

Technical Series  
Volume 10  
Number 1  
June 1990

State of Florida  
Department of  
Environmental Regulation

## Methods for Aquatic Biology

Landon T. Ross



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# Introduction

Biological methods, in part at least, are not as well standardized as those of many other disciplines. It is not possible to cite one source, a biological *Standard Methods*, for even the majority of the procedures in common use. This document refers to published methods when they are readily accessible, generally avoiding repeating them. Other methods have been developed, or at least modified, by the department, or are to be found only in specialized literature, and are presented in detail.

It will be noted that a few of the methods contained here are not typically "biological" in nature. Nonetheless, these are procedures often carried out by biologists in the Department of Environmental Regulation.

This manual is intended as a reference to the methods routinely being used by the biologists of the Florida Department of Environmental Regulation. It is written to provide a single location for method citation; to provide guidance to the department's biologists in carrying out procedures so that the results will be readily compared with other department data, as well as for other biologists who wish or need to use the same methods as the department; and to provide written documentation for quality assurance purposes.

Procedures relating to quality assurance have been grouped into a separate section. It would have been preferable for these to have been incorporated into the individual methods, but a good deal of duplication would have been necessary since many methods involve the same principals of quality assurance. Anyone using this manual should carefully check the listings under Quality Assurance.

# Acknowledgements

The course of development of this manual has extended over a period of some twenty years. Most of the biologists who have been employed by the Department of

Environmental Regulation or its predecessor agency, the Department of Pollution Control, have contributed to the methods listed. Special efforts by the following individuals are gratefully acknowledged: Bill Beck (Florida Index), Charlie Biedermann (Chlorophyll and Phaeophytin), Marshall Faircloth (Algal Assays), Russ Frydenborg (Chlorophyll and Phaeophytin), Jim Hulbert (Florida Index), Frank Phillips (Sediments), Harvey Rudolph (Marine Invertebrate Identification References), Leslee Williams (Bacteria and Quality Assurance), and Steve Wolfe (Toxicity Assays).

# General Methods

## Nomenclatural Guidelines

1. All organisms should be divided into separate species. *Xus* spp. should not be used to indicate more than one species of the genus *Xus*, except as a last resort.

2. The number of tallies reported for a sample should be equal to the number of species present. Different life stages, color patterns, etc., should not be tallied separately.

3. When species names are unknown, they should be reported in the form *Zus* sp. #2 - Doe (if John Doe identifies the reference specimen) and serially numbered within each genus and for a given authority, using whole numbers. The authority name is an important part of the name, to be used to distinguish the various possible *Zus* sp. #2's in the state. A specimen labeled in this manner should be kept in a reference collection and the name remain constant, regardless of personnel changes, until the correct binomial is learned. Existing reference collections should be converted to this format and exchange of reference material is encouraged to limit the number of duplicate (or multiply) designations attached to a single species.

4. The format, *Zus* sp. #2 - Doe, should be used whenever possible as this is more informative than *Zus* sp. The latter designation should only be used when dealing with an apparent single species with characteristics insufficiently distinct to create a numerical designation.

5. Uncertain identifications should be handled in the following manner:

a. *Zus* cf. *yus*, for uncertain species

b. cf. *Xus*, for uncertain genus

c. cf. *Xus yus*, for uncertain genus but if correct, the species is *yus*



d. cf. *Xus* cf. *yus*, for genus and species both uncertain

e. *Xus* (*Aus*) *yus*, for subgenera

The use of question marks, parentheses, etc., for uncertain identifications should be avoided as this can cause confusion.

## Identification References

The following reference lists have been developed over a number of years, and contain the identification literature which has been found to be most useful for the groups we work with in Florida. References marked with an asterisk (\*) are considered to be of the highest utility.

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## Macroinvertebrates

### Sample Size

Two major constraints are involved with respect to biological sample size; having a sample of adequate size and having a sample capable of being analyzed in a reasonable time. These factors are, to some extent, mutually contradictory, and no entirely satisfactory compromise is possible. The specifications listed below are

believed to be generally conservative with regard to adequacy of sample size. Compromising has been in the direction of sometimes needing to deal with extensive sample processing times. While these methods are adequate for the department's routine monitoring purposes, studies or investigations for special purposes may require some specific level of statistical accuracy. In such instances, other, more statistically based methods may need to be used.

A basic macroinvertebrate sample is made up of three individual replicates which are composited to yield the sample values. If the first replicate examined (either artificial substrate or grab sample) contains more than 200 individuals, however, that replicate may be considered to be the entire sample. The other two replicates may be discarded or not taken, as appropriate. In the case of grab samples, the first grab taken should be examined in the field to estimate the number of organisms present. If this number is less than 15, additional grabs should be taken until a total of at least 15 organisms is obtained. The number of grabs required to reach this total then becomes one replicate, and two more replicates should be taken, each consisting of the same number of grabs as the first replicate.

Even when limited to a single replicate, occasional samples may contain very large numbers of individuals. If, as is often the case, these are organisms requiring detailed individual preparation prior to identification, such as chironomid midge larvae, tubificids, or small polychaetes, and more than 100 of them are present, the number of individuals so prepared must be reduced to a fraction of those present in the replicate. As a first step, the organisms should be sorted into morphotypes using a dissecting microscope. Then, ten individuals of each type, if available, should be properly prepared and identified. If all of the identified individuals of a morphotype are found to consist of a single species, then that identification should be used for the remaining individuals of the morphotype. If the individuals are divided more or less evenly among two or three species, these proportions should be applied to the remaining specimens of that type. If more than three species are found, all of the remaining specimens of that type will need to be individually prepared and identified.

## **Artificial Substrates**

Macroinvertebrates acceptable for this method are those retained by a U.S. Standard No. 30 sieve, excluding Cladocera, Copepoda, Ostracoda, Nematoda, and



Foraminifera. Colonial invertebrates should be counted as colonial units according to the analyst's judgement. Individuals within a colony are not to be counted.

Artificial substrates should be put in place four weeks before other samples are to be taken. This should be done early enough to ensure that recovery is during the appropriate quarter. Three EPA-modified Hester-Dendy artificial substrates are to be used at each station, suspended from the surface at mid-depth or at 1 meter, whichever is less. Alternately, the substrates may be attached by stainless steel rods of about 30 cm in length to concrete blocks and placed on the bottom of the body of water. This second method of placement is particularly recommended for use in flowing waters with reasonably firm substrates. They should be located to avoid vandalism and accidental damage as well as possible. When retrieved, care should be taken to avoid dislodging any organisms and each substrate must be placed in a separate container.

All composite counts should include both numbers of organisms tallied and concentrations per square meter. The surface area of a metric EPA-modified Hester-Dendy artificial substrate is  $0.126 \text{ m}^2$  (large disc diameter = 75 mm).

## Natural Substrates

Samples should be taken using either an Ekman dredge (soft substrates) or a Petite Ponar dredge (hard substrates). Identifications and counts should be handled similarly to those for artificial substrates. The internal measurements of the dredge actually used for sampling should be determined to establish the surface area sampled per grab (approximately  $0.024 \text{ m}^2$ ).

## Qualitative

This method is preferably limited to rivers and streams, although useful data may also sometimes be obtained from lakes. The Florida Index (see below) is not applicable to marine waters. Collection should be primarily by D-frame nets although other apparatus, such as dredges, Surber samplers, and corers, may be used. All habitats at a station should be sampled, including submerged vegetation,

hard substrates of all types (including internal examination of wood fragments or pieces of clay), and all sediment types. Field sorting is usually required to determine when sampling is complete. Sampling is usually adequate if no new species are found after about 10 minutes of sampling. Two to six hours may ultimately be required to cover a station fully.

The macroinvertebrates collected should be identified, but not counted, and reported along with the Florida Index classes to which they belong. Only Classes I and II are required. Classes I and II organisms, acceptable as of 19 April 1977, are listed in Table I. Taxa listed to species in the table must be identified to species to be used for calculation of the Florida Index. Taxa listed to genera should at least be sorted into species to calculate the Index properly ( $F.I. = (\text{No. of spp. in Class I} \times 2) + (\text{No. of spp. in Class II})$ ).

**Table I**  
**Florida Index**

| Class I                                            | Class II                                                                                         |
|----------------------------------------------------|--------------------------------------------------------------------------------------------------|
| <i>Elimia</i> (= <i>Goniobasis</i> ) (all species) | <i>Asellus</i> (all species)                                                                     |
| <i>Plecoptera</i> (all species)                    | <i>Gammaridae</i> (all species)                                                                  |
|                                                    | <i>Palaemonetes paludosus</i>                                                                    |
| <i>Argia</i> (all species)                         | <i>Gomphus</i> (all species)                                                                     |
| <i>Boyeria</i> (all species)                       | <i>Neurocordulia</i> (all species)                                                               |
| <i>Calopteryx</i> (= <i>Agrion</i> ) (all species) |                                                                                                  |
| <i>Hetaerina americana</i>                         | <i>Stenonema mexicanum integrum</i>                                                              |
| <i>Hetaerina titia</i>                             | <i>Stenacron interpunctatum</i>                                                                  |
| <i>Macromia</i> (all species)                      | <i>Tricorythodes albilineatus</i>                                                                |
| <i>Progomphus</i> (all species)                    |                                                                                                  |
|                                                    | <i>Cheumatopsyche</i> (all species)                                                              |
| <i>Corydalus cornutus</i>                          | <i>Leptocella</i> (= <i>Nectopsyche</i> ) (all species)                                          |
|                                                    | <i>Oecetis</i> (all species)                                                                     |
| <i>Stenonema exiguum</i>                           |                                                                                                  |
| <i>Stenonema smithae</i>                           | <i>Ablabesmyia ramphe</i> group (= <i>A. janta</i> ,<br><i>A. parajanta</i> , <i>A. ramphe</i> ) |

*Brachycentrus* (all species)  
*Chimarra* spp. (all species)  
*Hydropsyche* (all species)  
*Hydroptila* (all species)  
*Macronemum carolina*  
*Oxyethira* (all species)  
*Polycentropus* (all species)

Simuliidae (all species)

*Ablabesmyia aspera*

*Ablabesmyia mallochi* (= *A. ornata*, *A. tarella*)

*Corynoneura* (all species)

*Cricotopus bicinctus*

*Eukiefferiella* (all species)

*Labrundinia johannseni*

*Labrundinia neopilosella*

*Labrundinia pilosella* (= *L. floridana*)

*Labrundinia virescens*

*Pentaneura inculta*

*Polypedilum fallax*

*Psectrocladius* (all species)

*Rheocricotopus robacki*

*Rheotanytarsus exiguus* group

*Stenochironomus* (all species)

*Stictochironomus devinctus*

*Thienemanniella* (all species)

*Xylotopus par* (= *Brillia par*)

*Ablabesmyia parajanta*

*Ablabesmyia peleensis*

*Clinotanypus* (all species)

*Cricotopus* (all species ex. *C. bicinctus*)

*Endochironomus nigricans*

*Larsia lurida*

*Polypedilum halterale*

*Polypedilum illinoense*

*Procladius* (all species)

## Periphyton

Samples should be collected by suspending eight 25 X 75 mm microscope slides vertically approximately 15 cm from the surface for two weeks before other samples are to be taken. Four of the slides are to be used for identification and counts, and

four for biomass measurements. In marine samples, chlorophyll a analysis should be substituted for ash-free dry weight biomass measurements (see below).

**Counts** - The slides should be scraped with a razor blade and rubber spatula and the scrapings dispersed in a known volume (usually 10 - 100 ml) of distilled water. Preservation, if required, should be with 5% buffered formalin.

Samples are not to be stirred in a blender as suggested in the EPA's *Biological Field and Laboratory Methods* (1973), as this breaks up filaments and colonies.

Counts should be made using the Sedgwick-Rafter strip count method or one similar. Unit counts, not cell counts, should be made and reported for the following groups: coccoid blue-greens, filamentous blue-greens, coccoid greens, filamentous greens, green flagellates, other pigmented flagellates, centric diatoms, pennate diatoms, and total live algae. Dead diatom frustules are not to be counted.

In the Cyanophyta, all non-filamentous forms are to be reported as coccoid. This includes non-filamentous colonies. In the Chlorophyta, genera that are not filamentous or flagellate are to be reported as coccoid. Care should be taken not to include colonial flagellates, such as *Volvox* sp. in the coccoid category.

Counts should be made and reported for all replicates and on a separate composite sheet in units per square centimeter.

**Biomass** - The four slides should be scraped and the scrapings suspended in the same manner as for counts. The sample should be washed several times by centrifugation or settling to reduce interference by dissolved solids. After washing, it should be concentrated by centrifugation or settling.

To determine dry weight, the concentrated sample should be placed in a tared crucible and dried to a constant weight (approximately 24 hours) at 105°C. The crucible should then be placed in a muffle furnace at 550°C for one hour, cooled, rewet with distilled water, and dried again at 105°C to obtain ash weight. The biomass (ash-free weight) of the sample is obtained by subtracting ash weight from dry weight and is to be reported in *grams per square centimeter* of substrate scraped.

## Phytoplankton

A grab sample should be taken from just below the water's surface (usually 0.2 - 0.5 m) using a clean collecting device which has been rinsed (including drainage tube) with water from the same station. The sample bottle should be cleaned with a laboratory detergent and rinsed thoroughly after each use. Generally, a 500 ml sample is ample and dilutions may be appropriate for very dense phytoplankton populations.

If "live" analysis is planned, the sample bottle should be kept away from light and thermal or physical shocks. There are several acceptable preservation methods (e.g., formalin, Lugol's solution, merthiolate) for algae. If formalin is used, it should be neutralized to avoid cytolysis or morphological distortion. Generally, 500 ml samples require about 10 ml of preservative.

Phytoplankton counts should be done using the Sedgwick-Rafter strip count method or one similar. Unit counts, not cell counts, should be used and reported to the same groups as for periphyton. Counts are to be reported in units per cubic centimeter (ml).

If sample concentration is appropriate, this can be done by sedimentation and/or centrifugation as described in *Standard Methods*. In either case, the supernatant should be checked occasionally to ensure thorough sample concentration. It is recommended that chambers and tubes used be cleaned periodically with hot chromic acid and rinsed thoroughly.

## Macrophytes

This is not intended to be a comprehensive study of plant communities, but rather a rough map or chart depicting major species composition and areal coverage

of the aquatic and wetland plants in the immediate vicinity of the sampling station. Not more than one to two hours should be spent on this task.

Generally, in rivers, streams, etc., the area covered should reach about 100 meters upstream and downstream from the station. In lakes and marine/estuarine locations, an area extending 100 meters from the point on shore that is nearest to the station will usually be sufficient. In lakes and marine/estuarine areas of less than 4 hectares or 250 meters in diameter, the entire shoreline should be mapped. The station location should be indicated on the map.

The types of communities recorded should include immergent, emergent, floating, marsh and flood plain. The depth of some marsh communities may have to be estimated by stepping-off distances. Estimates for near shore communities may be made by using boat length as a unit of measure. Species which are not easily recognizable should be recorded as "sp. 1" etc., with samples taken back to the laboratory for identification. Immergent vegetation in unclear waters should be sampled with a device such as an Ekman dredge.

## **Bacteria**

### **General**

Bacteriological samples are collected in conjunction with compliance monitoring, enforcement actions, ambient water quality monitoring, emergency response operations, and pollution identification surveys.

### **Methods**

Membrane filter (MF) methods are included here for laboratories which analyze samples for total coliform, fecal coliform, and fecal streptococci bacteria. Although

total and fecal coliform tests are required by Florida regulation, fecal streptococci are included in case separation of warm-blooded sources of pollution becomes necessary. Heterotrophic plate count (also referred to as Standard Plate Count and Total Aerobic Plate Count) is included since it is both a quality control requirement for determining acceptability of the laboratory's deionized water and measurement of the total heterotrophic bacterial population in an environmental water sample. Heterotrophic plate counts can be determined with either membrane filter or pour plate methods. Ambient or environmental samples can be processed by either method. Quality control analyses of deionized water is most frequently done by pour plate method.

## **Sample Collection and Handling**

Detailed instructions on this subject are available in *Standard Methods*, 16th Edition, 1985, pages 856-859 and in *Microbiological Methods for Monitoring the Environment*; EPA 600/8-78-016, pages 5-31. The following additional instructions should be observed.

1. Samples are to be labeled as to station number, date, time of collection, and name of sampler.

2. Samples are to be maintained in wet ice while in transport. Once in the laboratory they should be immediately processed. If a delay in processing cannot be avoided, samples should be stored in the dark at 4°C. This delay must not exceed 8 hours from collection time. Samples are not to be frozen since freezing and subsequent thawing destroys the bacteria.

3. For any sample(s) subject to litigation, complete chain-of-custody procedures should be followed.

4. Ambient monitoring samples which exceed the 8 hours holding time should have strict documentation regarding collection time and processing time. Data should be labelled as "Q" (= out of holding time) when entered into STORET.

## **General Laboratory Practices**

Incubators (low and high temperature types) and water bath temperatures are to be checked twice daily. Water baths are to be cleaned periodically to prevent scale.

Autoclaves are to be checked periodically for proper performance using acceptable quality assurance methods. Autoclave tape, sterilometers, or comparable

incubators are to be used at all times. Diack® tubes or comparable devices are to be used to ensure sterilization of large volume liquids, e.g., phosphate buffered water, with each batch.

Pre-sterilized membrane filters are to be 47 mm in diameter, 0.45  $\mu$  pore size and non-gas sterilized. Chemical sterilization may produce inhibitory by products that interfere with recovery. A number of acceptable commercial products are on the market. Petri dishes for MF work should be 50 X 12 mm in size with tight fitted lids.

Filtration apparatus can be glass, stainless steel, or autoclavable plastic. All units are to be completely wrapped and sterilized prior to use. Do not use aluminum foil with stainless steel funnels; brown paper with autoclave tape is preferred.

Dehydrated media or pre-prepared ampouled media are acceptable for membrane filter analyses. Media lots are variable and all appropriate quality control checks should be done to assure accurate analyses. Phosphate buffered water should be used when sample dilution is required. Check *Standard Methods* and *Microbiological Methods for Monitoring the Environment*, for additional information.

For any sample, at least three dilutions are to be run to bracket the countable membrane. The acceptable ranges for numbers of well-distributed and well-defined colonies are: Total Coliforms 20 to 80, Fecal Coliforms 20 to 60, Fecal Streptococci 20 to 100, and Heterotrophic Count 30 to 300.

Although membrane filter analysis has gained wide acceptance, certain limitation should be noted.

1. It should not be used in the examination of chlorinated waters. Chlorine injured bacteria are underestimated since they do not grow well on the membrane filter.
2. It cannot be used in high turbidity samples which cause clogging of the filter pores.
3. Injured bacteria in the sample, though viable, might fail to form colonies on the membrane filter.
4. There are some indications that the chemical used to produce the grid markings on the filter might be inhibitory.



There are no definite guideline as to what level of turbidity or impurities (algae, etc.) would cause clogging of membrane pores. It is therefore recommended that personal judgement be exercised in this regard. Either serial dilution of the sample in question should be employed to reduce the level, or the MPN method be used in place of the MF method.

The MPN method is also recommended as a quality control measure to be run in parallel with the MF method whenever a highly turbid sample is encountered. Chlorine and turbidity levels in the sample are the two major factors used in determining what methods (MPN vs. MF) are to be used.

References for the membrane filter analyses should be consulted for complete details of analyses, enumeration, verification, and interpretation of results.

|                     | <i>Std. Methods</i> 16th ed. | <i>Microbiolog. Methods</i> |
|---------------------|------------------------------|-----------------------------|
|                     | Section: Pages               | Section: Pages              |
| Heterotrophic Count | 907C: 869-870                | No reference                |
| Total Coliforms     | 909A: 887-894                | IIIB: 108-114               |
| Fecal Coliforms     | 909C: 895-898                | IIIC: 124-138               |
| Fecal Streptococci  | 910B: 905-907                | IIID: 136-138               |

The Pour Plate method for heterotrophic plate counts can be found in *Standard Methods*, 16th ed., Section 907A, pp. 864-866 and *Microbiological Methods for Monitoring the Environment*, Section IIIA, pp. 101-107.

Quality assurance protocols and quality control tests for the microbiology laboratory are addressed in *Standard Methods*, 16th ed., Section 902, pp. 830-849 and in *Microbiological Methods*, Part IV, pp. 192-242.

## Interpretation of Bacterial Indicators

**Heterotrophic Plate Counts.** Assays for heterotrophic plate count are often done in pollution identification surveys to detect the total aerobic bacterial population in a water body. All other bacterial populations discussed in this section are a subpopulation of this group.

**Total Coliform Bacteria.** Total coliform bacteria are aerobic and facultative anaerobic gram negative bacteria which ferment lactose within 48 hours at  $35 \pm 0.5^\circ\text{C}$ . These organisms appear as dark black-brown colonies with a green metallic sheen in 24 hours on M-Endo (membrane filter) media.

These organisms represent the presence of soil-associated bacteria which meet the definition above. Test for these organisms are done to investigate natural influences to a water body, e.g., rainfall runoff.

**Fecal Coliform Bacteria.** Fecal coliform bacteria are a subset of the total coliform group representing the presence of fecal material from warm blooded animals. These organisms are differentiated from the total coliform group by growing on a selective media (M-FC media) at an elevated temperature,  $44.5 \pm 0.2^\circ\text{C}$ . These organisms appear dark blue with a crystalline like center on M-FC media. This group of organisms determine the impact of fecal pollution and is usually the test of choice for pollution identification when time is limited.

**Fecal Streptococci Bacteria.** Fecal streptococci bacteria are not part of the fecal coliform group. These organisms do inhabit the intestinal tract of warm-blooded animals and, thus, are another type of fecal pollution indicator. KF streptococcus agar is the media of choice and organisms appear as ruby red colonies on the membrane filter. These organisms are found in greater densities in animals other than man. On this basis, Geldreich and Kenner (1969) proposed the fecal coliform-fecal streptococci ratio. Ratios greater than 4.0 strongly indicate the presence of human associated pollution in freshwaters. Ratios less than 0.1 strongly indicate the presence of agricultural or non-human animal pollution. Ratios between 4.0 and 0.1 indicate mixed sources. One caution in the application of this ratio must be noted. This ratio is not applicable to salt waters (estuarine or brackish). Fecal streptococci densities are artificially enhanced in saltwater since these organisms grow very well in the saltwater environment. Of similar concern, fecal coliform bacteria are inhibited in saltwaters and are therefore underestimated. As a result, the ratio is not a reliable indication of pollution sources in saltwaters.

# Bioassays

## Toxicity Assays

Acute and chronic toxicity bioassays are conducted following EPA methods (Peltier and Weber, 1985; Weber *et al.*, 1988, 1989). Our recommended method, when alternatives are presented by EPA, or exceptions to the methods are discussed further below.

### Test Species

**Acute toxicity testing.** The following species are recommended for use (Peltier and Weber, 1985):

Fresh water:

*Notropis leedsi* (bannerfin shiner) \*

*Ceriodaphnia dubia* (water flea)

*Daphnia pulex* (water flea)

Salt water:

*Menidia beryllina* (inland silversides)

*Mysidopsis bahia* (mysid or possum shrimp)

\* this species is not in the EPA manual

**Chronic toxicity testing.** The following species are recommended for use (Weber *et al.*, 1988, 1989):

Fresh water:

*Pimephales promelas* (fathead minnow)

*Ceriodaphnia dubia* (water flea)

*Selenastrum capricornutum* (green alga)

Salt water:

*Menidia beryllina* (inland silversides)

*Mysidopsis bahia* (mysid or possum shrimp)

*Arbacia punctulata* (sea urchin)

*Champia parvula* (red alga)

## Test Organism Culture

Test organisms are cultured according to EPA methods with the following exceptions:

*Notropis leedsi* is not yet included in the EPA acute toxicity manual. Bannerfin shiners are cultured by keeping approximately 100–150 mixed adults and juveniles, approximately half male and half female, in 125 gal. bare aquaria with flow-through water. When eggs are desired, stacks of 6 in. rectangular clay tiles, separated by approximately 3/16 in. silicone sealer beads, are placed in the tanks. The shiners are crevice spawners and readily use the spaces between the tiles as spawning sites when water temperatures are above about 20° C. The tiles are removed within 24 hours to provide fry for testing that are within 24 hours of the same age. The egg-carrying tiles are either placed in separate aquaria with *Maroxy*™ fungicide and aerated to keep them free of detritus, or the eggs are removed from the tiles and placed in vigorously aerated containers, also treated with *Maroxy*, to keep them suspended until hatching. At 25° C, hatching takes place in approximately 96 hours.

The newly hatched fry live off yolk sacs for the first day or two. On days two through four (after hatching), the fry are fed *Daphnia* chow. Beginning on day three they are also fed *Artemia* nauplii.

*Ceriodaphnia dubia* are used in both acute and chronic bioassays. They are cultured at 20° C in 2-gal. unaerated aquaria with a 16-hr light/8-hr dark light cycle. They are fed twice daily during the week and once daily on weekends with the *Ceriodaphnia* chow described in Weber *et al.*, 1989. Weekly, algae are scraped from the aquaria sides and approximately 75% of the water, along with detritus and the excess population, are siphoned out. The water is replaced with deep-well water.

## Test Methods

*Notropis leedsi*. The bannerfin shiner, a species indigenous to Florida, is used for acute toxicity testing. The protocol is identical to that for *Pimephales promelas*, the fathead minnow (Peltier and Weber, 1985).

*Ceriodaphnia dubia*. *Ceriodaphnia dubia* is used for acute as well as chronic toxicity testing. For both tests, a "conditioned" water is used for controls and diluent. Research has indicated that *Ceriodaphnia dubia* requires certain trace elements

(e.g., selenium) in the water for health. Deep-well water is conditioned by adding *Ceriodaphnia* chow (approximately 1.5 ml/l) and aerating for approximately one week.

With the exception of the dilution water, *C. dubia* chronic tests follow the EPA methods (Weber *et al.*, 1988). When used for acute toxicity testing, the protocols for *Daphnia pulex* (Peltier and Weber, 1985) are followed except that tests are carried out in the 1 oz. plastic vials used for the chronic test, and five test organisms are loaded into each of four replicates, so that 20 are exposed to each test concentration. Individuals of the correct age (<24 hours old) are obtained by sieving adults from a culture tank the day before a test begins and placing them into a Carolina dish of conditioned water. During loading of the test chambers, individuals suitable for testing are readily identified as the Carolina dish contains only adults or <24-hr old neonates. The test individuals are transferred directly from the Carolina dish to the test chambers. The number of neonates in each test vial is counted under a dissecting microscope after loading and at 24-hr intervals until the test ends. Counting is greatly simplified if the dissecting scope used has low enough power and great enough depth of field to permit the entire diameter and depth of water in the test vial to be in focus at once.

## Algal Assays

Algal assays provide a mechanism for empirically testing the growth responses of algae to water samples, with or without the addition of other substances.

### Algal Growth Potential

The algal growth potential (AGP) test measures the amount of growth that occurs in a test species of algae, over a fixed time interval, when it is placed in a water sample. Concurrent chemical tests of the nutrient concentrations in the sample, particularly soluble species, are normally made to aid in interpretation of the results of the AGP test. Methods followed are those specified in U.S. EPA, 1974 and 1978b, for marine and freshwater samples, respectively. Modifications or additions to the EPA methodology are specified below.

*Selenastrum capricornutum* is to be used as the test species in waters which are of less than 2‰ salinity. For more saline waters, *Dunaliella tertiolecta* is to be used.

If a test sample is between 2‰ and 5‰, it is to be adjusted to 5‰ using sea salts. Stock cultures should be transferred to new flasks every 21 days, and new cultures for tests started seven days prior to test initiation. Glassware for use in algal assay tests should be autoclaved prior to usage. Data are to be reported as mg/l dry weight of the 14-day standing crop.

### **Limiting Nutrient**

The limiting nutrient algal assay determines the growth response of an algal test species to the addition of various nutrients, singly and in combination. The results allow one to predict, to some extent, the impact that nutrient additions may have on ambient waters. The AGP test, in which no nutrients are added, makes up a portion of the limiting nutrient test. The same test methods are utilized as were specified above for the AGP test, U.S. EPA, 1974 and 1978b, however, a phosphorus spike of 0.1 mg/l is to be used rather than 0.05 mg/l.

## **Chlorophyll and Phaeophytin**

Chlorophyll *a* is found in all species of algae and has, therefore, been widely used as a measure of phytoplankton standing crop. Biomass determinations of this sort are considered relatively imprecise due to the variability of the chlorophyll content found between species and within a species depending on a variety of chemical, physical, and biological factors. Additional variation can also result with the ease of pigment extraction. For example, coccoid green forms extract with difficulty while diatoms extract rather easily, older cells were found to be more difficult to extract than young cells (U.S. EPA, 1973). To overcome these problems, a variety of techniques for pigment extraction have been proposed. Rather than simplifying the problem, however, these differing methods result in a further variability in the results depending on which method is chosen and makes comparisons of results impossible. Also the lack of specific procedural details allows for a high degree of experimental error. There is, therefore, an overwhelming need for the development and universal acceptance of one standard method for the extraction of phytoplankton chlorophyll. The procedure detailed here is a modification of the ones specified in

*EPA Biological Field and Laboratory Methods (1973), Standard Methods for the Examination of Water and Waste Water (1976), and Strickland and Parsons (1972), but includes specific details which will help eliminate procedural variations between laboratories.*

## **Reagent List and Preparation**

1. Reagent Grade Acetone

2. 2 N Hydrochloric Acid

3. Magnesium Carbonate ( $\text{MgCO}_3$ ) Aqueous Suspension: Add 1 g  $\text{MgCO}_3$  to 100 ml distilled water and mix well. Filter the mixture through a 0.45  $\mu$  effective pore size GF/C glass fiber filter and keep the filtrate.

4. 90% Aqueous Acetone–Magnesium Carbonate Suspension: Add 100 ml filtered magnesium carbonate aqueous suspension to 900 ml reagent grade acetone (the resulting suspension will be slightly less than 1 liter). Discard the suspension if a floc forms.

## **Sample Collection**

1. Collect a minimum of 500 ml of sample for analysis. In oligotrophic waters substantially larger sample quantities are needed.

2. To avoid chlorophyll degradation resulting from excessive heat and light, the samples should be stored in opaque, thoroughly cleaned bottles and iced during transportation from the sample site.

3. Since preservation of unprocessed chlorophyll samples is not recommended in the literature, a maximum refrigerated holding period of 24 hours prior to filtration is suggested.

## Filtration

1. Thoroughly mix the sample by completely inverting the collection bottle several times to ensure a uniform distribution of the algae within the sample.

2. Filter two replicate portions of the sample through GF/C, 5.5 cm diameter, glass fiber filters.

3. Add approximately 1 ml of aqueous magnesium carbonate suspension to the sample just prior to completion of the filtering process.

4. Dry the filters by maintaining the vacuum for 0.5 min. after all the water has been filtered.

5. Wrap the filters with aluminum foil and label (fold the filters in half so that the algae are on the inside). Store by freezing the wrapped filters.

Should completion of the analysis be delayed, the filter containing the algae may be stored for a maximum of 30 days.

## Pigment Extraction

Due to the instability of the chlorophyll molecule the following procedure should be conducted under subdued lighting.

1. Macerate the glass fiber filter in a 30 ml Potter Elvehjen glass tissue grinder tube with a teflon pestle (manufactured by Wheaton Scientific and available from Scientific Products, #T4028-30) using approximately 3 ml aqueous acetone-MgCO<sub>3</sub> suspension for 1 min. at 500 rpm. The teflon pestle should be fluted to facilitate complete grinding. This may be easily accomplished by filing the bottom of the pestle with a triangular cross-section file. To be sure that all parts of the filters are completely ground it is necessary to carefully fold the filters into quarters and push them completely to the bottom of the glass tube. The mixture should also be agitated several times



during the grinding procedure. To avoid excessive heating of the mixture during grinding, keep the grinding tube immersed in a jar of ice water.

2. Rinse the teflon pestle with 1 or 2 ml of the aqueous acetone-MgCO<sub>3</sub> suspension into the tissue grinder tube.

3. Carefully transfer the mixture to a 15 ml screw cap centrifuge tube. Rinse the tissue grinder tube with the aqueous acetone-MgCO<sub>3</sub> suspension and transfer the rinse to the centrifuge tube being careful not to exceed a final volume of 12 ml. Record the exact volume.

4. Wrap the centrifuge tubes with aluminum foil to exclude light from the mixture during pigment extraction. Label both the tube and wrapping.

5. Steep for 18 to 24 hours in refrigeration (4°C) prior to absorbance analysis.

6. Remove the centrifuge tubes from the refrigerator and clarify the extract by centrifugation for 10 minutes at 3000 rpm.

## **Spectrophotometer Calibration**

1. A narrow band (0.5 to 2 nm) double beam spectrophotometer is preferred.

2. Turn on the spectrophotometer according to the procedure set forth in the instrument manual. Be sure to allow for an adequate warm up period. Check for meter fluctuations due to vibration or power surges and minimize these effects.

3. Calibrate the instrument according to the manual directions. Record any anomalies peculiar to the spectrophotometer. Maintain a quality control data sheet for the instrument and up-date this sheet prior to each use.

4. Spectrophotometric cuvettes used for pigment analysis should be the quartz or "spectrosil" type and have a path length of 1 cm. Use the same two

cuvettes throughout the analysis to eliminate the error resulting from the optical properties peculiar to each cuvette.

5. Fill the two clean cuvettes selected for the analysis with approximately 4 ml of the 90% acetone-MgCO<sub>3</sub> solution and insert them into the sample and reference channels of the spectrophotometer. Be sure that the cuvette trade mark is oriented in the same direction each time the cuvette is inserted into the spectrophotometer.

6. Adjust the instrument to read 0.000 at 750 and 664 nm with both sample and reference cuvettes in place.

## Pigment Analysis

1. Transfer approximately 4 ml of the extracted clarified pigment into the sample cuvette (the reference cuvette containing the reference sample remains in the reference channel of the spectrophotometer).

2. Determine the optical density of the chlorophyll sample at 750, 664, and record the values.

3. Obtain a turbidity corrected value by subtracting the 750 nm optical density from the 664 value. Record the value corrected for turbidity as 664<sub>A</sub>. If the optical density at 750 nm is greater than the density at 664 nm (i. e., the calculated corrected value would be negative), too much interference is present to allow analysis.

4. Acidify the sample cuvette with two drops of 2 N HCl and gently invert three times (use stopper). Allow a minimum of 1 minutes, but not more than 2 minutes, for any reaction.

5. Determine the optical density at 750 and 665 nm.

6. Obtain a corrected, after-acidification value (665<sub>A</sub>) by subtracting the 750 nm after acidification absorbance from the value determined at 665 nm in step 5, above.

Be sure to stopper the cuvettes during absorbance determinations to prevent the evaporation of acetone. Rinse the sample cuvette between samples with the

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With respect to the chlorophyll calculations, L is the light path length measured in cm, *not*  $\mu\text{m}$ , and the correct equation for phaeophytin is:

$$\text{Phaeophytin } a \text{ (mg/m}^3\text{)} = \frac{26.73(1.7[665_A] - 664_B) \times V_1}{V_2 \times L}$$

acetone-MgCO<sub>3</sub> solution and with a small amount of the next sample to be analyzed. Be sure that the spectrophotometer remains zeroed for the aqueous acetone-MgCO<sub>3</sub> solution, checking after every four samples analyzed.

## Calculations

1. To obtain an estimate of the active photosynthetic pigments, chlorophyll *a* values should be calculated according to the monochromatic equation (Lorenzen, 1967) which corrects for phaeophytin *a* (the degradation product of chlorophyll *a*).

$$\text{Phaeophytin corrected Chlorophyll } a \text{ (mg/m}^3\text{)} = \frac{26.73 (664_B - 665_A) \times V_1}{V_2 \times L}$$

$$\text{Phaeophytin } a \text{ (mg/m}^3\text{)} = \frac{26.73 (1.7(664_B - 665_A)) \times V_1}{V_2 \times L}$$

664<sub>B</sub> and 665<sub>A</sub> are the corrected optical densities at 664 nm before and 665 nm after acidification, respectively, V<sub>1</sub> is the volume of extract (ml) and V<sub>2</sub> is the volume of sample filtered (liters). L is the light path length (μm).

## Sediments

Generally, an Ekman dredge should be used to sample soft substrates and a Petite Ponar dredge for hard substrates. If the dredge does not close completely, and small particles are lost upon retrieval, the sample should be discarded and another one taken. Triplicate samples should be taken and results averaged to constitute a composite.

If a relatively undisturbed sample is obviously stratified, aliquots should be taken from both layers and the two processed and reported separately. This should only be done in exceptional cases; that is, a thin organic layer over an otherwise homogeneous sand bottom does not qualify. In such a case, a comment on the data sheet will suffice.

Before removing an aliquot for analysis, the replicate should be thoroughly mixed by hand to eliminate bias due to settling during transport to the laboratory. Periodically, a second aliquot from a replicate sample should be processed to determine the efficacy of this mixing.

## Particle Size

1. a. Spread the sample aliquots evenly in drying pans and oven dry to constant weight (usually 12-24 hours). After drying, aggregates can be broken up by gentle tapping with gloved fingers or a clean cork stopper. Avoid grinding as this may break individual particles.

or

b. If the sample contains so much clay that a dense aggregate is formed which cannot be dispersed using gentle manipulation, Graham's Salt (see step "3." below), and agitation, dry weight may be estimated. Start with two aliquots of moist sediment of approximately 50 grams each. Accurately weigh both aliquots. Dry one aliquot to constant weight as described above, and accurately weigh the aliquot again. Determine dry weight as a fraction of the original weight: dry weight divided by moist weight. Multiply the weight of the second aliquot by this fraction to obtain an estimated dry weight, and proceed to step "3." using the second aliquot.

2. Accurately weigh out a convenient (for calculation purposes) amount of the dry sediment—not less than 25 grams. Save the remainder of the aliquot for determination of the organic fraction, below.

3. For every 25 g. of sample, disperse the sediment with 250 ml of distilled water and 10 ml of aqueous sodium hexametaphosphate ( $\text{NaPO}_3$ )<sub>6</sub>.

also known as Graham's Salt (6.2 g/l). Agitate the sediment-dispersant mixture with a glass rod for 10 to 15 minutes, leave overnight, and restir before continuing the analysis (12 to 15 minutes later). If a power stirrer is used, be careful not to fragment particles.

4. Wash the restirred sediment onto a 0.063 mm (U.S. Standard No. 230) sieve positioned in a white enamel pan or basin containing enough water to cover the sieve surface. Silts and clays will wash through as the sieve is agitated. A gentle stream of water will aid washing, but avoid forcing larger particles through the mesh. Change the wash water periodically to determine when separation is complete.

5. Oven dry the sieve and retained sands at 105°C.

6. After drying, any remaining silt-clay particles should be eliminated by gentle shaking and brushing. Weigh the remaining sands fraction so that the silt clay percentage can be computed as described below.

7. Wash the entire sands fraction through the nested, graded sieves (see below for sizes). A gentle stream of water from a wash bottle will aid in dislodging particles from the sides and mesh, but it is imperative that particles not be forced through sieves.

8. When washing is complete, oven dry the sediments and sieves at 105°C, restack them and dry-sieve to ensure complete separation of any aggregates formed during wet-sieving. Gentle brushing will also facilitate complete dry-sieving.

9. Clean each sieve thoroughly and weigh the trappings.

10. Compute the sediment composition (as % of weight) as follows:

a. Silt-clay component = total sediment dry weight minus washed sands component (materials retained on the No. 230 sieve in Step "6.", dry weight to be reported as < 63µm (STORET parameter code 80250).

b. Sand components = weight of material retained on nested sieves expressed as percentage of total dry weight.

**>2.0 mm = material retained on #10 sieve  
(STORET parameter code 80256)**

**0.5 to 2.0 mm = material retained on #35 sieve  
(STORET parameter code 80254)**

**0.125 to 0.5 mm = material retained on #120 sieve  
(STORET parameter code 46531)**

**0.063 to 0.125 mm = material retained on #230 sieve  
(STORET parameter code 80251)**

If the appropriate sieves are available, instead of the 0.125 to 0.5 mm size fraction, one may determine:

**0.25 to 0.5 mm = material retained on a #60 sieve  
(STORET parameter code 80253)**

**0.125 to 0.25 mm = material retained on a #120 sieve  
(STORET parameter code 80252)**

The total of the individual sand components' weights, determined above, may be compared to the weight of the total sand fraction determined in step "6.". If there is a significant difference between the two, the total of the individual sand components' weights will generally provide the more accurate value for use in calculations.

## **Organic Fraction**

1. Weigh out another aliquot of not less than 25 g and place in crucible.
2. Place crucible in muffle furnace at 550°C for 4 hours.

3. Remove crucible, cool and rewet with distilled water.
4. Oven dry at 105°C and weigh again:

$$\% \text{ organics} = \frac{(\text{initial weight} - \text{final weight}) \times 100}{\text{initial weight}}$$

(STORET parameter code 80096)

## Quality Assurance

With some exceptions, biological methods do not lend themselves to the classical types of quality assurance used for chemical/physical methods. Recommended QA techniques applicable to the methods discussed in this manual are listed here, although some techniques which were noted within the text of the individual methods (especially the bioassay methods and the chlorophyll and phaeophytin methods) will not be repeated. No specific recommendations are made at this time for the sediment methods, although the standard geological/sedimentological texts may be consulted.

## Sampling Design

In any investigation, it is necessary to ensure that the number and type of samples are adequate to meet the project requirements. In most cases, questions relating to spatial and temporal variation will also need to be addressed. Some technique for determining the precision of the chosen sampling design needs to be used. The statistical methods in Elliot (1971, p.126) are recommended for use in conjunction with a pilot sampling program.



## **Macroinvertebrate Sample Sorting**

Since macroinvertebrate samples, as originally collected, usually involve some mixture of organisms and sediment or organic debris, a sorting step is required to separate the organisms. Any failure to completely remove the macroinvertebrates will result in erroneous measurements, and should be closely guarded against. In many kinds of samples, the judicious use of a stain, typically rose bengal, will help eliminate such errors. In addition, a certain percentage of sorting dishes or trays should be examined by a second biologist after being processed. This step is particularly useful in eliminating the systematic bias of overlooking an entire group of organisms. When a new type of sample or a sample from a new location is first being examined, a higher percentage of rechecking is strongly encouraged.

## **Identification of Macroinvertebrates, Algae, and Macrophytes**

Identification may well be the single most important routinely performed function involved in biological monitoring. As such, it deserves the closest attention to quality assurance. Several factors are important in assuring the quality of identifications: the education and experience of the taxonomist and of consulting authorities, the identification references used, and the care with which specimens are archived. With regard to archiving, a series of type specimens of all species encountered should be properly kept and permanently maintained. These specimens are especially useful when verified by authoritative taxonomists. This step is particularly important with respect to organisms of uncertain or incomplete identification. In addition, it is strongly recommended that voucher specimens be kept of each species encountered from each location in which it is found.

Reference algal samples for evaluating identification ability are sometimes available from the U. S. Environmental Protection Agency, Office of Research and Development, Environmental Monitoring and Support Laboratory, Cincinnati, Ohio 45268. These should be incorporated into any algal identification program on as regular a basis as possible. (Similar macroinvertebrate reference samples have also been prepared in the past by the same source, but are no longer available.)

## **Algal Counts**

In counting algae, whether from plankton or periphyton samples, the counting device, usually a Whipple grid, should be calibrated for each microscope using a stage micrometer. Samples of simulated plankton should be processed on at least an annual basis to test counting ability. These samples are available from the U. S. EPA Cincinnati Laboratory mentioned above. In all cases, it is strongly advised to analyze three replicate samples from each station.

## **Bacteria**

A number of quality assurance techniques are specified under "Bacteria", above. Certain other QA procedures should also be followed. Sample holding time should be carefully documented, showing both time of collection and time of laboratory processing. This type of information is needed for proper data interpretation. In the absence of reference samples, it is recommended that samples be split with another laboratory on at least an annual basis.

## **Bioassays**

Quality assurance protocols have been outlined in the methods section. In addition to the procedures associated with the individual tests, current documentation in the form of bench sheets, culture data, and life history data must be maintained. For organisms not included in the cited EPA references, complete information on the method development and data validation should be maintained. Deviations from approved protocols must also be thoroughly documented and supported with the appropriate data.

## **Chlorophyll and Phaeophytin**

In addition to the quality assurance procedures specified in the methodology chapter, it is recommended that chlorophyll reference samples be analyzed periodi-

cally—preferably at least annually. Such samples can be obtained from the U. S. EPA Cincinnati Laboratory.

## Man-hour Requirements

The tabulation, below, is intended to allow the ready estimation of the workload involved for most of the biological analyses performed by the Department of Environmental Regulation. There is, of course, a large amount of variation in individual analyses; these values are intended to be averages. Travel time is not included in the "sampling" time allotments; it will need to be added for total workload determinations.

| Parameters                  | Sampling | Analysis | Total |
|-----------------------------|----------|----------|-------|
| Macroinvertebrates          |          |          |       |
| Freshwater                  |          |          |       |
| Artif. Substrate (per rep.) | .35      | 6.25     | 6.60  |
| Nat. Substrate (per rep.)   | .50      | 3.25     | 3.75  |
| Qualitative                 | 5.00     | 12.50    | 17.50 |
| Marine                      |          |          |       |
| Nat. Substrate (per rep.)   | .50      | 12.85    | 13.35 |
| Periphyton Counts           | .35      | 2.50     | 2.85  |
| Phytoplankton Counts        | .35      | 3.25     | 3.60  |
| Sediments                   | .50      | 2.50     | 3.00  |
| Macrophytes                 | .50      | .50      | 1.00  |
| Bacteria                    |          |          |       |
| Total Coliforms             | *        | .50      | .50   |
| Fecal Coliforms             | *        | .50      | .50   |

|                         |   |        |        |
|-------------------------|---|--------|--------|
| <b>Bioassays</b>        |   |        |        |
| Acute Toxicity          |   |        |        |
| Static Screening        | * | 35.00  | 35.00  |
| Static Definitive       | * | 70.00  | 70.00  |
| Static Reference        | 0 | 70.00  | 70.00  |
| Flow-through Definitive | 0 | 300.00 | 300.00 |
| Chronic Toxicity        |   |        |        |
| Static Definitive       | * | 125.00 | 125.00 |
| Algal Assay             |   |        |        |
| Algal Growth Potential  | * | 2.00   | 2.00   |
| Limiting Nutrient       | * | 8.00   | 8.00   |

\*Negligible

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