Reagents

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

5.1 *Alcohol, isopropyl*, 40-percent solution. Dilute 40 mL concentrated isopropyl alcohol to 100 mL using distilled water.

5.2 *Anesthesia, MS 222 (tricanemethane sulfonate).* Prepare a stock solution by dissolving 1 g MS 222 in 500 mL distilled water. Dilute the stock solution 1 part to 6 parts using distilled water before use.

5.3 *Distilled or deionized water.*

5.4 *Formaldehyde solution*, 4 percent. Dilute 10 mL 37- to 40-percent aqueous formaldehyde solution (formalin) to 100 mL using distilled water.

5.5 *Household borax.* Add about 3 g borax to 1 L 4-percent formaldehyde solution to prevent shrinkage of biological specimens.

6. Analysis

6.1 Preserve specimens in 4-percent formaldehyde solution (10-percent formalin) containing about 3 g borax per liter. Specimens more than 8 cm in length should be slit on the right side to ensure penetration of the preservative into the body cavity. After about a week in the formaldehyde solution, remove the specimens, wash thoroughly by several changes of tap water for at least 24 hours, and transfer the specimens to a 40-percent isopropyl alcohol solution. One change of alcohol is necessary to remove traces of formaldehyde before permanent preservation in 40-percent isopropyl alcohol solution (Needham and Needham, 1962).

6.2 Identify specimens using the best available taxonomic keys, such as Jordan and Everman (1890–1900) and Eddy (1978). Lagler (1956, p. 19-64) described the families of North American freshwater fish and listed local and regional publications about fish taxonomy. Weber (1973) also lists taxonomic references by region. Widely used regional fish keys include, for example, Schultz (1936), Simon (1946), Trautman (1957), and Hubbs and Lagler (1958). The recognized common and scientific names of North American fish are given in Bailey and others (1970). For the identification of other aquatic vertebrates, refer to Bishop (1947), Carr (1952), and Conant (1975).

6.3 When a tentative species identification has been made using a key, confirmation or rejection of the determination is based on: (1) A comparison with species characteristics listed in the key, (2) determination of correct geographic range, (3) comparison with photographs and drawings in various keys, and (4) identification by a specialist of individuals of questionable species.

6.4 Fish, amphibians, and other aquatic, cold-blooded animals can be handled easier and with less harm done to them if they are anesthetized. There also is less chance that the worker will be injured by sharp teeth or spines when the animal's reactions have been slowed. MS 222 (tricanemethane sulfonate), at the prescribed concentration, is the preferred anesthetic. Read label completely for directions and warnings about the use of this chemical.

6.5 Weigh each fish to the nearest gram after blotting dry using a paper towel or cheesecloth.

6.6 Measure the total length of each fish to the nearest millimeter. Fork length is preferred by some fisheries' biologists (fig. 56).

6.7 Food habits (optional). If the food habits of the fish are one of the study objectives, representative specimens usually must be killed. However, methods are available for removing food materials from the stomachs of living fish (Wales, 1962). Make a quantitative determination of the food present in the stomachs using a method appropriate to the study objectives. The usual methods are numerical, frequency of occurrence, percentage of bulk, gravimetric, and volumetric (Lagler, 1956, p. 120-128).

6.8 Age and growth by the length frequency method (optional). This method is based on the assumption that the lengths of individuals of a species of one age group will be normally distributed about the mean length, when collected at the same time. Accurate results using this method require fairly large samples of all age groups in the population (Carlander, 1969).

6.9 Age and growth by the scale-analysis method (optional). Using a knife blade or scalpel, remove a sample of scales from the left side of the fish (fig. 56). Place the scales in a folded piece of bond typing paper, and insert into an envelope. Record the following on the outside of the envelope: species, locality, method of capture, time, date, collector, length, weight, and sex (if known) of the fish. Using the collected scales, determine the age of the fish using the methods described in Lagler (1956, p. 131-158).

6.10 *Population density (optional)* is population size in relation to some unit of space. It generally is measured and expressed as the number of individuals or standing crop (biomass) per unit of area; for example, 53 brook trout per surface area, or 190 lb of fish per surface area.

The methods for determining population density can be divided into two general categories: (1) Direct or total count, and (2) indirect or sample count. The opportunity for total direct counting only occurs when the entire population can be concentrated, such as during a reclamation project or during a spawning run. More often the population must be estimated by sampling methods. The three most commonly used sampling methods include: (1) The area-density method, (2) the mark and recapture method, and (3) the catch-per-unit-effort method. The methods are described in Cooper and Lagler (1956) and Everhart and others (1975).

6.10.1 The area-density method consists of counting the number of fish in a series of random or stratified plots or in areas that are representative of the total area whose population is to be estimated. The sample count then is expanded to an estimate of the population by multiplying the aggregate sample count by the fraction: total area (or

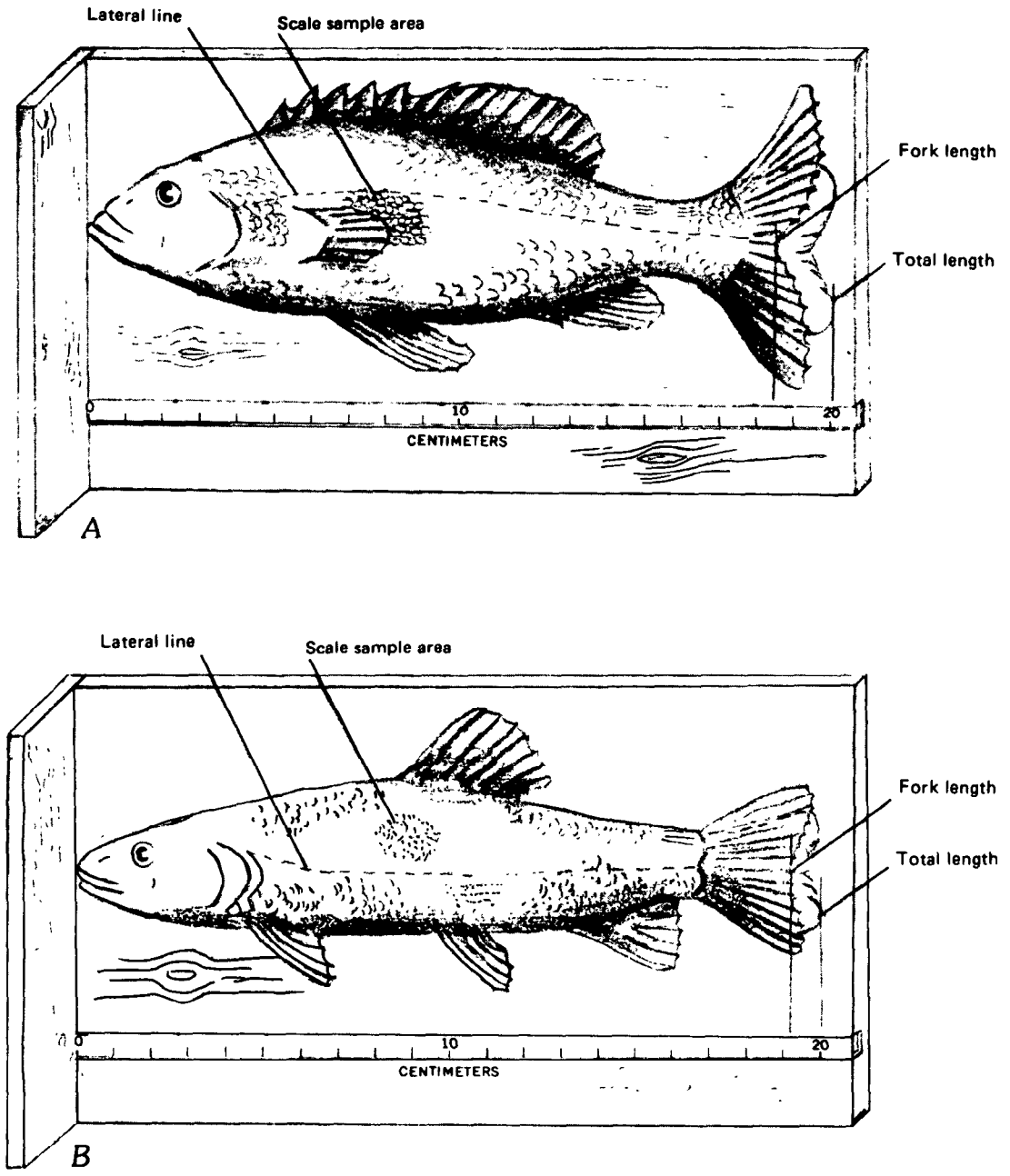

Figure 56.—Fish measurements and areas for scale collection on: (*A*) spiny-rayed and (*B*) soft-rayed fish.

time) divided by the sum of sample areas (Everhart and others, 1975).

6.10.2 The mark and recapture method of populations involves, first, the capture and release of a number of marked individuals into the population; and second, the subsequent recapture of marked individuals and the capture of unmarked individuals from the population.

6.10.3 The catch-per-unit-effort method requires a measurable decrease in the population by fishing and commonly is referred to as the DeLury (1947) regression method. The method of Moran (1951) and Zippin (1956, 1958) is appropriate when effort is constant. The DeLury (1947) and Leslie (1952) methods are appropriate when effort is variable. These methods are valid only if the population is closed, and the chance of capture is equal and remains constant from sample to sample. Examples of the application of data from the catch-per-unit-effort method to regression analyses are presented in Lagler (1956), Zippin (1956, 1958), and Everhart and others (1975).

Methods for measuring population density are numerous and too involved to go into detail here. The investigator should review the indicated literature and adapt proven techniques to fit a specific case.

7. Calculations

7.1 Percent species composition in sample

$$= \frac{\text{Number of individuals of a given species}}{\text{Total number of all fish collected}} \times 100.$$

7.2 Plot weight as a function of length, as described in Lagler (1956, p. 159-166, figs. 47, 48).

7.3 Plot age as a function of length, as described in Lagler (1956, p. 149-158).

7.4 The calculations required for food-habit studies are determined by the methods of analysis. The usual methods are described in Lagler (1956, p. 120-130).

7.5 Calculate the population-density estimate from area-density data using the equation

$$N = \frac{A}{a} \sum_{i=1}^{a} N_i,$$

where

N = the estimate of population size;
A = the number of equal units of area (or time) occupied by the total population;
a = the number of units sampled; and
N_i = the number counted in the i^{th} sample area.

The estimated variance ($\hat{v}$) is

$$\hat{v}(\hat{N}) = \frac{A^2 - aA}{a} \times \frac{a \sum_{i=1}^{a} N_i^2 - \sum_{i=1}^{a} N_i^2}{a(a-1)}.$$

7.6 Calculate the population-density estimate from mark and recapture data using the equation

$$N = MC/R$$

where

N = the estimate of population size;
M = the number of individuals marked and released into the population;
C = the recapture sample size that includes both marked and unmarked individuals; and
R = the number of marked individuals that are recaptured.

If the population density is large enough for multiple marking and recapture periods, use Schnable's equation (1938)

$$N = \frac{\sum_{t=1}^{n} C_t M_t}{\sum_{t=1}^{n} R_t}.$$

7.7 Calculate the population-density estimate from catch-per-unit-effort data using the line or regression technique where catch-per-unit effort is plotted against cumulative catch. In such a graph, the catch-per-unit effort is the ordinate and the cumulative catch is the abscissa. Fit the straight regression line to its intercept with the x axis. The intercept value is the approximation of the population density (Lagler, 1956).

8. Reporting of results

8.1 Report percent species composition in sample to the nearest whole number.

8.2 Report weight to the nearest gram, and length to the nearest millimeter.

8.3 Report age to the nearest year.

8.4 Report food-habit analyses by the method used and by study objectives.

9. Precision

No numerical precision data are available.

10. Sources of information

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Methods for investigation of fish and other aquatic vertebrate kills

(B-6040-85)

Parameter and Code: Not applicable

1. Applications

Methods of investigation and collection are applicable to all water.

2. Summary of method

2.1 Fish kills are obvious and important events related to water quality. The methods in this section describe what important facts need to be documented when making an onsite investigation and how to properly preserve specimens for laboratory examination to determine the probable cause of death. The collection of fish and other vertebrates from a natural or man-caused kill generally is only one phase of a more comprehensive investigation that involves onsite and laboratory chemical tests.

2.2 Because fish-kill investigations normally are the responsibility of State and Federal enforcement agencies, the U.S. Geological Survey's involvement usually is that of a supportive role. However, because many fish kills are due to a slug of toxic material of short duration, personnel from the first agency on the scene should be prepared to collect the necessary samples and information.

2.3 For additional information about the investigation of fish kills, see Smith and others (1956), Burdick (1965), Federal Water Pollution Control Administration (1966, 1967), and American Public Health Association and others (1985).

3. Interferences

Physical factors, such as stream velocity and depth of water, may make collection difficult. Access to affected waters also is a common problem. Some pollutants are toxic or hazardous to humans and require special precautions.

4. Apparatus

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

4.1 *Aluminum foil*, heavy weight type.

4.2 *Dip net*, long handle, and $\frac{3}{16}$-in. mesh.

4.3 *Plastic bags*, various sizes.

4.4 *Waterproof ink.*

4.5 *Waterproof labels*, or labels may be cut from sheets of plastic paper.

5. Reagents

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

5.1 *Distilled or deionized water.*

5.2 *Ethyl alcohol*, 75 percent. Dilute 750 mL commercial 95-percent denatured ethyl alcohol to 950 mL using distilled water.

6. Analysis

Samples should be shipped to an appropriate laboratory for histological or pathological examination. The nearest laboratory can be located by contacting the local office of the State Fish and Game Department or State Department of Health.

7. Calculations

No calculations are necessary.

8. Reporting of results

Report estimated number of distressed or dead fish, or other observed aquatic vertebrates, followed with an appropriate qualifying statement such as estimation based on 1 hour of observation or number of specimens observed per unit length of shoreline. Degrees of severity of fish kills have been based on the number of dead or dying fish per length of shoreline (American Public Health Association and others, 1985).

9. Precision

No numerical precision data are available.

10. Sources of information

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CELLULAR CONTENTS

Introduction

Chlorophyll *a* is the primary photosynthetic pigment of all oxygen-producing photosynthetic organisms and is present in all algae (phytoplankton and periphyton). Thus, measurement of this pigment can indicate the quantity of algae present and provide an estimate of the primary productivity (Lorenzen, 1970). Because environmental and nutritional factors may affect the chlorophyll concentration without affecting the total algal biomass, this measurement is only an estimate. Green algae and euglenophytes also contain chlorophyll *b* (Wetzel, 1975). Certain other algae contain chlorophylls *c* and *d*. Ratios between the different types of chlorophyll may indicate the taxonomic composition of an algal community.

An estimate of the quantity of living micro-organisms (biomass) in an aquatic environment can be useful when assessing water quality. The universal occurrence and central function of adenosine triphosphate (ATP) in living cells and its chemical stability make it an excellent indicator of the presence of living material. The level of endogenous ATP (that is, the quantity of ATP per unit biomass) in bacteria (Allen, 1973), in algae (Holm-Hansen, 1970), and in zooplankton (Holm-Hansen, 1973) is relatively constant when compared to cellular organic-carbon content in several species of organisms. Furthermore, its concentration in all phases of a growth cycle remains relatively constant. In studies where cell viability was determined (Hamilton and Holm-Hansen, 1967; Dawes and Large, 1970), the concentration of ATP per viable cell remained relatively constant during periods of starvation. The quantity of ATP, therefore, can be used to estimate total living biomass.

Collection

The sites and methods used for phytoplankton and periphyton sampling should correspond as closely as possible to those selected for chemical and microbiological sampling. The sample-collection method will be determined by the study objectives. In lakes, reservoirs, deep rivers, and estuaries, phytoplankton abundance may vary transversely, with depth and width, and with time of day. To collect a sample representative of the phytoplankton concentration at a particular depth, use a water-sampling bottle. To collect a sample representative of the entire flow of a stream, use a depth-integrating sampler (Guy and Norman, 1970; Goerlitz and Brown, 1972). For small streams, a depth-integrated sample or a point sample at a single transverse position at the centroid of flow is adequate. For further information about collection of phytoplankton samples, see the "Phytoplankton" section.

After collection of the phytoplankton sample, place a 47-mm glass-fiber filter on a filter funnel. Filter a measured volume of water sample at a vacuum of no more than 250 mm of mercury. Rinse the sides of the filter funnel with a few milliliters of distilled water. For estuarine samples, use rinse water that is near the salinity of the sample.

Roll the filter so the plankton is on the inside and proceed with the prescribed method of determination or place the rolled filter in a glass vial, 22×85 mm, and store frozen in the dark. Storage should not exceed 2 weeks. Dry ice is used for preserving samples while in transit (samples must not thaw before analysis begins).

Most analyses of the periphyton community have been adapted from long-established methods of phytoplankton analyses. The attached benthic nature of periphyton, however, causes special collection problems that adversely affect the accuracy of various estimates. Methods have been developed for collecting periphyton from natural substrates and from artificial substrates.

Natural submerged substrates commonly contain periphyton that can be sampled quantitatively. The periphyton should be removed from a known area of substrate onsite. Several devices for removing periphyton from a known area of natural substrates are shown in figure 18. Stockner and Armstrong (1971) sampled periphyton using a plastic hypodermic syringe that had a toothbrush attached to the end of the syringe piston. Holding the barrel of the syringe tightly against the substrate, the piston is pushed in until the brush contacts the periphyton. The piston then is rotated several times to dislodge the periphyton and then is withdrawn, pulling the periphyton up with it. A glass plate is placed immediately under the end of the barrel, and the syringe inverted. Four small holes at the base of the syringe enable the water to move freely when procuring the sample.

The device used by Douglas (1958) consists of a broad-necked polyethylene flask that has the bottom removed. The neck of the flask is held tightly against the surface to be sampled, and the periphyton inside the enclosed area is dislodged from the substrate using a stiff nylon brush. The loose periphyton is removed from the flask using a pipet. Ertl's (1971) apparatus consists of two concentric metal, or plastic, cylinders separated by spacers. The space between the cylinders is filled with modeling clay, and the sampler is pressed firmly against the substrate to be sampled. Using

a blunt stick or metal rod, the clay is forced down onto the substrate to isolate the sampling area of the inner circle. The periphyton within the inner circle is dislodged using a stiff brush and removed using a pipet.

Artificial substrates can be attached to a supporting object in a stream or lake (figs. 19, 20). The substrate must be submerged during the entire colonization period but may be near the surface of the water and can be suspended at several depths. The substrates may be attached to natural items, such as submerged trees, stumps, logs, or boulders, or they may be attached to stakes driven into the bottom. Floating samplers also may be used. The sampler should be secured so that it will not drift into any obstruction or become beached. In extremely shallow streams, a weir may have to be constructed to guarantee sufficient water to float the sampler. If such a weir is constructed, data from the sample should be compared only with data obtained from comparably placed samplers. A floating sampler is not recommended for any area that would have intermittent flow for any period during the exposure time.

The artificial substrates should be placed in areas of light that typify the streams, rivers, or lakes being studied. For example, if most of the stream is shaded, an area that receives a great deal of sunlight should not be selected as being representative. In general, substrate samples collected from similar lighting conditions need to be compared; but, depending on the study objective, this is not a requirement.

To ensure a continuous period of uniform substrate exposure to the environment being monitored, the sampler should be examined, periodically if possible, for any evidence of fouling or mechanical damage. If the sampler or substrate has been fouled or beached, the data for that sampling period should not be compared with data from any other substrate that has had free, continuous, and uninterrupted exposure to the aquatic environment.

The length of time required for colonization of the substrates by periphyton will depend on other environmental factors as well as water quality. Exposure times will vary and must be determined for each season and water type. The exposure period should be long enough to enable the development of a periphyton community large enough for measurement but, at the same time, should avoid so much growth that sloughing would occur. Test samplers can be used prior to the actual monitoring to determine the most desirable exposure time for the prevailing (that is, seasonal and environmental) conditions. The general exposure period for fresh to brackish waters, mesotrophic to eutrophic, within the thermal range of 15 to 35 °C, is 14 days. Exposure periods during special conditions of low productivity (that is, few nutrients, low temperature) or very high productivity may, by experience, be adjusted for the onsite conditions. Exposure periods should be identical for all sites in the entire study area.

The artificial substrates should be located so damage to the apparatus by floating debris is minimized. Vandalism is a common problem and placing the substrate away from frequently traveled areas is advisable. For further information on collection of periphyton samples, see the "Periphyton" section.

Place the detached periphyton from the natural substrate or the complete artificial substrate into a bottle containing water or preservative. Store frozen in the dark for no more than 2 weeks. Dry ice is used for preserving samples in transit.

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Chlorophyll in phytoplankton by spectroscopy

(B-6501-85)

Parameters and Codes:
Chlorophyll *a*, phytoplankton, spectrometric, uncorrected (μg/L): 32230
Chlorophyll *b*, phytoplankton, spectrometric (μg/L): 32231
Chlorophyll *c*, phytoplankton, spectrometric (μg/L): 32232
Chlorophyll, total, phytoplankton, spectrometric, uncorrected (μg/L): 32234

1. Applications

The method is suitable for all water.

2. Summary of method

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

3. Interferences

Suspended materials in the sample may clog the membrane filter. Erroneously large values may result from the presence of fragments of tree leaves and other plant materials. Exposure to light or acid at any stage of storage and analysis can result in photochemical and chemical degradation of the chlorophylls. Large populations of photosynthetic bacteria will result in an overestimation of phytoplankton chlorophyll (Hussaing, 1973).

4. Apparatus

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

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

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

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

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

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

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

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

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

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

4.10 *Tissue grinder.*

4.11 *Water-sampling bottle.* Depth-integrating samplers are described by Guy and Norman (1970).

5. Reagents

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

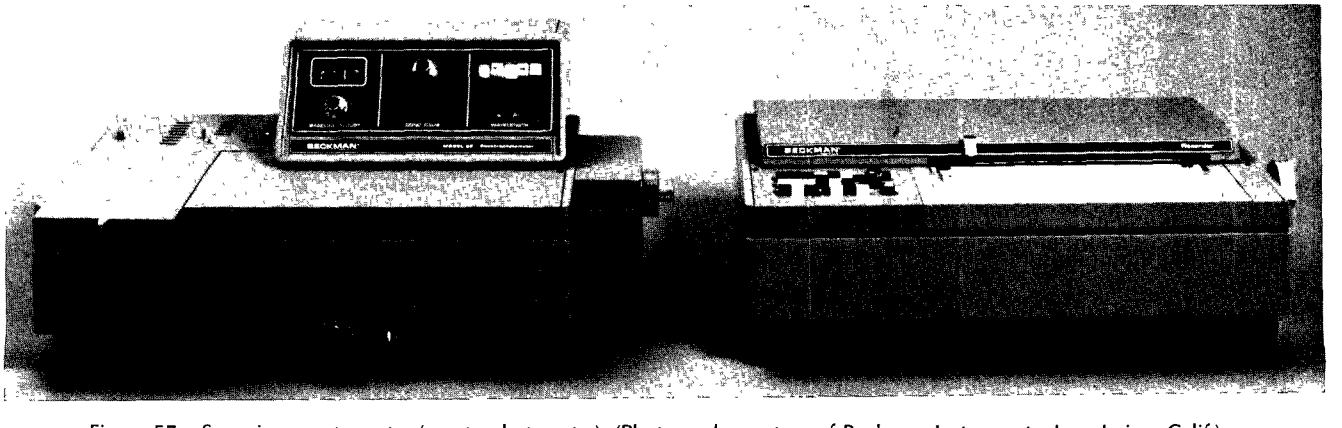

Figure 57.—Scanning spectrometer (spectrophotometer). (Photograph courtesy of Beckman Instruments, Inc., Irvine, Calif.)

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

5.2 *Distilled or deionized water.*

6. Analysis

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

6.2 Place the filter in a tissue grinder. Add 3 to 4 mL of 90-percent acetone, and grind at 500 r/min for 3 minutes. If multiple filters are used, use a 40-mL grinder.

6.3 Transfer the sample to a 15-mL graduated centrifuge tube, and wash the pestle and grinder two or three times using 90-percent acetone. Adjust to some convenient volume, such as 10±0.1 mL. Store for 10 minutes in the dark at room temperature.

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

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

6.6 Read the absorbances at 750, 664, 647, and 630 nm and compare to a 90-percent acetone blank. (Dilute the extract using 90-percent acetone if the absorbance is greater than 0.8.) If the 750-nm reading is greater than 0.005 absorbance unit per centimeter of light path, decrease the turbidity as in 6.5.

7. Calculations

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

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

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

and

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

where

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

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

and

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

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

$$\text{Chlorophyll } a \text{ (micrograms per liter)} = \frac{\text{Derived value (micrograms per milliliter)} \times \text{Extract volume (milliliters)}}{\text{Sample volume (liters)}}.$$

8. Reporting of results

Report concentrations of chlorophyll *a*, *b*, or *c*, in micrograms per liter, as follows: less than 1 μg/L, one decimal; 1 μg/L and greater, two significant figures.

9. Precision

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

9.2 The following precision estimates were reported by Strickland and Parsons (1972).

Chlorophyll *a* precision at the 5 μg level. The correct value is in the range: Mean of *n* determinations $\pm 0.26/n^{1/2}$ μg chlorophyll *a*.

Chlorophyll *b* precision at the 0.5 μg level. The correct value is in the range: Mean of *n* determinations $\pm 0.21/n^{1/2}$ μg chlorophyll *b*.

9.3 The precision of chlorophyll *c* determinations is variable and very poor, anywhere between ±10 and ±30 percent of the quantity being measured; results are not accurate.

10. Sources of information

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Strickland, J.D.H., and Parsons, T.R., 1972, A practical handbook of seawater analysis (2d ed.): Fisheries Research Board of Canada Bulletin 167, 311 p.

Chlorophyll in phytoplankton by chromatography and spectroscopy

(B-6520-85)

Parameters and Codes:

Chlorophyll *a*, phytoplankton, chromatographic/spectrometric (μg/L): 70951
Chlorophyll *b*, phytoplankton, chromatographic/spectrometric (μg/L): 70952

1. Applications

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

2. Summary of method

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

3. Interferences

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

4. Apparatus

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

4.1 *Air dryer.*

4.2 *Centrifuge.*

4.3 *Centrifuge tubes*, graduated, screwcap, 15- and 40-mL capacity.

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

4.5 *Developing tank and rack.*

4.6 *Evaporation device.*

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

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

4.9 *Glass pipets*, 10-mL capacity.

4.10 *Glass vials*, screwcap, 22×85 mm.

4.11 *Gloves*, long-service latex.

4.12 *Grinding motor*, that has 0.1 horsepower.

4.13 *Microdoser*, and 50-μL syringe.

4.14 *Pasteur pipets*, disposable.

4.15 *Propipet*, or equivalent suction device.

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

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

4.18 *Tissue grinder.*

5. Reagents

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

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

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

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

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

5.4 *Dimethyl sulfoxide* (DMSO).

5.5 *Distilled or deionized water.*

5.6 *Ethyl ether.*

5.7 *Methyl alcohol.*

5.8 *Nitrogen gas*, prepurified.

5.9 *Petroleum ether*, 30 to 60 °C.

6. Analysis

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

6.2 Place the filter in a tissue grinder. Add 3 to 4 mL DMSO and grind at 500 r/min for 3 minutes. If multiple filters are used, use a 40-mL grinder.

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

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

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

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

6.6 Cap and shake as in 6.4.

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

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

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

6.10 Add an equal volume of distilled water, and shake as in 6.4.

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

6.12 Remove the top ethyl ether layer using a capillary pipet, and place in the conical tube in the evaporation device.

Evaporate to dryness by blowing nitrogen gas over the ethyl ether surface.

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

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

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

6.16 Determine $R\hat{f}$ values (Note 2) for pure chlorophylls.

Note 2: $R\hat{f}$ value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

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

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

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

7. Calculations

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

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

where

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

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

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

$$\begin{array}{l}\text{Original sample (micrograms chlorophyll per liter)}\end{array} = \frac{\begin{array}{c}\text{Micrograms chlorophyll per milliliter (as in 6.19)} \\ \times 3\text{ mL}\end{array} \times \frac{\begin{array}{c}\text{Concentrate volume} \\ \text{(microliters)}\end{array}}{\begin{array}{c}\text{Volume streaked} \\ \text{(microliters)}\end{array}}}{\begin{array}{c}\text{Volume filtered onsite} \\ \text{(liters)}\end{array}}.$$

8. Reporting of results

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

9. Precision

No precision data are available.

10. Source of information

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

Chlorophyll in phytoplankton by high-pressure liquid chromatography

(B-6530-85)

Parameters and Codes:
Chlorophyll *a*, phytoplankton, chromatographic/fluorometric (μg/L): 70953
Chlorophyll *b*, phytoplankton, chromatographic/fluorometric (μg/L): 70954

1. Applications

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

2. Summary of method

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

3. Interferences

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

4. Apparatus

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

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

4.2 *Centrifuge.*

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

4.4 *Evaporation device.*

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

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

4.7 *Gloves*, long-service latex.

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

4.9 *Pasteur pipets*, disposable.

4.10 *Separatory funnels*, 125 mL.

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

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

5. Reagents

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

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

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

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

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

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

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

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

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

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

5.6 *Distilled or deionized water.*

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

5.8 *Dimethyl sulfoxide* (DMSO).

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

5.10 *Nitrogen gas*, prepurified.

6. Analysis

6.1 *Sample preparation.* Analyze only samples on glass-

fiber filters. Record the volume of water filtered for the phytoplankton sample. [If a biomass determination is required, save the DMSO layer (see 6.1.7).]

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

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

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

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

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

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

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

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

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

6.1.9 Drain and discard the bottom layer.

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

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

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

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

6.2 *High-pressure liquid-chromatographic analysis.*

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

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

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

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

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

7. Calculations

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

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

where

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

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

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

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

where

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

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

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

where

RF = response factor of chlorophyll *a* or *b* from 7.3, in milligrams per unit area;
I = integrated area of the chlorophyll *a* or *b* peak in the sample as determined in 6.2.5;
V_e = final volume of the sample extract from 6.1.13, in milliliters;
A_s = volume of water filtered in 6.1, in liters; and
V_i = volume of sample extract injected in 6.2.5, in microliters.

8. Reporting of results

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

9. Precision

No precision data are available.

10. Sources of information

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

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

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Chlorophyll in phytoplankton by chromatography and fluorometry

(B-6540-85)

Parameters and Codes:
Chlorophyll *a*, phytoplankton, chromatographic/fluorometric (μg/L): 70953
Chlorophyll *b*, phytoplankton, chromatographic/fluorometric (μg/L): 70954

1. Applications

The method is suitable for all water. The method is not suitable for determining chlorophyll *c*.

2. Summary of method

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

3. Interferences

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

4. Apparatus

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

4.1 *Air dryer.*

4.2 *Centrifuge.*

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

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

4.5 *Developing tank and rack.*

4.6 *Evaporation device.*

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

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

4.9 *Glass pipets*, 5- and 10-mL capacity.

4.10 *Glass vials*, screwcap, 22×85 mm.

4.11 *Gloves*, long-service latex.

4.12 *Grinding motor*, that has 0.1 horsepower.

4.13 *Microdoser*, and 50-μL syringe.

4.14 *Pasteur pipets*, disposable.

4.15 *Propipet*, or equivalent suction device.

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

4.17 *Spectrofluorometer* (fig. 58), that has redsensitive R446S photomultiplier, or equivalent. Use cells that have a light path of 1 cm.

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

4.19 *Tissue grinder.*

5. Reagents

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

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

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

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

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

5.4 *Dimethyl sulfoxide* (DMSO).

5.5 *Distilled or deionized water.*

5.6 *Ethyl ether.*

5.7 *Methyl alcohol.*

5.8 *Nitrogen gas*, prepurified.

5.9 *Petroleum ether*, 30 to 60 °C.

6. Analysis

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

6.2 Place the filter in a tissue grinder. Add 3 to 4 mL DMSO and grind at 500 r/min for 3 minutes. If multiple filters are used, use a 40-mL grinder.

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

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

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

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

6.6 Cap and shake as in 6.4.

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

6.8 During centrifugation, prepare chromatography tank by pouring 294 mL petroleum ether and 6 mL methyl alcohol into the tank. Mix well. Prepare fresh before each use. Use

two solvent-saturation pads and the developing rack to dry the chromatography sheet.

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

6.10 Add an equal volume of distilled water, and shake as in 6.4.

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

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

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

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

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

6.16 Determine $R\hat{f}$ values (Note 2) for pure chlorophylls.

Note 2: $R\hat{f}$ value = distance traveled by the chlorophyll from the point of application divided by the distance traveled by the solvent from the point of application.

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

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

6.19 Determine the concentration of chlorophyll *a* or *b* using the spectrofluorometer as follows. Curves are prepared daily to standardize the spectrofluorometer. Five standard solutions of each chlorophyll should be prepared at the concentrations of 0.25, 0.5, 1, 2, and 4 mg/L. These are prepared from the chlorophyll stock solutions by an appropriate dilution using 90-percent acetone. The absorbance then is read on a spectrometer at 664 nm for chlorophyll *a* and 647 nm for chlorophyll *b*. Determine concentrations of

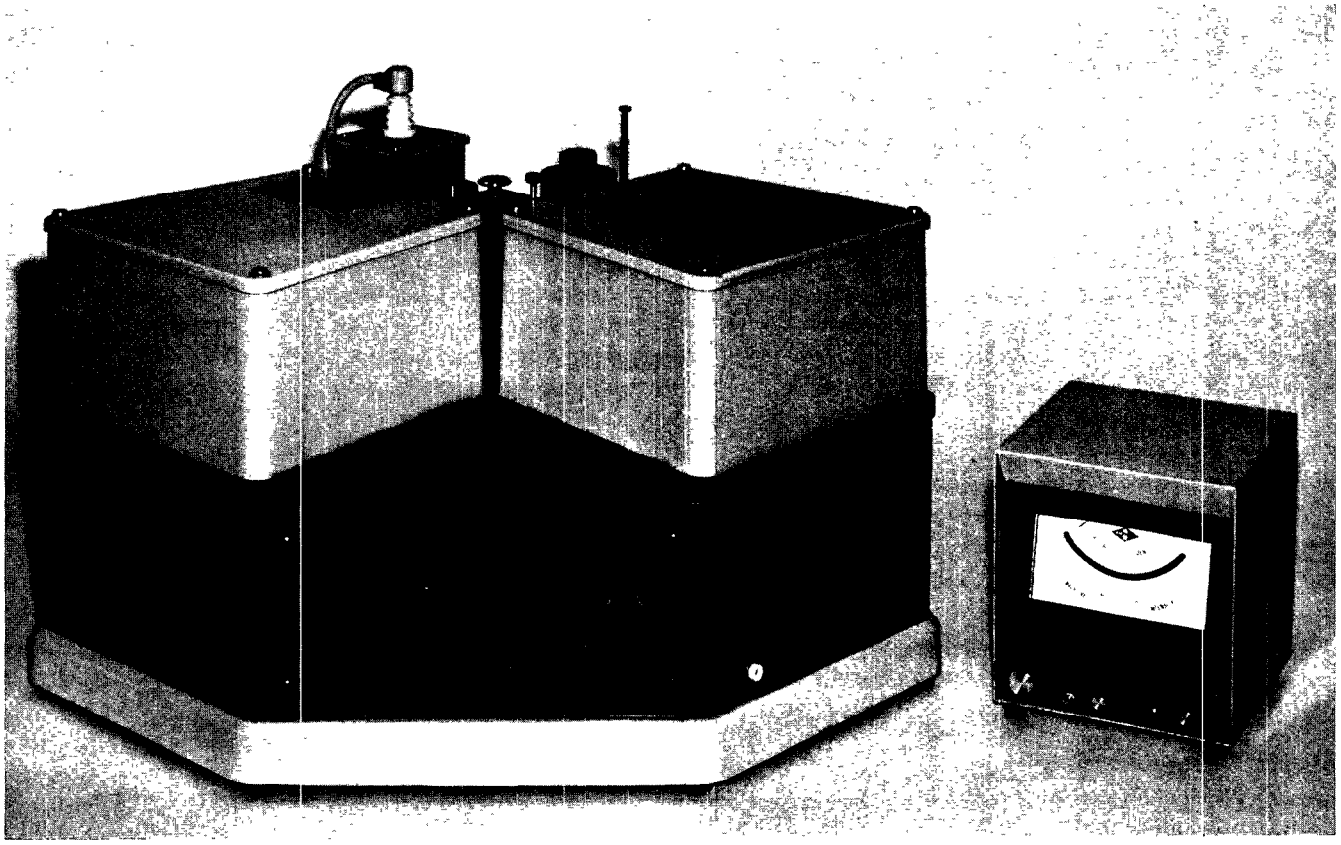

Figure 58.—Spectrofluorometer. (Photograph courtesy of AMINCO Division of SLM Instruments, Inc., Urbana, Ill.)

standard solutions and samples using the specific absorptivities of 0.0877 L/mg×cm for chlorophyll *a* and 0.0514 L/mg×cm for chlorophyll *b* from the following equation (Jeffrey and Humphrey, 1975):

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

where

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

6.20 These chlorophyll standard solutions are used to standardize the spectrofluorometer. For chlorophyll *a*, set the spectrofluorometer for an excitation wavelength of 430 nm and an emission wavelength of 670 nm. For chlorophyll *b*, the excitation wavelength is 460 nm and the emission wavelength is 650 nm. Set entrance and exit slits at 2 mm. Plot chlorophyll concentration versus relative fluorescence intensity. Determine unknown concentrations from the standard solution curve.

7. Calculations

The concentration of chlorophyll obtained in 6.20 is corrected for the concentration step onsite and in the determination:

$$\text{Original sample (micrograms chlorophyll per liter)} = \frac{\text{Micrograms chlorophyll per milliliter (as in 6.20)} \times 3\ \text{mL} \times \dfrac{\text{Concentrate volume (microliters)}}{\text{Volume streaked (microliters)}}}{\text{Volume filtered onsite (liters)}}.$$

8. Reporting of results

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

9. Precision

No precision data are available.

10. Source of information

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