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BIOLOGICAL FIELD AND LABORATORY METHODS FOR MEASURING THE QUALITY OF SURFACE WATERS AND EFFLUENTS

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Review Notice

This report has been reviewed by the National Environmental Research Center, Cincinnati, and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

FOREWORD

Man and his environment must be protected from the adverse effects of pesticides, radiation, noise and other forms of pollution, and the unwise management of solid waste. Efforts to protect the environment require a focus that recognizes the interplay between the components of our physical environment – air, water, and land. The National Environmental Research Centers provide this multidisciplinary focus through programs engaged in

- studies on the effects of environmental contaminants on man and the biosphere, and
- a search for ways to prevent contamination and to recycle valuable resources.

This manual was developed within the National Environmental Research Center – Cincinnati to provide pollution biologists with the most recent methods for measuring the effects of environmental contaminants on freshwater and marine organisms in field and laboratory studies which are carried out to establish water quality criteria for the recognized beneficial uses of water and to monitor surface water quality.

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PREFACE

This manual was published under Research Objective Achievement Plan 1BA027-05AEF, "Methods for Determining Biological Parameters of all Waters," as part of the National Analytical Methods Development Research Program. The manual was prepared largely by a standing committee of senior Agency biologists organized in 1970 to assist the Biological Methods Branch in the selection of methods for use in routine field and laboratory work in fresh and marine waters arising during short-term enforcement studies, water quality trend monitoring, effluent testing and research projects.

The methods contained in this manual are considered by the Committee to be the best available at this time. The manual will be revised and new methods will be recommended as the need arises.

The Committee attempted to avoid duplicating field and laboratory methods already adequately described for Agency use in *Standard Methods for the Examination of Water and Wastewater*, 13th edition, and frequent reference is made to this source throughout the manual.

Questions and comments regarding the contents of this manual should be directed to:

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INTRODUCTION

The role of aquatic biology in the water pollution control program of the U. S. Environmental Protection Agency includes field and laboratory studies carried out to establish water quality criteria for the recognized beneficial uses of water resources and to monitor water quality.

Field studies are employed to: measure the toxicity of specific pollutants or effluents to individual species or communities of aquatic organisms under natural conditions; detect violations of water quality standards; evaluate the trophic status of waters; and determine long-term trends in water quality.

Laboratory studies are employed to: measure the effects of known or potentially deleterious substances on aquatic organisms to estimate "safe" concentrations; and determine environmental requirements (such as temperature, pH, dissolved oxygen, etc.) of the more important and sensitive species of aquatic organisms. Field surveys and water quality monitoring are conducted principally by the regional surveillance and analysis and national enforcement programs. Laboratory studies of water quality requirements, toxicity testing, and methods development are conducted principally by the national research programs.

The effects of pollutants are reflected in the population density, species composition and diversity, physiological condition and metabolic rates of natural aquatic communities. Methods for field surveys and long-term water quality monitoring described in this manual, therefore, are directed primarily toward sample collection and processing, organism identification, and the measurement of biomass and metabolic rates. Guidelines are also provided for data evaluation and interpretation.

There are three basic types of biological field studies; reconnaissance surveys, synoptic surveys, and comparative evaluations. Although there is a considerable amount of overlap, each of the above types has specific requirements in terms of study design.

Reconnaissance surveys may range from a brief perusal of the study area by boat, plane, or car, to an actual field study in which samples are collected for the purpose of characterizing the physical boundaries of the various habitat types (substrate, current, depth, etc.) and obtaining cursory information on the flora and fauna. Although they may be an end in themselves, reconnaissance surveys are generally conducted with a view to obtaining information adequate to design more comprehensive studies. They may be quantitative or qualitative in approach. As discussed in the biometrics section, quantitative reconnaissance samples are very useful for evaluating the amount of sampling effort required to obtain the desired level of precision in more detailed studies.

Synoptic surveys generally involve an attempt to determine the kinds and relative abundance of organisms present in the environment being studied. This type of study may be expanded to include quantitative estimates of standing crop or production of biomass, but is generally more qualitative in approach. Systematic sampling, in which a deliberate attempt is made to collect specimens from all recognizable habitats, is generally utilized in synoptic surveys. Synoptic surveys provide useful background data, are valuable for evaluating seasonal changes in species present, and provide useful information for long-term surveillance programs.

The more usual type of field studies involve *comparative evaluations,* which may take various forms including: comparisons of the flora and fauna in different areas of the same body of water, such as conventional "upstream-downstream" studies; comparisons of the flora and fauna at a given location in a body of water over time, such as is the case in trend monitoring; and comparisons of the flora and fauna in different bodies of water.

Comparative studies frequently involve both quantitative and qualitative approaches. However, as previously pointed out, the choice is often dependent upon such factors as available resources, time limitations, and characteristics of the habitat to be studied. The latter factor may be quite important because the habitat to be studied may not be amenable to the use of quantitative sampling devices.

A special field method that warrants a brief notation is scuba (Self Contained Underwater Breathing Apparatus). Scuba enables the biologist to observe, first hand, conditions that otherwise could be described only from sediment, chemical, physical, and biological samples taken with various surface-operated equipment. Equipment modified from standard sampling equipment or prefabricated, installed, and/or operated by scuba divers has proven very valuable in assessing the environmental conditions where surface sampling gear was inadequate. Underwater photography presents visual evidence of existing conditions and permits the monitoring of long-term changes in an aquatic environment.*

By utilizing such underwater habitats as Tektite and Sublimnos, biologists can observe, collect, and analyze samples without leaving the aquatic environment. Scuba is a very effective tool available to the aquatic biologist, and methods incorporating scuba should be considered for use in situations where equipment operated at the surface does not provide sufficient information.

*Braidech, T.E., P.E. Gehring, and C.O. Kleveno. Biological studies related to oxygen depletion and nutrient regeneration processes in the Lake Erie Basin. Project Hypo-Canada Centre for Inland Waters, Paper No. 6, U. S. Environmental Protection Agency Technical Report TS05-71-208-24, February 1972.

SAFETY

The hazards associated with work on or near water require special consideration. Personnel should not be assigned to duty alone in boats, and should be competent in the use of boating equipment (courses are offered by the U. S. Coast Guard). Field training should also include instructions on the proper rigging and handling of biological sampling gear.

Life preservers (jacket type work vests) should be worn at all times when on or near deep water. Boats should have air-tight or foam-filled compartments for flotation and be equipped with fire extinguishers, running lights, oars, and anchor. The use of inflatable plastic or rubber boats is discouraged.

All boat trailers should have two rear running and stop lights and turn signals and a license plate illuminator. Trailers 80 inches (wheel to wheel) or more wide should be equipped with amber marker lights on the front and rear of the frame on both sides.

Laboratories should be provided with fire extinguishers, fume hoods, and eye fountains. Safety glasses should be worn when mixing dangerous chemicals and preservatives.

A copy of the *EPA Safety Manual* is available from the Office of Administration, Washington, D.C.

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1.0 INTRODUCTION

Field and laboratory studies should be well-planned in advance to assure the collection of unbiased and precise data which are technically defensible and amenable to statistical evaluation. The purpose of this chapter is to present some of the basic concepts and techniques of sampling design and data evaluation that can be easily applied by biologists.

An attempt has been made to present the material in a format comfortable to the non-statistician, and examples are used to illustrate most of the techniques.

1.1 Terminology

To avoid ambiguity in the following discussions, the basic terms must be defined. Most of the terms are widely used in everyday language, but in biometry may be used in a very restricted sense.

1.1.1 Experiment

An experiment is often considered to be a rigidly controlled laboratory investigation, but in this chapter the terms experiment, study, and field study are used interchangeably as the context seems to require. A general definition which will usually fit either of these terms is "any scientific endeavor where observations or measurements are made in order to draw inferences about the real world."

1.1.2 Observation

This term is used here in much the same manner as it is in everyday language. Often the context will suggest using the term "measurement" in place of "observation." This will imply a quantified observation. For statistical purposes, an observation is a record representing some property or characteristic of a real-world object.

This may be a numeric value representing the weight of a fish, a check mark indicating the presence of some species in a bottom quadrat – in short, any type of observation.

1.1.3 Characteristics of interest

In any experiment or sampling study, many types of observations or measurements could be made. Usually, however, there are few types of measurements that are related to the purpose of the study. The measurement of chlorophyll or ATP in a plankton haul may be of interest, whereas the cell count or detritus content may not be of interest. Thus, the characteristic of interest is the characteristic to be observed or measured, the measurements recorded, analyzed and interpreted in order to draw an inference about the real world.

1.1.4 Universe and experimental unit

The experimental unit is the object upon which an observation is made. The characteristic of interest to the study is observed and recorded for each unit. The experimental unit may be referred to in some cases as the sampling unit. For example, a fish, an entire catch, a liter of pond water, or a square meter of bottom may each be an experimental unit. The experimental unit must be clearly defined so as to restrict measurements to only those units of interest to the study. The set of all experimental units of interest to the study is termed the "universe."

1.1.5 Population and sample

In biology, a population is considered to be a group of individuals of the same species. The statistical use of the term *population*, however, refers to the set of values for the characteristic of interest for the entire group of experimental units about which the inferences are to be made (universe).

When studies are made, observations are not usually taken for all possible experimental units. Only a sample is taken. A sample is a *set of observations,* usually only a small fraction of the total number of observations that conceivably could be taken, and is a subset of the population. The term sample is often used in everyday language to mean a portion of the real world which has been selected for measurement, such as a water sample or a plankton haul. However, in this section the term "sample" will be used to denote *"a set of observations"* – the written records themselves.

1.1.6 Parameter and statistic

When we attempt to characterize a population, we realize that we can never obtain a perfect answer, so we settle for whatever accuracy and precision that is required. We try to take an adequately-sized sample and compute a number from our sample that is representative of the population. For example, if we are interested in the population mean, we take a sample and compute the sample mean. The sample mean is referred to as a statistic, whereas the population mean is referred to as a parameter. In general, the statistic is related to the parameter in much the same way as the sample is related to the population. Hence, we speak of population parameters and sample statistics.

Obviously many samples may be selected from most populations. If there is variability in the population, a statistic computed from one sample will differ somewhat from the same statistic computed from another sample. Hence, whereas a parameter such as the population mean is fixed, the statistic or sample mean is a variable, and there is uncertainty associated with it as an estimator of the population parameter which derives from the variation among samples.

2.0 STUDY DESIGN

2.1 Randomization

In biological studies, the experimental units (sampling units or sampling points) must be selected with known probability. Usually, random selection is the only feasible means of satisfying the "known probability" criterion. The question of why known probability is required is a valid one. The answer is that only by knowing the probability of selection of a sample can we extrapolate from the sample to the population in an objective way. The probability allows us to place a weight upon an observation in making our extrapolation to the population. There is no other quantifiable measure of "how well" the selected sample represents the population.

Thus our efforts to select a "good" sample should include an appropriate effort to define the problem in such a way as to allow us to estimate the parameter of interest using a sample of known probability; i.e., a random sample.

The preceding discussion should leave little doubt that there is a fundamental distinction between a "haphazardly-selected" sample and a "randomly-selected" sample. The distinction is that a haphazardly-selected sample is one where there is *no conscious bias,* whereas a randomly-selected sample is one where there is *consciously no bias.* There is consciously no bias because the randomization is planned, and therefore bias is planned out of the study. This is usually accomplished with the aid of a table of random numbers. A sample selected according to a plan that includes random selection of experimental units is the only sample validly called a random sample.

Reference to the definition of the term, sample, at the beginning of the chapter will remind us that a sample consists of a set of observations, each made upon an experimental or sampling unit. To sample randomly, the entire set of sampling units (population) must be identifiable and enumerated. Sometimes the task of enumeration may be considerable, but often it may be minimized by such conveniences as maps, that allow easier access to adequate representation of the entity to be sampled.

The comment has frequently been made that random sampling causes effort to be put into drawing samples of little meaning or utility to the study. This need not be the case. Sampling units should be defined by the investigator so as to eliminate those units which are potentially of no interest. Stratification can be used to place less emphasis on those units which are of less interest.

Much of the work done in biological field studies is aimed at explaining spatial distributions of population densities or of some parameter related to population densities and the measurement of rates of change which permit prediction of some future course of a biologically-related parameter. In these cases the sampling unit is a unit of space (volume, area). Even in cases where the sampling unit is not a unit of space, the problem may often be stated in such a manner that a unit of space may be used, so that random sampling may be more easily carried out.

For example, suppose the problem is to estimate the chlorophyll content of algae in a pond at a particular time of year. The measurement is upon algae, yet the sample consists of a volume of water. We could use our knowledge of the way the algae are spatially distributed or make some reasonable assumptions, then construct a random sampling scheme based upon a unit of volume (liter) as the basic sampling unit.

It is not always a simple or straightforward matter to define sampling units, because of the dynamic nature of living populations. Many aquatic organisms are mobile, and even rooted or sessile forms change with time, so that changes occurring during the study often make data interpretation difficult. Thus the benefit to be derived from any attempt to consider such factors in the planning stage will be considerable.

Random sample selection is a subject apart from the selection of the study site. It is of use only after the study objectives have been defined, the type of measurements have been selected, and the sampling units have been defined. At this point, random sampling provides an objective means of obtaining information to achieve the objectives of the study.

One satisfactory method of random sample selection is described. First, number the universe or entire set of sampling units from which the sample will be selected. This number is N. Then from a table of random numbers select as many random numbers, n, as there will be sampling units selected for the sample. Random numbers tables are available in most applied statistics texts or books of mathematical tables. Select a starting point in the table and read the numbers consecutively in any direction (across, diagonal, down, up). The number of observations, n (sample size), must be determined prior to sampling. For example, if n is a two-digit number, select two-digit numbers ignoring any number greater than n or any number that has already been selected. These numbers will be the numbers of the sampling units to be selected.

To obtain reliable data, information about the statistical population is needed in advance of the full scale study. This information may be obtained from prior related studies, gained by pre-study reconnaissance, or if no direct information is available, professional opinion about the characteristics of the population may be relied upon.

2.1.1 Simple random sampling

Simple (or unrestricted) random sampling is used when there is no reason to subdivide the population from which the sample is drawn. The sample is drawn such that every unit of the population has an equal chance of being selected. This may be accomplished by using the random selection scheme already described.

2.1.2 Stratified random sampling

If any knowledge of the expected size or variation of the observations is available, it can often be used as a guide in subdividing the population into subpopulations (strata) with a resulting increase in efficiency of estimation. Perhaps the most profitable means of obtaining information for stratification is through a pre-study reconnaissance (a pilot study). The pilot study planning should be done carefully, perhaps stratifying based upon suspected variability. The results of the pilot study may be used to obtain estimates of variances needed to establish sample size. Other advantages of the pilot study are that it accomplishes a detailed reconnaissance, and it provides the opportunity to obtain experience in the actual field situation where the final study will be made. Information obtained and difficulties encountered may often be used to set up a more realistic study and avoid costly and needless expenditures. To maximize precision, strata should be constructed such that the observations are most alike within strata and most different among strata, i.e., minimum variance within strata and maximum variance among strata. In practice, the information used to form strata will usually be from previously obtained data, or information about characteristics correlated with the characteristic of interest. In aquatic field situations, stratification may be based upon depth, bottom type, isotherms, and numerous other variables suspected of being correlated with the characteristic of interest. Stratification is often done on other bases such as convenience or administrative imperative, but except where these correspond with criteria which minimize the variation within strata, no gain in precision may be expected.

Number of Strata

In aquatic biological field studies, the use of knowledge of biological cause-and-effect may help define reasonable strata (e.g., thermoclines, sediment types, etc., may markedly affect the organisms so that the environmental feature may be the obvious choice for the strata divisions). Where a gradient is suspected and where stratification is based on a factor correlated to an unknown degree with the characteristic of interest, the answer to the question of how many strata to form and where to locate their boundaries is not clear. Usually as many strata are selected as may be handled in the study. In practice, gains in efficiency due to stratification usually become negligible after only a few divisions unless the characteristic used as the basis of stratification is very highly correlated with the characteristic of interest.

2.1.3 Systematic random sampling

In field studies, the biologist frequently wishes to use some sort of transect, perhaps to be assured of including an adequate cross section while maintaining relative ease of sampling. The use of transects is an example of systematic sampling. However, a random starting point is chosen along the transect to introduce the randomness needed to guarantee freedom from bias and allow statistical inference.

The method of placement of the transect should be given a great deal of thought. Often transects are set up arbitrarily, but they should not be. To avoid arbitrariness, randomization should be employed in transect placement.

2.2 Sample Size

2.2.1 Simple random sampling

In any study, one important early question is that of the size of the sample. The question is important because if, on the one hand, a sample is too large, the effort is wasteful, and if, on the other hand, a sample is too small, the question of importance to the study may not be properly answered.

Case 1 – Estimation of a Binomial Proportion

An estimate of the proportion of occurrence of the two categories must be available. If the categories are presence and absence, let the probability of observing a presence be P $(0 < P < 1)$ and the probability of observing an absence be Q $(0 < Q < 1, P + Q = 1)$. The second type of information which is needed is an acceptable magnitude of error, d, in estimating P (and hence Q). With this information, together with the size, n, of the population, the formula for n as an initial approximation (n_0), is:

$$n_0 = \frac{t^2PQ}{d^2} \qquad (1)$$

The value for t is obtained from tables of "Student's t" distribution, but for the initial computation the value 2 may be used to obtain a sample size, n_0, that will ensure with a .95 probability, that P is within d of its true value. If n_0 is less than 30, use a second calculation where t is obtained from a table of "Student's t" with $n_0 - 1$ degrees of freedom. If the calculation results in an n_0, where $\frac{n_0}{N} < .05$, no further calculation is warranted. Use n_0 as the sample size. If $\frac{n_0}{N} > .05$, make the following computation:

$$n = \frac{n_0}{1 + \frac{n_0 - 1}{N}} \qquad (2)$$

Case 2 – Estimation of a Population Mean for Measurement Data

In this case an estimate of the variance, s^2, must be obtained from some source, and a statement of the margin of error, d, must be expressed in the same units as are the sample observations. To calculate an initial sample size:

$$n_0 = \frac{t^2 s^2}{d^2} \qquad (3)$$

If $n_0 < 30$, recalculate using t from the tables, and if $\frac{n_0}{N} > .05$, a further calculation is in order:

$$n = \frac{n_0}{1 + \frac{n_0}{N}} \tag{4}$$

After a sample of size, n, is obtained from the population, the basic sample statistics may be calculated. The calculations are the same as for equations (11) through (15) unless the sample size, n, is greater than 5 percent of the population N. If $\frac{n}{N} > .05$, a correction factor is used so that the calculation for the sample variance is:

$$s^2 = \left(\frac{N-n}{N}\right) \frac{\Sigma X_i^2 - \frac{(\Sigma X_i)^2}{n}}{n - 1} \tag{5}$$

The other calculations make use of, s^2, as calculated above, wherever s^2 appears in the formulas.

2.2.2 Stratified random sampling

To compute the sample size required to obtain an estimate of the mean within a specified acceptable error, computations can be made similar to those for simple random sampling: a probability level must be specified; an estimate of the variance within each stratum must be available; and the number of sampling units in each stratum must be known. Although this involves a good deal of work, it illustrates the need for a pilot study and indicates that we must know something about the phenomena we are studying if we are to plan an effective sampling program.

If the pilot study or other sources of information have resulted in what are considered to be reliable estimates of the variance within strata, the sampling can be optimally allocated to strata. Otherwise proportional allocation should be used. Optimal allocation, properly used, will result in more precise estimates for a given sample size.

For proportional allocation the calculation for sample size is:

$$n = \frac{\frac{t^2 \Sigma N_k s_k^2}{Nd^2}}{1 + \frac{\Sigma N_k s_k^2}{N^2 d^2}} \tag{6}$$

where t = the entry for the desired probability level from a table of "Student's t" (use 2 for a rough estimate); N_k = the number of sampling units in stratum k; s_k^2 = the variance of stratum k; N = the total number of sampling units in all strata; and d = the acceptable error expressed in the same units as the observations.

For optimal allocation, the calculation is:

$$n = \frac{\frac{t^2 (\Sigma N_k s_k)^2}{N^2 d^2}}{1 + \frac{t^2 \Sigma N_k s_k^2}{N^2 d^2}} \tag{7}$$

where the symbols are the same as above and where $s_k = \sqrt{s_k^2}$, the standard deviation of stratum k [see Equations (16) to (19)].

Having established sample size, it remains to determine the portion of the sample to be allocated to each stratum.

For proportional allocation:

$$n_k = \frac{nN_k}{N} \tag{8}$$

where n_k = the number of observations to be made in stratum k.

For optimal allocation:

$$n_k = \frac{nN_k s_k}{\Sigma N_k s_k} \tag{9}$$

Sample selection within each stratum is performed in the same manner as for simple random sampling.

2.2.3 Systematic random sampling

After the location of a transect line is selected, the number of experimental units (the number of possible sampling points) along this line must be determined. This may be done in many ways depending upon the particular situation. Possible examples are the number of square meter plots of bottom centered along a 100-meter transect (N = 100); or the meters of distance along a 400-meter transect as points of departure for making a plankton haul of some predetermined duration perpendicular to the transect. (In the second example, a question of subsampling or some assumption about local, homogeneous distribution might arise since the plankton net has a radius less than one meter). The interval of sampling, C, determines sample size: n = N/C. The mean is estimated as usual; the variance as for a simple random sample if there are no trends, periodicities, or other non-random effects.

2.3 Subsampling

Situations often arise where it is natural or imperative that the sampling units are defined in a two-step manner. For example: colonies of benthic organisms might be the first step, and the measurement of some characteristic on the individuals within the colony might be the second step; or streams might be the first (primary) step, and reaches, riffles or pools as the second step (or element) within the unit. When a sample of primary units is selected, and then for each primary unit a sample is selected by observing some element of the primary unit, the sampling scheme is known as subsampling or two-stage sampling. The computations are straight forward, but somewhat more involved.

The method of selection of the primary units must be established. It may be a simple random sample (equal probabilities), a stratified random sample (equal probabilities within strata), or other scheme such as probability proportional to size (or estimated size) of primary unit. In any case, let us call the probability of selection of the $i^{\underline{th}}$ primary unit, Z_i. For simple random sampling, $Z_i = \frac{1}{N}$, where N is the number of primary units in the universe. For stratified random sampling, $Z_{ki} = \frac{1}{N_k}$, where k signifies the $k^{\underline{th}}$ stratum. For selection in which the primary units are selected with probability proportional to their size, the probability of selection of the $j^{\underline{th}}$ primary unit is

$$Z_j = \frac{L_j}{\sum_{i=1}^{n} L_i} \qquad (10)$$

where L equals the number of elements in the primary unit indicated by its subscript. If stratification is used with the latter scheme, merely apply the rule to each stratum. Other methods of assigning probability of selection may be used. The important thing is to establish the probability of selection for each primary unit.

3.0 GRAPHIC EXAMINATION OF DATA

Often the most elementary techniques are of the greatest use in data interpretation. Visual examination of data can point the way for more discriminatory analyses, or on the other hand, interpretations may become so obvious that further analysis is superfluous. In either case, graphical examination of data is often the most effortless way to obtain an initial examination of data and affords the chance to organize the data. Therefore, it is often done as a first step. Some commonly used techniques are presented below. Cell counts (algal cells per milliliter) will serve as the numeric example (Table 1).

3.1 Raw Data

As brought out in other chapters of this manual, it is of utmost importance that raw data be recorded in a careful, logical, interpretable manner together with appropriate, but not superfluous, annotations. Note that although some annotations may be considered superfluous to the immediate intent of the data, they may not be so for other purposes. Any note that might aid in determining whether the data are comparable to other similar data, etc., should be recorded if possible.

3.2 Frequency Histograms

To construct a frequency histogram from the data of Table 1, examine the raw data to determine the range, then establish intervals. Choose the intervals with care so they will be optimally integrative and differentiative. If the intervals are too wide, too many observations will be integrated into one interval and the picture will be hidden; if too narrow, too few will fall into one interval and a confusing overdifferentiation or overspreading of the data will result. It is often enlightening if the same data are plotted with the use of several interval sizes. Construct the intervals so that no doubt exist as to which interval an observation belongs, i.e., the end of one interval must not be the same number as the beginning of the next.

The algal count data in Tables 2 and 3 were grouped by two interval sizes (10,000 cells/ml and 20,000 cells/ml). It is easy to see that the data are grouped largely in the range 0 to 6 x 10^4 cells/ml and that the frequency of occurrence is

TABLE 1. RAW DATA ON PLANKTON COUNTS

Date	Count	Date	Count	Date	Count
June		June		July	
8	23,077	25	7,692	11	44,231
9	36,538	26	23,077	12	50,000
10	26,923	27	134,615	13	26,923
11	23,077	28	32,692	14	44,231
12	13,462	29	25,000	15	46,154
13	19,231	30	146,154	16	55,768
14	21,154	July		17	9,615
15	61,538	1	107,692	18	13,462
16	96,154	2	13,462	19	3,846
17	23,077	3	9,615	20	3,846
18	46,154	4	148,077	21	11,538
19	48,077	5	53,846	22	7,692
20	51,923	6	103,846	23	13,462
21	50,000	7	78,846	24	21,154
22	292,308	8	132,692	25	17,308
23	165,385	9	228,846		
24	42,308	10	307,692		

lesser, the larger the value. Closer inspection will reveal that with the finer interval width (Table 2), the frequency of occurrence does not increase monotonically as cell count decreases. Rather, the frequency peak is found in the interval 20,000 to 30,000 cells/ml. This observation was not possible using the coarser interval width; the frequencies were "overintegrated" and did not reveal this part of the pattern. Finer interval widths could further change the picture presented by each of these groupings.

Although a frequency table contains all the information that a comparable histogram contains, the graphical value of a histogram is usually worth the small effort required for its construction. Figures 1 and 2 are frequency histograms corresponding to Tables 2 and 3, respectively. It can be seen that the histograms are more immediately interpretable. The height of each bar is the frequency of the interval; the width is the interval width.

3.3 Frequency Polygon

Another way to present essentially the same information as that in a frequency histogram is the use of a frequency polygon. Plot points at the height of the frequency and at the midpoint of the interval, and connect the points with straight lines. The data of Table 3 are used to

TABLE 2. FREQUENCY TABLE FOR DATA IN TABLE 1 GROUPED AT AN INTERVAL WIDTH OF 10,000 CELLS/ML

Interval	Frequency	Interval	Frequency
0 - 10	6	200 - 210	0
10 - 20	7	210 - 220	0
20 - 30	9	220 - 230	1
30 - 40	2	230 - 240	0
40 - 50	6	240 - 250	0
50 - 60	5	250 - 260	0
60 - 70	1	260 - 270	0
70 - 80	1	270 - 280	0
80 - 90	0	280 - 290	0
90 - 100	1	290 - 300	1
100 - 110	2	300 - 310	1
110 - 120	0	310 - 320	0
120 - 130	0	320 - 330	0
130 - 140	2	330 - 340	0
140 - 150	2	340 - 350	0
150 - 160	0	350 - 360	0
160 - 170	1	360 - 370	0
170 - 180	0	370 - 380	0
180 - 190	0	380 - 390	0
190 - 200	0	390 - 400	0

illustrate the frequency polygon in Figure 3.

3.4 Cumulative Frequency

Cumulative frequency plots are often useful in data interpretation. As an example, a cumulative frequency histogram (Figure 4) was constructed using the frequency table (Table 2 or 3). The height of a bar (frequency) is the sum of all frequencies up to and including the one being plotted. Thus, the first bar will be the same as the frequency histogram, the second bar equals the sum of the first and second bars of the frequency histogram, etc., and the last bar is the sum of all frequencies.

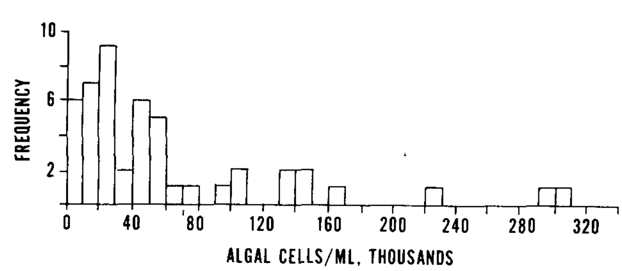

Figure 1. Frequency histogram; interval width is 10,000 cells/ml.

TABLE 3. FREQUENCY TABLE FOR DATA IN TABLE 1 GROUPED AT AN INTERVAL WIDTH OF 20,000 CELLS/ML

Interval	Frequency	Interval	Frequency
0 - 20	13	200 - 220	0
20 - 40	11	220 - 240	1
40 - 60	11	240 - 260	0
60 - 80	2	260 - 280	0
80 - 100	1	280 - 300	1
100 - 120	2	300 - 320	1
120 - 140	2	320 - 340	0
140 - 160	2	340 - 360	0
160 - 180	1	360 - 380	0
180 - 200	0	380 - 400	0

Closely related to the cumulative frequency histogram is the cumulative frequency distribution graph, a graph of relative frequencies. To obtain the cumulative graph, merely change the scale of the frequency axis on the cumulative frequency histogram. The scale change is made by dividing all values on the scale by the highest value on the scale (in this case the number of observations or 48).

The value of the cumulative frequency distribution graph is to allow relative frequency to be read, i.e., the fraction of observations less than or equal to some chosen value. Exercise caution in extrapolating from a cumulative frequency distribution to other situations. Always bear in mind that in spite of a planned lack of bias, each sample, or restricted set of samples, is subject to influences not accounted for and is therefore unique. This caution is all the more pertinent for cumulative frequency plots because they tend to smooth out some of the variation noticed in the frequency histogram. In addition, the phrase "fraction of observations less than or equal to some chosen value" can easily be read "fraction of time the observation is less than or equal to some chosen value." It is tempting to generalize from this reading and extend these results beyond their range of applicability.

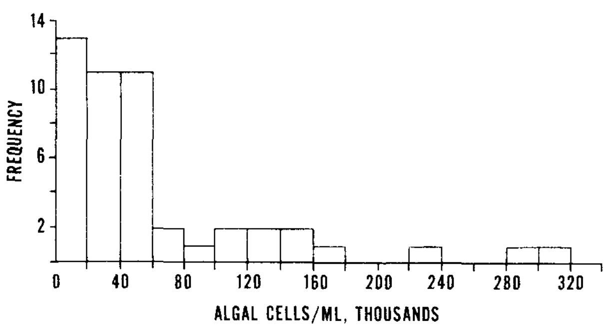

Figure 2. Frequency histogram; interval width is 20,000 cells/ml.

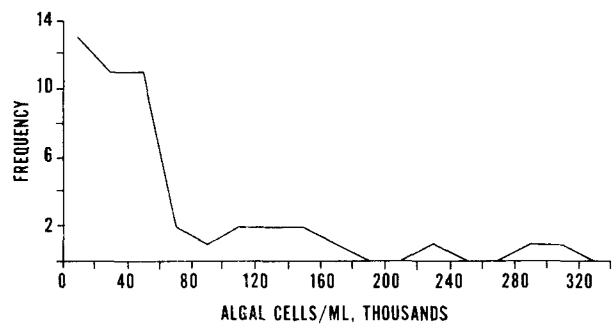

Figure 3. Frequency polygon; interval width is 20,000 cells/ml.

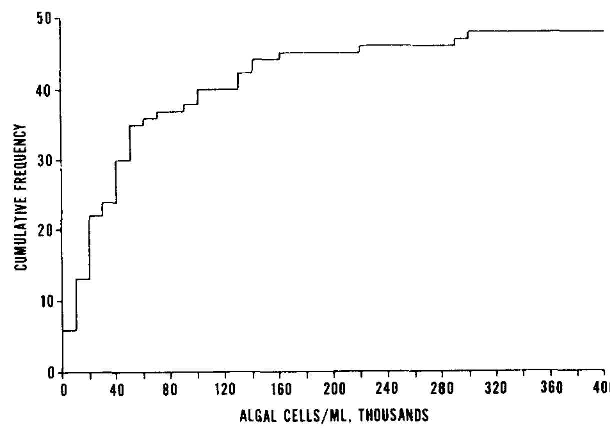

Figure 4. Cumulative frequency histogram; interval width is 10,000 cells/ml.

3.5 Two-dimensional Graphs

Often data are taken where the observations are recorded as a pair (cell count and time), (biomass and nutrient concentration). Here a quick plot of the set of pairs will usually be of value. Figure 5 is such a graph of data taken from Table 1. Each point is plotted at a height corresponding to cell count and at a distance from the ordinate axis corresponding to the number of days since the beginning observation. The peaks and troughs, their frequency, together with intimate knowledge of the conditions of the study, might suggest something of biological interest, further statistical analysis, or further field or laboratory work.

In summary, carefully prepared tables and graphs may be important and informative steps in data analysis. The added effort is usually small, whereas gains in interpretive insight may be large. Therefore, graphic examination of data is a recommended procedure in the course of most investigations.

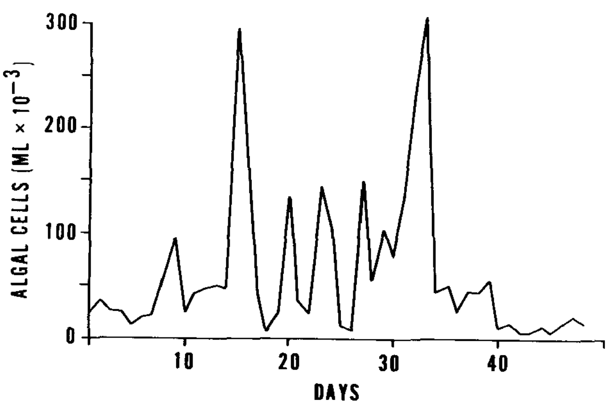

Figure 5. An example of a two-dimensional graph plotted from algal-count data in Table 1.

4.0 SAMPLE MEAN AND VARIANCE

4.1 General Application

Knowledge of certain computations and computational notations is essential to the use of statistical techniques. Some of the more basic of these will be briefly reviewed here.

To illustrate the computations, let us assume we have a set of data, i.e., a list of numeric values written down. Each of these values can be labeled by a set of numerals beginning with 1. Thus, the *first* of these values can be called X_1, the *second* X_2, etc., and the *last* one we call X_n.

The data values are labeled with consecutive numbers (recall from the definitions that these numeric values are observations), and there are n values in the set of data. A typical observation is X_i, where i may take any value between 1 and n, inclusive, and the subscript indicates which X is being referenced.

The sum of the numbers in a data set, such as our sample, is indicated in statistical computations by capital sigma, Σ. Associated with Σ are an operand (here, X_i), a subscript (here, $i = 1$), and a superscript (here, n), $\sum_{i=1}^{n} X_i$. The subscript $i = 1$ indicates that the value of the operand X is to be the number labeled X_1 in our data set and that this is to be the first observation of the sum. The superscript n indicates that the last number of the summation is to be the value of X_n, the last X in our data set.

Computations for the mean, variance, standard deviation, variance of the mean, and standard deviation of the mean (standard error) are presented below. Note that these are computations for a sample of n observations, i.e., they are statistics.

Mean ($\overline{X}$):

$$\overline{X} = \frac{\sum_{i=1}^{n} X_i}{n} \tag{11}$$

Variance (s^2):

$$s^2 = \frac{\sum_{i=1}^{n} X_i^2 - \frac{\left(\sum_{i=1}^{n} X_i\right)^2}{n}}{n-1} \tag{12}$$

Note: The X_i's are squared, then the summation is performed in the first term of the numerator; in the second term, the sum of the X_i's is first formed, then the sum is squared, as indicated by the parentheses.

Standard deviation (s):

$$s = \sqrt{s^2} \tag{13}$$

Variance of the mean ($s^2_{\overline{X}}$):

$$s^2_{\overline{X}} = \frac{s^2}{n} \tag{14}$$

Standard deviation of the mean or standard error ($s_{\bar{X}}$):

$$s_{\bar{X}} = \sqrt{s^2_{\bar{X}}} = \frac{s}{\sqrt{n}} \qquad (15)$$

4.2 Statistics for Stratified Random Samples

The calculations of the sample statistics for stratified random sampling are as follows (see 2.2.2 Stratified random samples):

For the mean of stratum k:

$$\bar{y} = \frac{\sum_{i=1}^{n_k} y_{ki}}{n_k} \qquad (16)$$

i.e., simply compute an arithmetic average for the measurements of stratum k.

For the variance of stratum k:

$$s^2 = \frac{\sum_{i=1}^{n_k} y_{ki}^2 - \frac{\left(\sum_{i=1}^{n_k} y_{ki}\right)^2}{n_k}}{n_k - 1} \qquad (17)$$

i.e., simply Equation 12 applied to the data of the k^{th} stratum.

For the mean of the stratified sample:

$$\bar{y}_{st} = \frac{\sum_{k=1}^{m} N_k \bar{y}_k}{N} \qquad (18)$$

for either type allocation or alternatively for proportional allocation:

$$\bar{y}_{st} = \frac{\sum_{k=1}^{m} n_k \bar{y}_k}{n} \qquad (19)$$

Note that Equations (18) and (19) are identical only for proportional allocation.

4.3 Statistics for Subsamples

If simple random sampling is used to select a subsample, the following formulas are used to calculate the sample statistics (see 2.3 Subsampling):

For the sample mean:

$$\bar{\bar{y}} = \frac{1}{n \sum_{i=1}^{n} L_i} \cdot \sum_{i=1}^{n} \left(\frac{L_i \bar{y}_i}{Z_i}\right) \qquad (20)$$

where $\bar{\bar{y}}$ is the average, computed over subsamples as well as for the sample

$$\bar{y}_i = \frac{\sum_{j=1}^{L_i} y_{ij}}{n} \qquad (21)$$

where y_{ij} equals the observation for the j^{th} element in the i^{th} primary unit, and L_i is the number of observations upon elements for primary unit i.

For the variance of the sample mean:

$$s^2(\bar{\bar{y}}) = \frac{1}{n(n-1)\left(\sum_{i=1}^{n} L_i\right)^2} \sum_{i=1}^{n} (\hat{Y}_i - \hat{Y}_n)^2 \qquad (22)$$

where $\hat{Y}_i$ is computed as

$$\hat{Y}_i = \frac{L_i \bar{y}_i}{Z_i} \qquad (23)$$

where $\hat{Y}_n$ is computed as

$$\hat{Y}_n = \frac{1}{n} \sum_{i=1}^{n} \hat{Y}_i = \bar{\bar{y}} \sum_{i=1}^{n} L_i \qquad (24)$$

or alternatively

$$s^2(\bar{\bar{y}}) = \frac{1}{n(n-1)\left(\sum_{i=1}^{n} L_i\right)^2} \cdot \Sigma \hat{Y}_i - \frac{(\Sigma \hat{Y}_i)^2}{n} \qquad (25)$$

4.4 Rounding

The questions of rounding and the number of digits to carry through the calculations always arise in making statistical computations. Measurement data are approximations, since they are rounded when the measurements were taken; count data and binomial data are not subject to this type of approximation.

Observe the following rules when working with measurement or continuous data.

- When rounding numbers to some number of decimal places, first look at the digit to the right of the last place to be retained. If this number is greater than 5, the last place to be retained is rounded up by 1; if it is less than 5, do not change the last place – merely drop the extra places. To round to 2 decimal places:

Unrounded	Rounded
1.239	1.24
28.5849	28.58

- If the digit to the right of the last place to be retained is 5, then look at the second digit to the right of the last place to be kept, provided that the unrounded number is recorded with that digit as a significant digit. If the second digit to the right is greater than 0, then round the number up by 1 in the last place to be kept; if the second digit is 0, then look at the third digit, etc. To round to 1 place:

Unrounded	Rounded
13.251	13.3
13.25001	13.3

- If the number is recorded to only one place to the right of the last place to be kept, and that digit is 0, or if the significant digits two or more places beyond the last place to be kept are all 0, a special rule (odd-even rule) is followed to ensure that upward rounding occurs as frequently as downward rounding. The rule is: if the digit to the right of the last place to be kept is 5, and is the last digit of significance, or if all following significant digits are 0, round up when the last digit to be retained is *odd* and drop the 5 when the last digit to be retained is *even*. To round to 1 place:

Unrounded	Rounded
13.2500	13.2
13.3500	13.4

Caution: all rounding must be made in 1 step to avoid introducing bias. For example the number 5.451 rounded to a whole number is clearly 5, but if the rounding were done in two steps it would first be rounded to 5.5 then to 6.

Retaining Significant Figures

Retention of significant figures in statistical computations can be summarized in three rules:

- Never use more significance for a raw data value than is warranted.
- During intermediate computations keep all significant figures for each data value, and carry the computations out in full.
- Round the final result to the accuracy set by the least accurate data value.

5.0 TESTS OF HYPOTHESES

Often in biological field studies some aspect of the study is directed to answering a hypothetical question about a population. If the hypothesis is quantifiable, such as: "At the time of sampling, the standing crop of plankton biomass per liter in lake A was the same as the standing crop per liter in lake B," then the hypothesis can be tested statistically. The question of drawing a sample in such a way that there is freedom from bias, so that such a test may be made, was discussed in the section on sampling (2.0).

Three standard types of tests of hypotheses will be described: the "t-test," the "χ^2-test," and the "F-test."

5.1 T-test

The t-test is used to compare a sample statistic (such as the mean) with some value for the purpose of making a judgment about the population as indicated by the sample. The comparison value may be the mean of another sample (in which case we are using the two samples to judge whether the two populations are the same). The form of the t-statistic is

$$t = \frac{\theta - \Theta}{S_\theta} \qquad (26)$$

where θ = some sample statistic; S_θ = the standard deviation of the sample statistic; and Θ = the value to which the sample statistic is compared (the value of the null hypothesis).

The use of the t-test requires the use of t-tables. The t-table is a two-way table usually arranged with the column headings being the probability, α, of rejecting the null hypothesis when it is true, and the row headings being the degrees of freedom. Entry of the table at the correct probability level requires a discussion of two types of hypotheses testable using the t-statistic.

The null hypothesis is a hypothesis of no difference between a population parameter and another value. Suppose the hypothesis to be tested is that the mean, μ, of some population equals 10. Then we would write the null hypothesis (symbolized H_o) as

$$H_o : \mu = 10$$

Here 10 is the value of Θ in the general form for the t-statistic. An alternative to the null hypothesis is now required. The investigator, viewing the experimental situation, determines the way in which this is stated. If the investigator merely wants to answer whether the sample indicates that $\mu = 10$ or not, then the alternate hypothesis, H_a, is

$$H_a : \mu \neq 10$$

If it is known, for example, that μ cannot be less than 10, then H_a is

$$H_a : \mu > 10$$

and by similar reasoning the other possible H_a is

$$H_a : \mu < 10$$

Hence, there are two types of alternate hypotheses: one where the alternative is simply that the null hypothesis is false ($H_a : \mu \neq 10$); the other, that the null hypothesis is false and, in addition, that the population parameter lies to one side or the other of the hypothesized value [$H_a : \mu$ (> or <) 10]. In the case of $H_a : \mu \neq 10$, the test is called a two-tailed test; in the case of either of the second types of alternate hypotheses, the t-test is called a one-tailed test.

To use a t-table, it must be determined whether the column headings (probability of a larger value, or percentage points, or other means of expressing α) are set for one-tailed or two-tailed tests. Some tables are presented with both headings, and the terms "sign ignored" and "sign considered" are used. "Sign ignored" implies a two-tailed test, and "sign considered" implies a one-tailed test. Where tables are given for one-tailed tests, the column for any probability (or percentage) is the column appropriate to twice the probability for a two-tailed test. Hence, if a column heading is .025 and the table is for one-tailed tests, use this same column for .05 in a two-tailed test (double any one-tailed test heading to get the proper two-tailed test heading; or conversely, halve the two-tailed test heading to obtain proper headings for one-tailed tests).

Testing $H_o : \mu = M$ (the population mean equals some value M):

$$t = \frac{\bar{X} - M}{s_{\bar{X}}} \tag{27}$$

where $\bar{X}$ is given by equation (11) or other appropriate equation; M = the hypothesized population mean; and $s_{\bar{X}}$ is given by equation (15). The t-table is entered at the chosen probability level (often .05) and n-1 degrees of freedom, where n is the number of observations in the sample.

When the computed t-statistic exceeds the tabular value there is said to be a $1 - \alpha$ probability that H_o is false.

Testing $H_o : \mu_1 = \mu_2$ (the mean of the population from which sample 1 was taken equals the mean of the population from which sample 2 was taken):

$$t = \frac{\bar{X}_1 - \bar{X}_2}{s_{\bar{X}_1 - \bar{X}_2}} \tag{28}$$

where $s_{\bar{X}_1 - \bar{X}_2}$ = the pooled standard error obtained by adding the corrected sums of squares for sample 1 to the corrected sums of squares for sample 2, and dividing by the sum of the degrees of freedom for each times the sum of the numbers of observations, i.e.,

$$s_{\bar{X}_1 - \bar{X}_2} = \sqrt{\frac{\Sigma X_1^2 - \frac{(\Sigma X_1)^2}{n_1} + \Sigma X_2^2 - \frac{(\Sigma X_2)^2}{n_2}}{(n_1 + n_2)\,[(n_1 - 1) + (n_2 - 1)]}} \quad (29)^*$$

An alternative and frequently useful form is

$$s_{\bar{X}_1 - \bar{X}_2} = \sqrt{\frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{(n_1 + n_2)(n_1 + n_2 - 2)}} \tag{30}$$

where s_1^2 and s_2^2 are each computed according to equation (12).

For all conditions to be met where the t-test is applicable, the sample should have been selected

*Σ sign, when unsubscripted, will indicate summation for all observations, hence ΣX_1 means sum of all observations in sample 1.

from a population distributed as a normal distribution. Even if the population is not distributed normally, however, as sample size increases, the t-test approaches to applicability. If it is suspected that the population deviates too drastically from the normal, exercise care in the use of the t-test. One method of checking whether the data are normally distributed is to plot the observations on normal probability graph paper. If the plot approximates a straight line, using the t-test is acceptable.

The t-test is used in certain cases where it is known that the parent distribution is not normal. One case commonly encountered in field studies is the binomial. The binomial may describe presence or absence, dead or alive, male or female, etc.

Testing H_o : P = K (the population proportion equals some value K):

$$t = \frac{P - K}{\sqrt{\frac{pq}{n}}} \tag{31}$$

where P = the symbol for the population proportion (e.g., proportion of males in the population); K = a constant positive fraction as the hypothesized proportion; p = the proportion observed in the sample; q = the complementary proportion (e.g., the proportion of females in the sample or 1 - p); and n = the number of observations in the sample. Note that since p is computed as (number of males in the sample) / (total number of individuals in the sample), it will always be a positive number less than one, and hence, so will q. Again α must be chosen; H_a can be any of the types previously discussed; and the degrees of freedom are n - 1.

Count data, where the objects counted are distributed randomly, follow a Poisson distribution. If the Poisson can be used as an adequate description of the distribution of the population, an approximate t may be computed.

Testing H_o : μ = M for the Poisson (the mean of the population distributed as a Poisson equals some hypothesized value M):

$$t = \frac{X - M}{\sqrt{\frac{\bar{X}}{n}}} \tag{32}$$

Note that $\bar{X} = \sigma^2$ for the Poisson, thus $\sqrt{\frac{\bar{X}}{n}}$ is the standard deviation of the mean, $s_{\bar{X}}$.

5.2 Chi Square Test (χ^2-test)

Like t, χ^2 values may be found in mathematical and statistical tables tabulated in a two-way arrangement. Usually, as with t, the column headings are probabilities of obtaining a larger χ^2 value when H_o is true, and the row headings are degrees of freedom. If the calculated χ^2 exceeds the tabular value, then the null hypothesis is rejected. The chi square test is often used with the assumption of approximate normality in the population.

Chi square appears in two forms that differ not only in appearance, but that provide formats for different applications.

- One form:

$$\chi^2 = \frac{(n-1)s^2}{\sigma^2} \tag{33}$$

is useful in tests regarding hypotheses about σ^2.

- The other form:

$$\chi^2 = \Sigma \frac{(0 - E)^2}{E} \tag{34}$$

where 0 = an observed value, and E = an expected (hypothesized) value, is especially useful in sampling from binomial and multinomial distribution, i.e., where the data may be classified into two or more categories.

Consider first a binomial situation. Suppose the data from fish collections from three lakes are to be pooled and the hypothesis of an equal sex ratio tested (Table 4).

TABLE 4. POOLED FISH SEX DATA FROM 3 LAKES

No. males	No. females	Total
892* (919)†	946 (919)	1838

*Observed values.
†Expected, or hypothesized, values.

To compute the hypothesized values (919 above), it is necessary to have formulated a null hypothesis. In this case, it was

H_o : No. males = No. females = (.5) (total)

Expected values are always computed based upon the null hypothesis. The computation for χ^2 is

$$\chi^2 = \frac{(892 - 919)^2 + (946 - 919)^2}{919} = 1.59 \text{ n.s.*}$$

*n.s. = not significant

There is one degree of freedom for this test. Since computed χ^2 is not greater than tabulated χ^2 (3.84), the null hypothesis is not rejected. This test, of course, applies equally well to data that has not been pooled, i.e., where the values are from two unpooled categories.

The information contained in each of the collections is partially obliterated by pooling. If the identity of the collections is maintained, two types of test may be made: a test of the null hypothesis for each collection separately; and a test of interaction, i.e., whether the ratio depends upon the lake from which the sample was obtained (Table 5).

TABLE 5. FISH SEX DATA FROM 3 LAKES

Lake	No Males	No Females	Total	χ^2
1	346* (354)†	362 (354)	708	.36 n.s.
2	302 (288)	274 (288)	576	1.30 n.s.
3	244 (277)	310 (277)	554	7.88 P = .005
Total	892 (919)	946 (919)	1838	1.59 n.s.

*Observed values.
†Expected, or hypothesized values.

With the use of the same null hypothesis, the following results are obtained.

The individual χ^2's were computed in the same manner as equation (34), in separate tests of the hypothesis for each lake. Note that the first two are not significant whereas the third is significant. This points to probable ecological differences among lakes, a possibility that would not have been discerned by pooling the data.

The test for interaction (dependence) is made by summing the individual χ^2's and subtracting the χ^2 obtained using totals, i.e.,

$$\chi^2 \text{ (interactions)} = \Sigma\chi^2 \text{ (individuals)} - \chi^2 \text{ (total)}$$
$$= .36 + 1.30 + 7.88 - 1.59 = 7.95$$

The degrees of freedom for the interaction χ^2 are the number of individual χ^2's minus one; in this case, two. This interaction χ^2 is significant ($P > .025$), which indicates that the sex ratio is indeed dependent upon the lake.

Another χ^2 test may be illustrated by the following example. Suppose that comparable techniques were used to collect from four streams. With the use of three species common to all streams, it is desired to test the hypothesis that the three species occur in the same ratio regardless of stream, i.e., that their ratio is independent of stream (Table 6).

TABLE 6. OCCURRENCE OF THREE SPECIES OF FISH

Stream	Number of organisms			Frequency
	Species 1	Species 2	Species 3	
1	24* (21.7)†	12 (12.5)	30 (31.7)	66
2	15 (18.5)	14 (10.6)	27 (26.9)	56
3	28 (27.4)	15 (15.7)	40 (39.9)	83
4	20 (19.4)	9 (11.2)	30 (28.4)	59
Total	87	50	127	264
Expected ratio	87/264	50/264	127/264	

*Observed values.
†Expected, or hypothesized

To discuss the table above, $O_{i\,j}$ = the observation for the $i^{\underline{th}}$ stream and the $j^{\underline{th}}$ species. Hence, $O_{2\,3}$ is the observation for stream two and species three, or 27. A similar indexing scheme applies to the expected values, $E_{i\,j}$. For the totals, a subscript replaced by a dot (.) symbolizes that summation has occurred for the observations indicated by that subscript. Hence, $O_{.2}$ is the total for species two (50); $O_{3.}$ is the total for stream three (93); and $O_{..}$ is the grand total (264).

Computations of expected values make use of the null hypothesis that the ratios are the same regardless of stream. The best estimate of this ratio for any species is $\frac{O_{.j}}{O_{..}}$, the ratio of the sum for species j to the total of all species. This ratio multiplied by the total for stream i gives the expected number of organisms of species j in stream i:

$$E_{i\,j} = \left(\frac{O_{.j}}{O_{..}}\right)(O_{i\,.}) \qquad (35)$$

For example,

$$E_{12} = \left(\frac{O_{.2}}{O_{..}}\right)(O_{1.})$$
$$= \frac{50}{264}\,(66)$$
$$= 12.5$$

χ^2 is computed as

$$\chi^2 = \sum_{ij} \frac{(O_{ij} - E_{ij})^2}{E_{ij}} = 2.69 \text{ (n.s.)}$$

For this type of hypothesis, there are (rows − 1) (colums − 1) degrees of freedom, in this case

$$(3)(2) = 6$$

In the example, χ^2 is nonsignificant. Thus, there is no evidence that the ratios among the organisms are different for different streams.

Tests of two types of hypotheses by χ^2 have been illustrated. The first type of hypothesis was one where there was a theoretical ratio, i.e., the ratio of males to females is 1:1. The second type of hypothesis was one where equal ratios were hypothesized, but the values of the ratios themselves were computed from the data. To draw the proper inference, it is important to make a distinction between these two types of hypotheses. Because the ratios are derived from the data in the later case, a better fit to these ratios (smaller χ^2) is expected. This is compensated for by loss of degrees of freedom. Thus, smaller computed χ^2's may be judged significant than would be in the case where the ratios are hypothesized independently of the data.

5.3 F-test

The F distribution is used for testing equality of variance. Values of F are found in books of mathematical and statistical tables as well as in most statistics texts. Computation of the F statistic involves the ratio of two variances, each with associated degrees of freedom. Both of these are used to enter the table. At any entry of the F tables for $(n_1 - 1)$ and $(n_2 - 1)$ degrees of freedom, there are usually two or more entries. These entries are for various levels of probability of rejection of the null hypothesis when in fact it is true.

The simplest F may be computed by forming the ratio of two variances. The null hypothesis is $H_o : \sigma_1{}^2 = \sigma_2{}^2$. The F statistic is

$$F = \frac{s_1{}^2}{s_2{}^2} \qquad (36)$$

where $s_1{}^2$ is computed from n_1 observations and $s_2{}^2$ from n_2. For simple variances, the degrees of freedom, f, will be $f_1 = n_1 - 1$ and $f_2 = n_2 - 1$. The table is entered at the chosen probability level, α, and if F exceeds the tabulated value, it is said that there is a $1 - \alpha$ probability that $\sigma_1{}^2$ exceeds $\sigma_2{}^2$.

5.4 Analysis of Variance

Two simple but potentially useful examples of the analysis of variance are presented to illustrate the use of this technique. The analysis of variance is a powerful and general technique applicable to data from virtually any experimental or field study. There are restrictions, however, in the use of the technique. Experimental errors are assumed to be normally (or approximately normally) distributed about a mean of zero and have a common variance; they are also assumed to be independent (i.e., there should be no correlations among responses that are unaccounted for by the identifiable factors of the study or by the model). The effects tested must be assumed to be linearly additive. In practice these assumptions are rarely completely fulfilled, but the analysis of variance can be used unless significant departures from normality, or correlations among adjacent observations, or other types of measurement bias are suspected. It would be prudent, however, to check with a statistician regarding any uncertainties about the applicability of the test before issuing final reports or publications.

5.4.1 Randomized design

The analysis of variance for completely randomized designs provides a technique often useful in field studies. This test is commonly used for data derived from highly-controlled laboratory or field experiments where treatments are applied randomly to all experimental units, and the interest lies in whether or not the treatments significantly affected the response of the experimental units. This case may be of use in water quality studies, but in these studies the *treatments* are the conditions found, or are classifications based upon ecological criteria. Here the desire is to detect any differences in some type of measurement that might exist in conjunction with the field situation or the classifications or criteria.

For example, suppose it is desired to test whether the biomass of organisms attaching to slides suspended in streams varies from stream to stream. A simple analysis such as this could precede a more in-depth biological study of the comparative productivity of the streams. Data from such a study are presented in Table 7.

TABLE 7. PERIPHYTON PRODUCTIVITY DATA

Stream	Slide	Biomass (mg dry wt.)
1	1	26
	2	20
	3	14
	4	25
2	1	34
	2	28
	3	Lost
	4	23
3	1	31
	2	35
	3	40
	4	28

In testing with the analysis of variance, as with other methods, a null hypothesis should be formulated. In this case the null hypothesis could be:

H_o: There are no differences in the biomass of organisms attached to the slides that may be attributed to differences among streams.

In utilizing the analysis of variance, the test for whether there are differences among streams is made by comparing two types of variances, most often called "mean squares" in this context. Two mean squares are computed: one based upon the means for streams; and one that is free of the effect of the means. In our example, a *mean square for streams* is computed with the use of the averages (or totals) from the streams. The magnitude of this mean square is affected both by differences among the means and by differences among slides of the same stream. The *mean square for slides* is computed that has no contribution due to stream differences. If the null hypothesis is true, then differences among streams do not exist and, therefore, they make no contribution to the *mean square for streams*. Thus, both mean squares (for streams and for slides) are estimates of the same variance, and with repeated sampling, they would be expected to average to the same value. If the null hypothesis (H_o) is true, the ratio of these values is expected to equal one. If H_o is not true, i.e., if there *are* real differences due to the effect of streams, then the mean square for streams is affected by these differences and is expected to be the larger. The ratio in the second case is expected to be greater than one. The ratio of these two variances forms an F-test.

The analysis of variance is presented in Table 8.

The computations are:

$$C = \frac{(85 + 85 + 134)^2}{11} = 8401.45$$

$$\sum_{ij} X_{ij}^2 = 26^2 + 20^2 + \cdots + 40^2 + 28^2 = 8936$$

$$\text{Total SS} = 8936 - 8401.45 = 534.55$$

$$\sum_i \left(\frac{X_{i.}^2}{r_i}\right) = \frac{85^2}{4} + \frac{85^2}{3} + \frac{134^2}{4} = 8703.58$$

$$\text{Streams SS} = 8703.58 - 8401.45 = 302.13$$

$$\begin{aligned}\text{Slides w/i streams SS} &= \text{Total SS} - \text{Streams SS}\\ &= 534.55 - 302.13\\ &= 232.42\end{aligned}$$

The mean squares (MS column) are computed by dividing the sums of squares (SS column) by its corresponding degrees of freedom (df column). (Nothing is usually learned in this context by computing a total MS.) The F-test is

TABLE 8. F-TEST USING PERIPHTON DATA

Source	df	SS
Total	N-1*	$\sum_{ij} X_{ij}^2 - C$
Streams	t-1	$\sum_i \frac{X_{i.}^2}{r_i} - C$
Slides w/i streams	$\sum_i (r_i\text{-}1)$	Total SS - Stream SS

*The symbols are defined as: N = total number of observations (slides); t = number of streams; r_i = number of slides in stream i; X_{ij} = an observation (biomass of a slide); $X_{i.}$ = sum of the observations for stream i; and C = correction for mean = $\frac{(\sum_{ij} X_{ij})^2}{N}$

Source	df	SS	MS	F
Total	10	534.55		
Streams	2	302.13	151.065	5.20*
Slides w/i streams	8	232.42	29.055	

*Significant at the 0.05 probability level.

performed by computing the ratio, (*mean square for streams*)/(*mean square for slides*), in this case, $\frac{151.065}{29.055} = 5.20$.

When the calculated F value (5.20) is compared with the F values in the table (tabular F values) where df = 2 for the numerator and df = 8 for the denominator, we find that the calculated F exceeds the value of the tabular F for probability .05. Thus, the experiment indicates a high probability (greater than 0.95) of there being a difference in biomass attached to the slides, a difference attributable to differences in streams.

Note that this analysis presumes good biological procedure and obviously cannot discriminate differences in streams from differences arising, for example, from the slides having been placed in a riffle in one stream and a pool in the next. In general, the form of any analysis of variance derives from a model describing an observation in the experiment. In the example, the model, although not stated explicitly, assumed only two factors affecting a biomass measurement – streams and slides within streams. If the model had included other factors, a more complicated analysis of variance would have resulted.

5.4.2 Factorial design

Another application of a simple analysis of variance may be made where the factors are arranged factorially. Suppose a field study where the effect of a suspected toxic effluent upon the fish fauna of a river was in question (Tables 9 and 10). Five samples were taken about one-quarter mile upstream and five, one-quarter mile downstream in August of the summer before the plant began operation, and the sampling scheme was repeated in August of the summer after operations began.

Standard statistical terminology refers to each of the combinations P_1T_1, P_2T_1, P_1T_2, and P_2T_2 as treatments or treatment combinations. Of use in the analysis is a table of treatment totals.

In planning for this field study, a null and alternate hypothesis should have been formed. In fact, whether stated explicitly or not, the null hypothesis was:

H_0: The toxic effluent has no effect upon the weight of fish caught

This hypothesis is not stated in statistical terms and, therefore, only implicitly tells us what test to make. Let us look further at the analysis before attempting to state a null hypothesis in statistical terms.

In this study two factors are identifiable: times and positions. A study could have been done on each of the two factors separately, i.e., an attempt could have been made to distinguish whether there was a difference associated with times, assuming all other factors insignificant, and likewise with the positions. The example, used here, however, includes both factors simultaneously. Data are given for times and for positions but with the complication that we cannot assume that one is insignificant when studying the other. For the purpose of this study, whether there is a significant difference with times or on the other hand with positions, are questions that are of little interest. Of interest to this study is whether the upstream-downstream difference varies with times. This type of contrast is termed a positions-times *interaction*. Thus, our null hypothesis is, in statistical

TABLE 9. POUNDS OF FISH CAUGHT PER 10 HOURS OVERNIGHT SET OF A 125-FOOT, 1½-INCH-MESH GILL NET

Times	Positions	
	Upstream (P_1)	Downstream (P_2)
Before (T_1)	28.3	29.0
	33.7	28.9
	38.2	20.3
	41.1	36.5
	17.6	29.4
After (T_2)	15.9	19.2
	29.5	22.8
	22.1	24.4
	37.6	16.7
	26.7	11.3

TABLE 10. TREATMENT TOTALS FOR THE DATA OF TABLE 9

Total	Positions		Times totals
	Upstream	Downstream	
Before	158.9	144.1	303.0
After	131.8	94.4	226.2
Positions totals	290.7	238.5	Grand total 529.2

terminology.

Ho: There is no significant interaction effect

Computations for testing this hypothesis with the use of an analysis of variance table are presented below.

Symbolically, an observation must have three indices specified to be completely identified: position, time, and sample number. Thus there are three subscripts: X_{ijk} is an observation at position i, time j, and from sample k. A value of 1 for i is upstream; 2, downstream; 1 for j is before; 2, after. A particular example is X_{123}, the third sample upstream after the plant began operation, or 22.1 pounds. A total (Table 10) is specified by using the dot notation. For the value of $X_{ij.}$, then the individually sampled values for position i, time j are totaled. It is a total for a treatment combination. For example, the value of $X_{11.}$ is 158.9, and the value of $X_{1..}$, where samplings and times are both totaled to give the total for upstream, is 290.7.

For a slight advantage in generality, let the following additional symbols apply: t = number of times of sampling (in this case t = 2); p = number of positions samples (in this case p = 2); s = number of samples per treatment combination; and n = the total number of observations.

The computations are:

Correction for mean (CT):

$$\frac{(\Sigma X_{ijk})^2}{n} = \frac{(529.2)^2}{20}$$
$$= 14002.63$$

Treatment Sum of Squares (SSTMT):

$$\frac{(\Sigma X_{ij.}^2)}{s} - CT$$

$$\frac{(158.9)^2}{5} + \frac{(131\ 8)^2}{5} + \frac{(144.1)^2}{5} + \frac{(94.4)^2}{5} - 14002.63 = 456.69$$

(Note that the divisor (5) may be factored out here, if desired, but where a different number of samples is taken for each treatment combination it should be left as above.)

Positions Sum of Squares (SSP):

$$\frac{\Sigma X_{i..}^2}{st} - CT$$

$$\frac{(250.7)^2}{10} + \frac{(238.5)^2}{10} - 14002.63 = 136.24$$

Times Sum of Squares (SST):

$$\frac{\Sigma X_{.j.}^2}{sp} - CT$$

$$\frac{(303.0)^2}{10} + \frac{(226.2)^2}{10} - 14002.63 = 294.91$$

Interaction of Positions and Times Sum of Squares (SSPT):

SSTMT - SSP - SST

456.69 - 136.24 - 294.91 = 25.54

Error Sums of Squares:

ΣX_{ijk}^2 - SSTMT - CT

15308.24 - 456.69 - 14002.63 = 848.92

Although not important to this example, the main effects, positions and times, are tested for significance. The F table is entered with df = 1 for effect tested, and df = 16 for error. The positions effect is not significant at any probability usually employed. The times effect is significant with probability greater than .95. The interaction effect is not significant, and we, therefore, conclude that no effect of the suspected toxic effluent can be distinguished in this data. Had the F value for interaction been large enough, we would have rejected the null hypothesis, and concluded that the effluent had a significant effect (Table 11).

TABLE 11. ANALYSIS OF VARIANCE TABLE FOR FIELD STUDY DATA OF TABLE 9

Source	df	SS	MS	F
Treatments	3	456.69		
Positions	1	136.24	136.24	2.56
Times	1	294.91	294.91	5.55*
Positions X times	1	25.54	25.54	<1
Error	16	848.92	53.05	

6.0 CONFIDENCE INTERVALS FOR MEANS AND VARIANCES

When means are computed in field studies, the desire often is to report them as intervals rather than as fixed numbers. This is entirely reasonable because computed means are virtually always derived from samples and are subject to the same uncertainty that is associated with the sample.

The correct computation of confidence intervals requires that the distribution of the observations be known. But very often approximations are close enough to correctness to be of use, and often are, or may be made to be, conservative. For computation of confidence intervals for the mean, the normal distribution is usually assumed to apply for several reasons: the central limit theorem assures us that with large samples the mean is likely to be approximately normally distributed; the required computations are well known and are easily applied; and when the normal distribution is known not to apply, suitable transformation of the data often is available to allow a valid application.

The confidence interval for a mean is an interval within which the true mean is said to have some stated probability of being found. If the probability of the mean *not* being in the interval is α (α could equal .1, .05, .01 or any probability value), then the statement may be written

$$P(CL_1 < \mu < CL_2) = 1 - \alpha$$

This is read, "The probability that the lower confidence limit (CL_1) is less than the true mean (μ) and that the upper confidence limit (CL_2) is greater than the true mean, equals 1 - α." However, we never know whether or not the true mean is actually included in the interval. So the confidence interval statement is really a statement about our procedure rather than about μ. It says that if we follow the procedure for repeated experiments, a proportion of those experiments equal to α will, by chance alone, fail to include the true mean between our limits. For example, if α = .05, we can expect 5 of 100 confidence intervals to fail to include the true mean.

To compute the limits, the sample mean, $\bar{X}$; the standard error, $s_{\bar{X}}$; and the degrees of freedom, n-1; must be known. A $t_{\alpha,\ n-1}$ value from tables of Student's t is obtained corresponding to n-1 degrees of freedom and probability α. The computation is

$$CL_1 = \bar{X} - (t_\alpha)(s_{\bar{X}})$$

$$CL_2 = \bar{X} + (t_\alpha)(s_{\bar{X}})$$

Other confidence limits may be computed, and one additional confidence limit is given in this section – the confidence limits for the true variance, σ^2. The information needed here is similar to that needed for the mean, namely, the estimated variance, s^2; the degrees of freedom, n-1; and values from χ^2 tables. The values from χ^2 depend upon the degrees of freedom and upon the probability level, α. The confidence interval is

$$P\left[\frac{(n-1)s^2}{\chi^2_{\frac{\alpha}{2}}} \leqq \sigma^2 \leqq \frac{(n-1)s^2}{\chi^2_{1-\frac{\alpha}{2}}}\right] = 1-\alpha$$

This will be illustrated for α = .05; (n-1) = 30; and s^2 = 5. Since α = .05; $1 - \frac{\alpha}{2} = 0.975$; the associated $\chi^2_{.975}$ = 16.8 and the $\chi^2_{0.025}$ = 47.25. Thus, the probability statement for the variance in this case is

$$P(3.19 \leqq \sigma^2 \leqq 16.8) = .95$$

7.0 LINEAR REGRESSION AND CORRELATION

7.1 Basic Concepts

It is often desired to investigate relationships between variables, i.e., rate of change of biomass and concentration of some nutrient; mortality per unit of time and concentration of some toxic substance; chlorophyll and biomass; or growth rate and temperature. As biologists, we appreciate the incredible complexity of the real-world relationships between such variables, but, simultaneously, we may wish to investigate the desirability of approximating these relationships with a straight line. Such an approximation may prove invaluable if used judiciously within the limits of the conditions where the relation holds. It is important to recognize that no matter how well the straight line describes the data, a causal relationship between the variables is never implied. Causality is much more difficult to establish than mere description by a statistical relation.

When studying the relationship between two variables, the data may be taken in one of two ways. One way is to measure two variables, e.g., measure dry weight biomass and an associated chlorophyll measurement. Where two variables are measured, the data are termed bivariate. The other way is to choose the level of one variable and measure the associated magnitude of the other variable.

Straight line equations may be obtained for each of these situations by the technique of *linear regression* analysis, and if the object is to predict one variable from the other, it is desirable to obtain such a relation. When the degree of (linear) association is to be examined, no straight line need be derived – only a measure of the strength of the relationship. This measure is the *correlation coefficient*, and the analysis is termed correlation analysis.

Thus, linear regression analysis and linear correlation analysis are two ways in which linear relationships between two variables may be examined.

7.2 Basic Computations

7.2.1 Regression equation

The regression equation is the equation for a straight line,

$$Y = a + bx$$

A graphic representation of this function is a straight line plotted on a two-axis graph. The line intercepts the y-axis a distance, a, away from the origin and has a slope whose value is b. Both a and b can be negative, zero, or positive. Figure 6 illustrates various possible graphs of a regression equation.

The regression equation is obtained by "least-squares," a technique ensuring that a "best" line will be objectively obtained. The application of least-squares to the simple case of a straight line relation between two variables is extremely simple.

In Table 12 is a set of data that are used to illustrate the use of regression analysis. Figure 7 is a plot of these data along with fitted line and confidence bands.

In fitting the regression equation, it is convenient to compute at least the following quantities:

(1) n = the number of pairs of observation of X and Y,
(2) ΣX = the total for X,
(3) ΣY = the total for Y,

TABLE 12. PERCENT SURVIVAL TO FRY STAGE OF EGGS OF GOGGLE-EYED WYKE VERSUS CONCENTRATION OF SUPERCHLOROKILL IN PARENTS' AQUARIUM WATER

Percent survival (Y)	Concentration, ppb (X)
74.	1.
82.	1.
68.	1.
65.	2.
60.	2.
72.	2.
64.	3.
60.	3.
57.	3.
51.	4.
50.	4.
55.	4.
24.	6.
28.	6.
36.	6.
0.	10.
10.	10.
4.	10.

(4) ΣX^2 = the total of the squared X's,
(5) ΣY^2 = the total of the squared Y's,
(6) ΣXY = the total of the products of the X,Y pairs,
(7) $(\Sigma X)^2$ = the square of quantity (2),
(8) $(\Sigma Y)^2$ = the square of quantity (3),
(9) $(\Sigma X)(\Sigma Y)$ = the product of quantities (2) and (3),
(10) CT_x = quantity (7) divided by quantity (1),
(11) CT_y = quantity (8) divided by quantity (1),
(12) CT_{xy} = quantity (9) divided by quantity (1).

With the calculation of these quantities, most of the work associated with using linear regression is complete. Often calculating machine characteristics may be so utilized that when one quantity is calculated the calculation of another is partly accomplished. Modern calculators and computers greatly simplify this task.

In Table 13 are the computed values of quantities (1) through (12) for the data of Table 12.

The estimated value for the slope of the line, b, is computed using

$$b = \frac{\Sigma XY - CT_{xy}}{\Sigma X^2 - CT_x} = \frac{(6) - (12)}{(4) - (10)} \qquad (37)$$

For the example, this is

$$b = \frac{2453 - 3726.67}{498 - 338}$$
$$= -8$$

rounded to the nearest whole number.

Computation of the estimated intercept, a, is as follows:

$$a = \bar{y} - b\bar{x} \qquad (38)$$
$$= \frac{\Sigma Y}{n} - b\frac{\Sigma X}{n}$$
$$= \frac{(3)}{(1)} - b\frac{(2)}{(1)}$$

which for the example

$$= \frac{860}{18} - (-8)\frac{78}{18}$$
$$= 82$$

rounded to the nearest whole number.

Thus, the regression equation for this data is

$$\hat{Y} = 82 - 8X$$

where $\hat{Y}$ = the percent survival, and X = concentration of pesticide.

Figure 7 shows the regression line, plotted along with the data points. Note that this line appears to be a good fit but that an eye fit might have been slightly different and still appear to be a "good fit." This indicates that some uncertainty is associated with the line. If a value for y is obtained with the use of the regression equation with a given x, another experiment, however well controlled, could easily produce a different value. The predicted values for y are subject to some uncertainty, and a statement of that uncertainty should invariably accompany the use of the predicted y.

TABLE 13. COMPUTED VALUES OF QUANTITIES (1) THROUGH (12) FOR THE DATA OF TABLE 12

Quantity	Value
(1) n	18
(2) ΣX	78
(3) ΣY	860
(4) ΣX^2	498
(5) ΣY^2	51,676
(6) ΣXY	2,453
(7) $(\Sigma X)^2$	6,084
(8) $(\Sigma Y)^2$	739,600
(9) $(\Sigma X)(\Sigma Y)$	67,080
(10) CT_x	338
(11) CT_y	41,088.89
(12) CT_{xy}	3,726.67

7.2.2 Confidence intervals

The proper statement of the uncertainty is an interval estimate, the same type as those previously discussed for means and variances. The probability statement for a predicted y depends upon the type of prediction being made. The regression equation is perhaps most often used to predict the mean y to be expected when x is some value, but it may also be used to predict the value of a particular observation of y when x is some value. These two types of predictions differ only in the width of the confidence intervals. A confidence interval for a predicted observation will be the wider of the two types because of uncertainty associated with variations among observations of y for a given x.

To compute the confidence intervals, first compute a variance estimate. This is the variance due to deviations of the observed values from the regression line. This computation is:

$$s^2_{y.x} = \frac{\Sigma Y^2 - CT_y - \frac{(\Sigma XY - CT_{xy})^2}{(\Sigma X^2 - CT_x)}}{n-2} \qquad (39)$$

For this example:

$$s^2_{y.x} = \frac{51{,}676 - 41{,}089 - \frac{(2{,}453 - 3{,}727)^2}{(498 - 338)^2}}{18-2} = 28$$

This statistic is useful in other computations as will become apparent.

For the confidence interval, the square root of the above statistic, or the standard error of deviations from regression is required, i.e.,

$$s_{y.x} = \sqrt{s^2_{y.x}} = 5 \qquad (40)$$

The confidence limits are computed as follows for a predicted mean:

$$CL(\hat{Y}) = a + bX_p \pm (t_\alpha)(s_{y.x})\sqrt{\frac{1}{n} + \frac{(X_p - \bar{X})^2}{(\Sigma X^2 - CT_x)}} \qquad (41)$$

where t_α is chosen from a table of t values using n−2 degrees of freedom and probability level α; $\hat{Y}$ = the computed Y for which the confidence interval is sought, a mean $\hat{Y}$ predicted to be

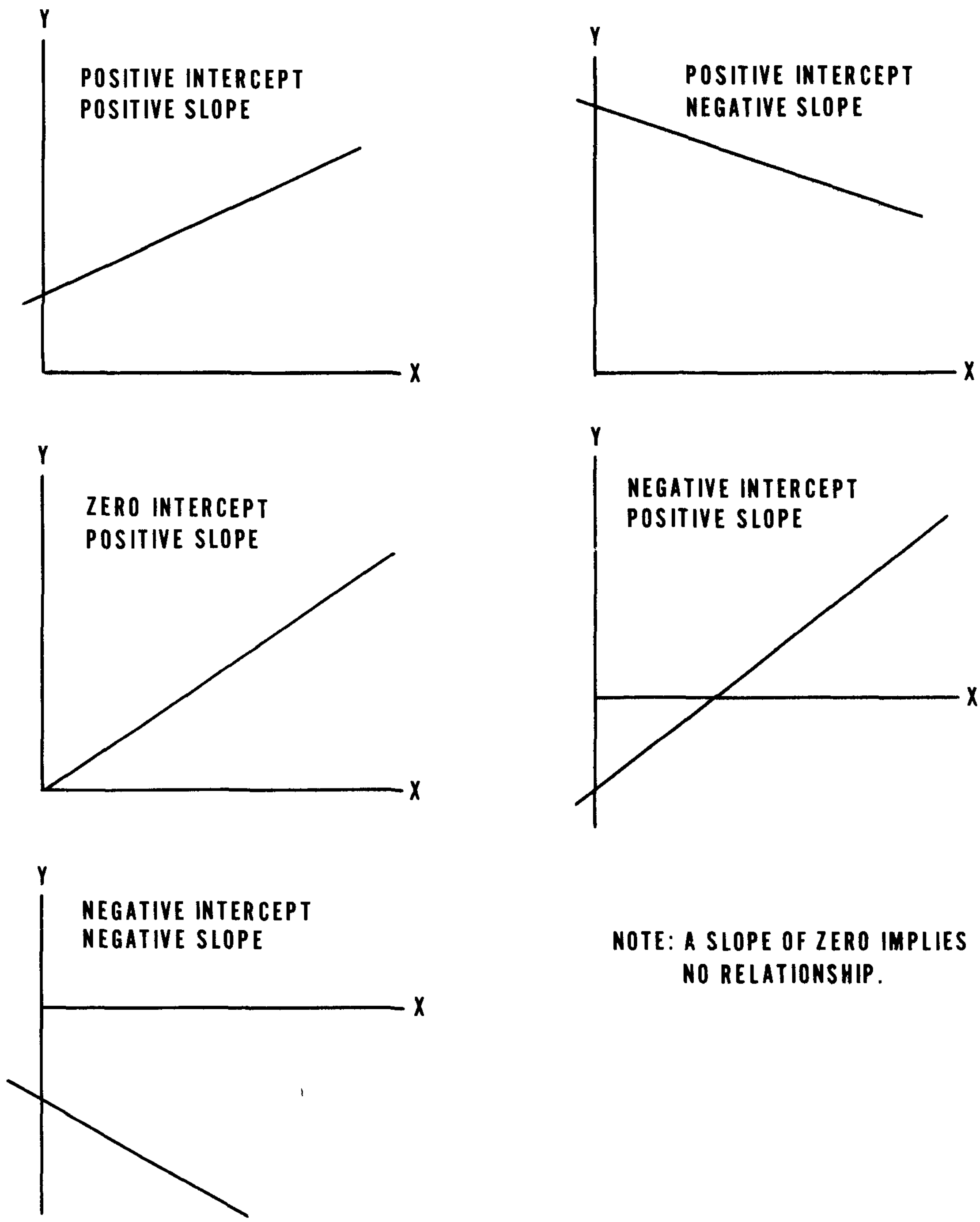

Figure 6. Examples of straight-line graphs illustrating regression equations.

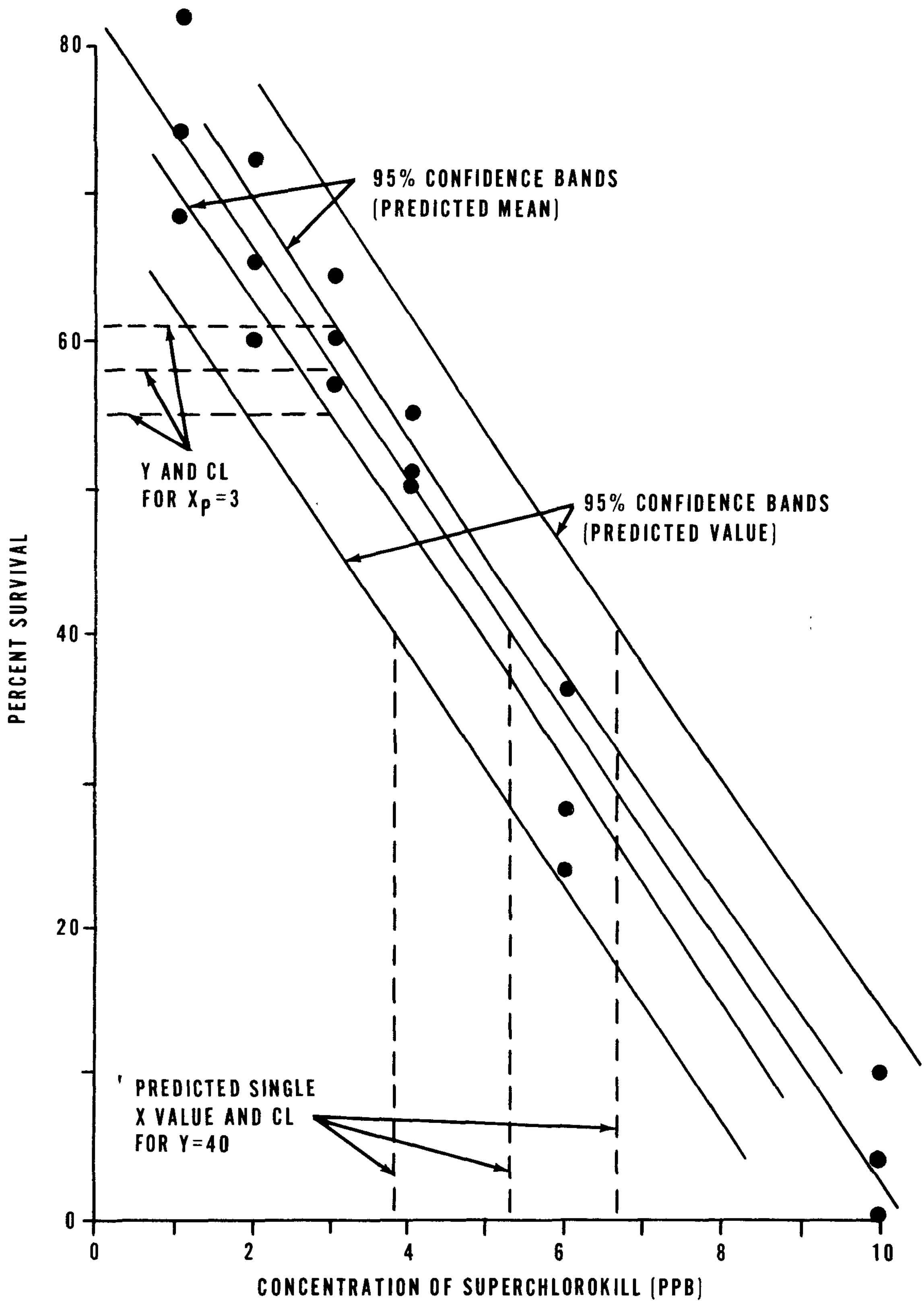

Figure 7. Regression analysis of data in Table 12.

observed on the average when the X value is X_p; X_p = the particular X value used to compute $\hat{Y}$; $\bar{X}$ = the mean of the X's used in these computations; $\frac{\Sigma X}{n} = \frac{(2)}{(1)}$; ΣX^2 = relation (4) in the computations; and CT_x = relation (10) in the computations. Note that in using Equation (41) where the signs (±) are shown, the minus (−) sign is used when computing the lower confidence limit and the plus (+) for the upper.

If a confidence interval for a particular Y (given a particular X, i.e., $\hat{Y}$) is desired, the confidence limits are computed using

$$CL(\hat{Y}) = a + bX_p \pm (t_\alpha)(s_{y.x})\sqrt{1 + \frac{1}{n} + \frac{(X_p - \bar{X})^2}{(\Sigma X^2 - CT_x)}} \quad (42)$$

Note that Equation (42) differs from Equation (41) only by the addition of 1 under the radical. All the symbols are the same as for Equation (41). Again these confidence intervals will be wider than those for $\hat{\bar{Y}}$.

If a graphical representation of the confidence interval for $\hat{\bar{Y}}$ or $\hat{Y}$ over a range of X is desired, merely compute the confidence interval for several (usually about 5) values of X, plot them on the same graph as the regression line, and draw a smooth curve through them. The intervals at the extremes of the data will be wider than the intervals near the mean values. This is because the uncertainty in the estimated slope is greater for the extreme values than for the central ones.

With such a plot, the predicted value of Y and its associated confidence interval for a given X can be read (see Figure 7, vertical line corresponding to X = 3 and notation).

7.2.3 Calibration curve

Often with data such as that given in Table 12, a calibration curve is needed from which to predict X when Y is given. That is, the linear relation is established from the data where values of X (say pesticide) are fixed and then Y (survival of eggs) is observed, where this relation predicts Y given X; then unknown concentrations of the pesticide are used, egg survival measured, and the relation is worked backwards to obtain pesticide concentration from egg survival. This may be done graphically from a plot such as that illustrated in Figure 7. Predicted X's and associated confidence intervals may be read from the plot (see horizontal line corresponding to y = 40 and notation).

Calibration curves and confidence intervals may also be worked algebraically. Where the problem has fixed X's, as in the example, the equation for X should be obtained algebraically, i.e.,

$$X = \frac{(Y-a)}{b} \quad (43)$$

for a predicted X ($\hat{X}$) given a mean value $\bar{Y}_m$ from a sample of m observations, the confidence limits may be computed as follows:

compute the quantity

$$A = b^2 - \frac{t_\alpha^2 s_{y.x}^2}{(\Sigma X^2 - CT_x)}$$

compute the confidence limits as (44)

$$CL(\hat{X}) = \bar{X} + \frac{b(\bar{Y}_m - \bar{Y})}{A} \pm \frac{t_\alpha s_{y.x}}{A}\sqrt{A\left(\frac{1}{m} + \frac{1}{n}\right) + \frac{(\bar{Y}_m - \bar{Y})^2}{(\Sigma X^2 - CT_x)}}$$

where $\bar{Y}_m$ = the average of m newly observed Y values; $\bar{X}$, b, $\bar{Y}$, $s_{y.x}$, ΣX^2, CT_x, and n = values obtained from the original set of data and whose meanings are unchanged. Note that m may equal one, and $\bar{Y}_m$ would therefore be a single observation.

7.3 Tests of Hypotheses

If it is not clear that a relationship exists between Y and X, a test should be made to determine whether the slope differs from zero. The test is a t-test with n−2 degrees of freedom. The t value is computed as

$$t = \frac{b - \beta_0}{s_b}, \quad (45)$$

where

$$s_b = \frac{s_{y.x}}{\sqrt{\Sigma X^2 - CT_x}}$$

Since the null hypothesis is

$$H_0 : \beta_0 = 0$$

set β_0 = 0 in the t-test and it becomes

$$t = \frac{b}{s_b}$$

If the computed t exceeds the tabular t, then the null hypothesis is rejected and the estimated slope, b, is tentatively accepted. Other values of β_o may be tested in the null hypothesis and in the t-test statistic.

With data such as those in Table 12, another hypothesis may be tested – that of lack of fit of the model to the data, or bias. This idea must be distinguished from random deviations from the straight line. Lack of fit implies a nonlinear trend as the true model, whereas random deviations from the model imply that the model adequately represents the trend. If more than one Y observation is available for each X (3 in the example Table 12), random fluctuations can be separated from deviations from the model, i.e., a random error may be computed at each point so that deviations from regression may be partitioned into random error and lack of fit.

The test is in the form of an analysis of variance and is illustrated in brief form symbolically in Table 14. Here, the F ratio MSL/MSE tests linearity, i.e., whether a linear model is sufficient; the ratio MSR/MSD tests whether the slope is significantly different from zero.

TABLE 14. ILLUSTRATION OF ANALYSIS OF VARIANCE TESTING LINEARITY OF REGRESSION AND SIGNIFICANCE OF REGRESSION

Source	df	MS	F
Total	n-1		
Regression	1	MSR	MSR/MSD
Deviations from regression	n-2	MSD	
Lack of fit	m-2	MSL	MSL/MSE
Error	n-m	MSE	

To use this analysis, one set of computations must be made in addition to those of Table 13. The computation is the same as that for treatment sums of squares in the analysis of variance previously discussed; in this case, levels of X are comparable to treatments. First compute the sum of the Y's, T_i, for each level of X. For $X = 1, T_1 = 224$, etc. Then compute:

$$\Sigma \frac{T_i^2}{k_i}$$

where k_i = the number of observations for the level of X; in this case always 3. For the example,

$$\Sigma \frac{T_i^2}{k_i} = 51341$$

With this, the analysis of variance table (Table 15) may be constructed. In the first part of Table 15, the sums of squares and degrees of freedom are given symbolically to relate to the computations of Table 13 and to the above computations. The mean squares (MS) are always obtained by dividing SS by df.

When the data for Table 12 are analyzed (second part of Table 15), there is a very unusual coincidence in the values of MS for deviations from regression, lack of fit, and error. *Note that this is coincidence and they must always be computed separately.*

As already known from the graph, t-test, etc., the regression is highly significant. A negative result from the test for nonlinearity (lack of fit) was also suspected from the visually-satisfactory fit of Figure 7. Therefore, for this range of data, we can conclude that a *linear* (straight line) *rela-*

TABLE 15. ANALYSIS OF VARIANCE OF THE DATA OF TABLE 12; TESTS FOR LINEARITY AND SIGNIFICANCE OF REGRESSION*

Source	df	SS
Total	n-1	$\Sigma Y^2 - CT_y$
Regression	1	$\frac{(\Sigma XY - CT_{xy})^2}{(\Sigma X^2 - CT_x)}$
Deviations from regression	n-2	Total SS - Regression SS
Lack of fit	m-2	Deviation SS - Error SS
Error	n-m	$\Sigma Y^2 - \frac{\Sigma T_i^2}{k_i}$

*Symbols refer to quantities of Table 13 or to symbols defined in the text immediately preceding this table.

For the data of Table 12:

Source	df	SS	MS	F
Total	17	10,587		
Regression	1	10,139	10,139	362**
Deviations from regression	16	448	28	
Lack of fit	4	113	28	1 n.s.
Error	12	335	28	

**Significant at the 0.01 probability level.

tionship exists, with estimated slope and intercept as computed.

7.4 Regression for Bivariate Data

As mentioned, where two associated measurements are taken without restrictions on either, the data are called bivariate. Linear regression is sometimes used to predict one of the variables by using a value from the other. Because no attempt is usually made to test bivariate data for lack of fit, a test for deviation from regression is as far as an analysis of variance table is taken. Linearity is assumed. Large deviations from linearity will appear in deviations from regression and cause the F values that are used to test for the significance of regression to appear to be nonsignificant.

Computations for the bivariate case exactly follow those for the univariate case [quantities (1) to (12) and as illustrated for the univariate case, Table 13]. The major operating difference is that, for bivariate data, the dependent variable is chosen as the variable to be predicted, whereas for univariate data, the dependent variable is fixed in advance. For example, if the bivariate data are pairs of observations on algal biomass and chlorophyll, either could be considered the dependent variable. If biomass is being predicted, then it is dependent. For the univariate case, such as for the data of Table 12, percent survival is the dependent variable by virtue of the nature of the experiment.

In the preceding section, it was seen that X and its confidence interval could be predicted from Y for univariate data (Equations 43, 44, and 45). But note that Equation (43) is merely an algebraic rearrangement of the regression of Y on X. For the bivariate case, this approach is not appropriate. If a regression of Y on X is fitted for bivariate data, and subsequently a prediction of X rather than Y is desired, a new regression must be computed. This is a simple task, and all the basic quantities are contained in a set of computations similar to computations in Table 13. A summary of the types of computations for univariate and bivariate data is given in Table 16.

TABLE 16. TYPES OF COMPUTATIONS ACCORDING TO VARIABLE PREDICTED AND DATA TYPE*

Predicted variable	Bivariate data	Univariate data (fixed X's)
Y	$y = R_1\ (X)$	$y = R_1\ (X)$
X	$x = R_2\ (Y)$	$X = R_1^{-1}\ (y)$

*R_1 symbolizes the regression using Y as dependent variable, R_2 a regression computed using X as dependent variable, R_1^{-1} is a algebraic rearrangement solving for X when the regression was R_1.

Since the computations for the bivariate regression of Y on X are the same as those for the univariate case, they will not be repeated. Where X is to be predicted, all computations proceed simply by interchanging X and Y in the notation. The computations for b and a are:

for the slope:

$$b_{x.y} = \frac{\Sigma XY - CT_{xy}}{\Sigma Y^2 - CT_y} = \frac{(6) - (12)}{(5) - (11)} \tag{46}$$

for the intercept:

$$a_{x.y} = \frac{(\Sigma X)}{n} - b_{x.y}\frac{(\Sigma Y)}{n} = \frac{(2)}{(1)} - b_{x.y}\frac{(3)}{(1)} \tag{47}$$

7.5 Linear Correlation

If a linear relationship is known to exist or can be assumed, the degree of association of two variables can be examined by linear correlation analysis. The data must be bivariate.

The correlation coefficient, r, is computed by the following:

$$r = \frac{\Sigma XY - CT_{xy}}{\sqrt{(\Sigma X^2 - CT_x)(\Sigma Y^2 - CT_y)}} \tag{48}$$

A perfect correlation (all points falling on a straight line with a nonzero slope) is indicated by a correlation coefficient of, r = 1, or r = -1. The negative value implies a decrease in one of the variables with an increase in the other. Correlation coefficients of r = 0 implies no linear relationship between the variables. Any real data will result in correlation coefficients between the extremes.

If a correlation coefficient is computed and is of low magnitude, test it to determine whether it is significantly different from zero. The test, a t-test, is computed as follows:

$$t = \frac{r}{\sqrt{\frac{(1-r^2)}{(n-2)}}} \tag{49}$$

The computed t is compared with the tabular t with n-2 degrees of freedom and chosen probability level. If the computed t exceeds the tabular t, the null hypothesis that the true correlation coefficient equals zero is rejected, and the computed r may be used.

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PLANKTON

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1.0 INTRODUCTION

Plankton are defined here as organisms suspended in a body of water and because of their physical characteristics or size, are incapable of sustained mobility in directions counter to the water currents. Most of the plankton are microscopic and of essentially neutral buoyancy. All of them drift with the currents.

Plankton consists of both plants (phytoplankton) and animals (zooplankton), and complex interrelationships exist among the various components of these groups. Chlorophyll-bearing plants such as algae usually constitute the greatest portion of the biomass of the plankton. Phytoplankton use the energy of sunlight to metabolize inorganic nutrients and convert them to complex organic materials. Zooplankton and other herbivores graze upon the phytoplankton and, in turn, are preyed upon by other organisms, thus passing the stored energy along to larger and usually more complex organisms. In this manner nutrients become available to large organisms such as macroinvertebrates and fish.

Organic materials excreted by plankton, and products of plankton decomposition, provide nutrients for heterotrophic microorganisms (many of which are also members of the plankton assemblage). The heterotrophs break down organic matter and release inorganic nutrients which become available again for use by the "primary producers." In waters severely polluted by organic matter, such as sewage, heterotrophs may be extremely abundant, sometimes having a mass exceeding that of the algae. As a result of heterotrophic metabolism, high concentrations of inorganic nutrients become available and massive algal blooms may develop.

Plankton may form the base of the food pyramid and drift with the pollutants; therefore, data concerning them may be particularly significant to the pollution biologist. Plankton blooms often cause extreme fluctuations of the dissolved oxygen content of the water, may be one of the causes of tastes and odors in the water and, if present in large numbers, are aesthetically objectionable. In some cases, plankton may be of limited value as indicator organisms because the plankton move with the water currents; thus, the origin of the plankton may be obscure and the duration of exposure to pollutants may be unknown.

The quantity of phytoplankton occurring at a particular station depends upon many factors including sampling depth, time of day, season of year, nutrient content of water, and the presence of toxic materials.

2.0 SAMPLE COLLECTION AND PRESERVATION

2.1 General Considerations

Before plankton samples are collected, a study design must be formulated. The objectives must be clearly defined, and the scope of the study must remain within the limitations of available manpower, time, and money. Historical, biological, chemical, and physical (especially hydrological) data should be examined when planning a study. Examination of biological and chemical data often reveals areas that warrant intensive sampling and other areas where periodic or seasonal sampling will suffice.

Physical data are extremely useful in the design of plankton studies; of particular importance are data concerning volume of flow, currents, prevailing wind direction, temperature, turbidity (light penetration), depths of reservoir penstock releases, and estuarine salinity "wedges."

After historical data have been examined, the study site should be visited for reconnaissance and preliminary sampling. Based on the results of this reconnaissance and on the preliminary plankton data, the survey plan can be modified to better fulfill study objectives and to facilitate efficient sampling.

2.1.1 Influential factors

In planning and conducting a plankton survey, a number of factors influence decisions and often alter collection routines. Since water currents determine the directions of plankton movements, knowing the directions, intensity, and complexity of currents in the sampling area is important. Some factors that influence currents are winds, flow, solar heating, and tides.

Sunlight influences both the movements of plankton and primary production. Daily vertical plankton migrations are common in many waters. Cloud cover, turbidity, and shading (e.g., from ice cover and dense growths of vegetation) influence the amount of light available to plankton.

Chemical factors, such as salinity, nutrients, and toxic agents, can profoundly affect plankton production and survival.

The nutrients most frequently mentioned in the literature as stimulators of algal growth are nitrogen and phosphorus; however, a paucity of any vital nutrient can limit algal production. The third category of chemical factors, toxic agents, is almost limitless in its components and combinations of effects. Toxic compounds may be synergistic or antagonistic to one another and may either kill planktonic organisms or alter their life cycles. Many chemicals discharged in industrial effluents are toxic to plankton.

2.1.2 Sampling frequency

The objectives of the study and time and manpower limitations dictate the frequency at which plankton samples are taken. If it is necessary to know the year-round plankton population in a body of water, it is necessary to sample weekly through spring and summer and monthly through fall and winter. However, more frequent sampling is often necessary. Because numerous plankton samples are usually needed to characterize the plankton, take daily samples whenever possible. Ideally, collections include one or two subsurface samples per day at each river sampling station and additional samples at various depths in lakes, estuaries, and oceans.

2.1.3 Sampling locations

In long-term programs, such as ambient trend monitoring, sampling should be sufficiently frequent and widespread to define the nature and quantity of all plankton in the body of water being studied. In short-term studies designed to show the effects of specific pollution sources on the plankton, sampling station locations and sampling depths may be more restricted because of limitations in time and manpower.

The physical nature of the water greatly influences the selection of sampling sites. On small streams, a great deal of planning is not usually required; here, locate the stations upstream from a suspected pollution source and as far downstream as pollutional effects are expected. Take great care, however, in interpreting plankton data from small streams, where much of the "plankton" may be derived from the scouring of periphyton from the stream bed. These attached organisms may have been exposed to pollution at fixed points for unknown time periods. On rivers, locate sampling stations, both upstream and downstream from pollution sources and, because lateral mixing often does not occur for great distances downstream, sample on both sides of the river. In both rivers and streams, care should be taken to account for confusing interferences such as contributions of plankton from lakes, reservoirs, and backwater areas. Plankton sampling stations in lakes, reservoirs, estuaries, and the oceans are generally located in grid networks or along longitudinal transects.

The location, magnitude, and temperature of pollutional discharges affect their dispersal, dilution, and effects on the plankton. Pollutants discharged from various sources may be antagonistic, synergistic, or additive in their effects on plankton. If possible, locate sampling stations in such a manner as to separate these effects.

In choosing sampling station locations, include areas from which plankton have been collected in the past. Contemporary plankton data can then be compared with historical data, thus documenting long-term pollutional effects.

2.1.4 Sampling depth

The waters of streams and rivers are generally well mixed, and subsurface sampling is sufficient. Sample in the main channel and avoid backwater areas. In lakes and reservoirs where plankton composition and density may vary with depth, take samples from several depths. The depth at the station and the depth of the thermocline (or sometimes the euphotic zone) generally determines sampling depths. In shallow areas (2 to 3 meters, 5 to 10 feet), subsurface sampling is usually sufficient. In deeper areas, take samples at regular intervals with depth. If only phytoplankton are to be examined, samples may be taken at three depths, evenly spaced from the surface to the thermocline. When collecting zooplankton, however, sample at 1-meter intervals from the surface to the lake bottom.

Because many factors influence the nature and distribution of plankton in estuaries, intensive sampling is necessary. Here, marine and freshwater plankton may be found along with brackish-water organisms that are neither strictly marine nor strictly freshwater inhabitants. In addition to the influences of the thermocline and light penetration on plankton depth distribution, the layering of waters of different salinities may inhibit the complete mixing of freshwater plankton with marine forms. In estuaries with extreme tides, the dimensions of these layers may change considerably during the course of the tidal cycle. However, the natural buoyancy of the plankton generally facilitates the mixing of forms. Estuarine plankton should be sampled at regular intervals from the surface to the bottom three or four times during one or more tidal cycles.

In deep marine waters or lakes, collect plankton samples at 3- to 6-meter intervals throughout the euphotic zone (it is neither practical nor profitable to sample the entire water column in very deep waters). The limits of sampling depth in these waters may be an arbitrary depth below the thermocline or the euphotic zone, or both. Perform tow or net sampling at 90° to the wind direction.

2.1.5 Field notes

Keep a record book containing all information written on the sample label, plus pertinent additional notes. These additional notes may include, but need not be restricted to:

- Weather information – especially direction and intensity of wind
- Cloud cover
- Water surface condition – smooth? Is plankton clumping at surface?
- Water color and turbidity
- Total depth at station
- A list of all types of samples taken at station.
- General descriptive information (e.g., direction, distance, and description of effluents in the vicinity). Sampling stations should be plotted on a map.

2.1.6 Sample labelling

Both labels and marker should be water proof (a soft-lead pencil is recommended). Insert the labels into sample containers immediately as plankton samples are collected. Record the following information on all labels:

- Location
  name of river, lake, etc.
  distance and direction to nearest city
  state and county
  river mile, latitude, and longitude, or other description
- Date and time
- Depth
- Type of sample (e.g., grab, vertical plankton net haul, etc.)
- Sample volume, tow length
- Preservatives used and concentration
- Name of collector

2.2 Phytoplankton

2.2.1 Sampling equipment

The type of samping equipment used is highly dependent upon where and how the sample is being taken (i.e., from a small lake, large deep lake, small stream, large stream, from the shore, from a bridge, from a small boat, or from a large boat) and how it is to be used.

The cylindrical type of sampler with stoppers that leave the ends open to allow free passage of water through the cylinder while it's being lowered is recommended. A messenger is released at the desired depth to close the stoppers in the ends. The Kemmerer, Juday, and Van Dorn samplers have such a design and can be obtained in a variety of sizes and materials. Use only nonmetallic samplers when metal analysis, algal assays, or primary productivity measurements are being performed. In shallow waters and when surface samples are desired, the sampler can be held in a horizontal position and operated manually. For sampling in deep waters, the Nansen reversing water bottle is often used and a boat equipped with a winch is desirable. Take caution when sampling from bridges with a Kemmerer type water bottle; if the messenger is dropped from the height of a bridge, it can batter and destroy the triggering device. To avoid this, support a messenger a few feet above the sampler by an attached string and drop it when the sampler is in place.

Net collection of phytoplankton is not recommended for quantitative work. Nannoplankton and even larger algae, such as some pennate diatoms, are thin enough to pass through the meshes of the net if oriented properly. Using a pump also presents problems: when the water is stratified, the tubing must be flushed between samplings and delicate algae may be harmed.

2.2.2 Sample volume

No fixed rule can be followed concerning the volume of sample to be taken – sampling personnel must use their own judgment. The volume of the sample needed depends on the numbers and kinds of analyses to be carried out, e.g., cell counts, chlorophyll, dry weight. When phytoplankton densities are less than 500 per ml, approximately 6 liters of sample are required for Sedgwick-Rafter and diatom species proportional counts. In most cases, a 1- to 2-liter sample will suffice for more productive waters.

2.2.3 Sample preservation

Biologists use a variety of preservatives, and each has advantages. If samples are to be stored for more than 1 year, the preferred preservative is formalin (40 percent formaldehyde = 100 percent formalin), which has been neutralized with sodium tetraborate (pH 7.0 to 7.3). Five milliliters of the neutralized formalin are added for each 100 ml of sample. This preservative will cause many flagellated forms to lose flagella. Adding saturated cupric sulfate solution to the preserved samples maintains the green color of phytoplankton samples and aids in distinguishing phytoplankton from detritus. One milliliter of the saturated solution per liter of sample is adequate. Adding detergent solution prevents clumping of settled organisms. One part of surgical detergent to five parts of water makes a convenient stock solution. Add 5 ml of stock solution per liter of sample. Do not use detergent when diatom slides are to be made.

Merthiolate is less desirable as a preservative, but offers the advantage of staining cell parts and simplifying identification. It also causes some of the algae, such as blue-greens, to lose gas from their vacuoles and, therefore, enhances settling. Samples preserved with merthiolate are not sterile, and should not be stored for more than 1 year. After that time formalin should be used. Merthiolate solution is prepared by dissolving the following in 1 liter of distilled water.

- 1.0 gram of merthiolate (sodium ethylmercury thiosalicylate).
- 1.0 ml of aqueous saturated iodine–potassium iodide solution prepared by dissolving 40 grams of iodine and 60 grams of potassium iodide in 1 liter of distilled water.
- 1.5 gram of Borax (sodium borate)

Dissolve each of the components separately in approximately 300 ml of distilled water, combine, and make up to 1 liter with distilled water. Add the resulting stock solution to samples to give a final concentration (V/V) of 36 mg/liter (i.e., 37.3 ml added to 1 liter of sample).

2.3 Zooplankton

2.3.1 Sampling equipment

Zooplankton analyses require larger samples than those needed for phytoplankton analyses. Collect quantitative samples with a messenger-operated water bottle, plankton trap, or metered plankton net. Obtain semi-quantitative samples by filtering surface water samples through nylon netting or by towing an unmetered plankton net through the water. In moderately and highly productive waters, a 6-liter water sample is usually sufficient. In oligotrophic, estuarine, and coastal waters, remove zooplankters from several hundred liters of the waters being sampled with the use of towed nets. Take duplicate samples if chemical analyses are desired.

Several sampling methods can be used.

Towing

An outboard motor boat fitted with a small davit, meter wheel, wire-angle indicator, and hand-operated winch is desirable. A 3- to 5-kg weight attached to the line is used to sink the net. Maintain speed to ensure a wire angle near 60° for easy calculation of the actual sampling depth of the net. The actual sampling depth equals the amount of wire extended times the cosine of the wire angle.

Oblique tow--Make an 8-minute tow at four levels in the water column (2 minutes at each level: just above the bottom, 1/3 total depth, 2/3 total depth, and just below the surface) to estimate zooplankton abundance.

Horizontal tow--Take samples for estimating zooplankton distribution and abundance within a particular layer of water with a messenger-operated net equipped with a flow-through measuring vane (such as the Clarke-Bumpus sampler). Each tow lasts from 5 to 8 minutes.

Vertical two--Lower a weighted net to the desired depth, record the amount of line extended, and retrieve at a rate of 0.5 to 1.0 meters per second. The volume of water strained can be estimated. Duplicate vertical tows are suggested at each station.

To sample most sizes of zooplankters, two nets of different mesh size can be attached a short distance apart on the same line.

Net casting

Zooplankton can also be sampled from shore by casting a weighted net as far as possible, allowing the net to reach depth, and hauling to shore at the rate of 0.5 to 1.0 meters per second. Take several samples to obtain a qualitative estimate of relative abundance and species present.

Suggested net sizes are: No. 6 (0.239 mm aperture) for adult copepods in estuarine and coastal waters; No. 10 (0.158 mm) for copepodites in saline water or microcrustacea in fresh water; and No. 20 (0.076 mm) for rotifers and nauplii. The No. 20 net clogs easily with phytoplankton because of its small aperture size.

Rinse messenger-operated samplers with clean water, allow to dry, and lubricate all moving parts with light machine oil. Clean nylon netting material thoroughly, rinse with clean water, and allow to dry (out of direct sunlight) before storing.

2.3.2 Sample volume

The sample volume varies with the specific purpose of the study. Twenty-liter surface samples obtained by bucket and filtered through a No. 20 net are large enough to obtain an estimate of zooplankton present in flowing streams and ponds. In lakes, large rivers, estuaries and coastal waters, filter 1.5 m^3 (horizontal tow) to 5 m^3 (oblique tow) of water through nets for adequate representation of species present.

2.3.3 Sample preservation

For identification and enumeration, preserve grab samples in a final concentration of 5 percent neutral (add sodium tetraborate to obtain a pH of 7.0 to 7.3) formalin. Adding either 70 percent ethanol or 5 percent neutral formalin, each with 5 percent glycern (glycerol) added, to preserve the concentrated net samples. Formalin is usually used for preserving samples obtained from coastal waters. In detritus-laden samples, add 0.04 percent Rose Bengal stain to help differentiate zooplankters from plant material.

For chemical analysis (taken, in part, from Recommended Procedures for Measuring the Productivity of Plankton Standing Stock and Related Oceanic Properties, National Academy of Sciences, Washington, D.C. 1960), the concentrated sample is placed in a fine-meshed (bolting silk or nylon) bag, drained of excess water, placed in a plastic bag, and frozen for laboratory processing. If the sample is taken from an estuarine or coastal station, the nylon bag is dipped several times in distilled water to remove the chloride from interstitial seawater which can interfere with carbon analysis.

3.0 SAMPLE PREPARATION

3.1 Phytoplankton

As the phytoplankton density decreases, the amount of concentration must be increased and, accordingly, larger sample volumes are required.

As a rule of thumb, concentrate samples when phytoplankton densities are below 500 per ml; approximately 6 liters of sample are required at that cell concentration. Generally, 1 liter is an adequate routine sample volume.

The following three methods may be used for concentrating preserved phytoplankton, but sedimentation is preferred.

3.1.1 Sedimentation

Preserved phytoplankton samples can often be settled in the original storage containers. Settling time is directly related to the depth of the sample in the bottle or settling tube. On the average, allow 4 hours per 10 mm of depth. After settling, siphon off the supernatant (Figure 1) or decant through a side drain. The use of a detergent aids in settling. Exercise caution because of the different sedimentation rates of the diverse sizes and shapes of phytoplankton.

3.1.2 Centrifugation

During centrifugation, some of the more fragile forms may be destroyed or flagella may become detached. In using plankton centrifuges, many of the cells may be lost; modern continuous-flow centrifuges avoid this.

3.1.3 Filtration

To concentrate samples by filtration, pass through a membrane filter. A special filter apparatus and a vacuum source are required. Samples containing large amounts of suspended material (other than phytoplankton) are difficult to enumerate by this method, because the suspended matter tends to crush the phytoplankters or obscure them from view. The vacuum should not exceed 0.5 atmospheres. Concentration by filtration is particularly useful for samples low in plankton and silt content.

3.2 Zooplankton

The zooplankton in grab samples are concentrated prior to counting by allowing them to settle for 24 hours in laboratory cylinders of appropriate size or in specially constructed settling tubes (Figure 1).

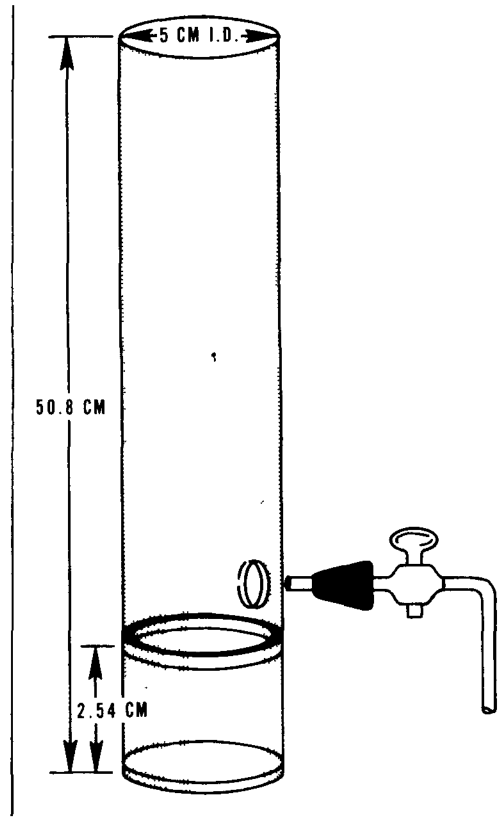

Figure 1. Plexiglas plankton settling tube with side drain and detachable cup. Not drawn to scale.

Take care to recover organisms (especially the Cladocera) that cling to the surface of the water in the settling tube.

4.0 SAMPLE ANALYSIS

4.1 Phytoplankton

4.1.1 Qualitative analysis of phytoplankton

The optical equipment needed includes a good quality compound binocular microscope with a mechanical stage. For high magnification, a substage condenser is required. The ocular lens should be 8X to 12X. Binocular eyepieces are generally preferred over a monocular eyepiece because of reduced fatigue. Four turret-mounted objective lenses should be provided with magnifications of approximately 10, 20, 45, and

100X. When combined with the oculars, the following characteristics are approximately correct.

Objective lens	Ocular lens	Subject magnification	Maximum working distance between objective and cover slip, mm	Depth of focus, μ
10X	10X	100X	7	8
20X	10X	200X	1.3	2
45X	10X	450X	0.5-0.7	1
100X	10X	1000X	0.2	0.4

An initial examination is needed because most phytoplankton samples will contain a diverse assemblage of organisms. Carry out the identification to species whenever possible. Because the size range of the individual organisms may extend over several orders of magnitude, no single magnification is completely satisfactory for identification. For the initial examination, place one or two drops of a concentrate on a glass slide and cover with a No. 1 or No. 1-1/2 cover slip. Use the 10X objective to examine the entire area under the cover slip and record all identifiable organisms. Then examine with the 20 and 45X objectives. Some very small organisms may require the use of the 100X objective (oil immersion) for identification. The initial examination helps to obtain an estimate of population density and may indicate the need for subsequent dilution or concentration of the sample, to recognize characteristics of small forms not obvious during the routine counting procedure, and to decide if more than one type of counting procedure must be used.

When identifying phytoplankton, it is useful to examine fresh, unpreserved samples. Preservation may cause some forms to become distorted, lose flagella, or be lost together. These can be determined by a comparison between fresh and preserved samples.

As the sample is examined under the microscope, identify the phytoplankton and tally under the following categories: coccoid blue-green, filamentous blue-green, coccoid green, filamentous green, green flagellates, other pigmented flagellates, centric diatoms, and pennate diatoms. In tallying diatoms, distinguish between "live" cells, i.e., those that contain any part of a protoplast, and empty frustules or shells.

The availability of taxonomic bench references and the skill of the biologist will govern the sophistication of identification efforts. No single reference is completely adequate for all phytoplankton. Some general references that should be available are listed below. Those marked with an asterisk are considered essential.

American Public Health Association, 1971. Standard methods for the examination of water and wastewater. 13th edition. Washington, D.C.

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*Patrick, R., and C. W. Reimer. 1966. The diatoms of the United States. Vol. I. Academy of Natural Sciences, Philadelphia.

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4.1.2 Quantitative analysis of phytoplankton

To calibrate the microscope, the ocular must be equipped with a Whipple grid-type micrometer. The exact magnification with any set of oculars varies, and therefore, each combination of oculars and objectives must be calibrated by matching the ocular micrometer against a stage micrometer. Details of the procedure are given in Standard Methods, 13th Edition.

When counting and identifying phytoplankton, analysts will find that samples from most natural waters seldom need dilution or concentration and that they can be enumerated directly. In those samples where algal concentrations are extreme, or where silt or detritus may interfere, carefully dilute a 10-ml portion of the sample 5 to 10 times with distilled water. In samples with very low populations, it may be necessary to concentrate organisms to minimize statistical counting errors. The analyst should recognize, however, that manipulations involved in dilution and concentration may introduce error.

Among the various taxa are forms that live as solitary cells, as components of natural groups or aggregates (colonies), or as both. Although every cell, whether solitary or in a group, can be individually tallied, this procedure is difficult, time consuming, and seldom worth the effort. The unit or clump count is easier and faster and is the system used commonly within this Agency. In this procedure, all unicellular or colonial (multi-cellular) organisms are tallied as single units and have equal numerical weight on the bench sheet.

The apparatus and techniques used in counting phytoplankton are described here.

Sedgwick-Rafter (S-R) Counting Chamber

The S-R cell is 50 mm long by 20 mm wide by 1 mm deep; thus, the total area of the bottom of the cell is 1000 mm^2 and the total volume is 1000 mm^3 or one ml. Check the volume of each counting chamber with a vernier caliper and micrometer. Because the depth of the chamber normally precludes the use of the 45X or 100X objectives, the 20X objective is generally used. However, special long-working-distance, higher-power objectives can be obtained.

For the procedure, see Standard Methods, 13th Edition. Place a 24 by 60 mm, No. 1 coverglass diagonally across the cell, and with a large-bore pipet or eyedropper, quickly transfer a 1-ml aliquot of well-mixed sample into the open corner of the chamber. The sample should be directed diagonally across the bottom of the cell. Usually, the cover slip will rotate into place as the cell is filled. Allow the S-R cell to stand for at least 15 minutes to permit settling. Because some organisms, notably blue-green algae, may float, examine the underside of the cover slip and add these organisms to the total count. Lower the objective lens carefully into position with the coarse focus adjustment to ensure that the cover slip will not be broken. Fine focus should always be up from the cover slip.

When making the strip count, examine two to four "strips" the length of the cell, depending upon the density of organisms. Enumerate all forms that are totally or partially covered by the image of the Whipple grid.

When making the field count, examine a minimum of 10 random Whipple fields in at least two identically prepared S-R cells. Be sure to adopt a consistent system of counting organisms that lie only partially within the grid or that touch one of the edges.

To calculate the concentration of organisms with the S-R cell, for the strip count:

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

where:

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

To calculate the concentration of organisms with the field count:

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

where:

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

Multiply or divide the number of cells per milliliter by a correction factor for dilution (including that resulting from the preservative) or for concentration.

Palmer-Maloney (P-M) Nannoplankton Cell

The P-M cell was especially designed for enumerating nannoplankton with a high-dry objective (45X). It has a circular chamber 17.9 mm in diameter and 0.4 mm deep, with a volume of 0.1 ml. Although useful for examining samples containing a high percentage of nannoplankton, more counts may be required to obtain a valid estimate of the larger, but less numerous, organisms present. Do not use this cell for routine counting unless the samples have high counts.

Pipet an aliquot of well-mixed sample into one of the 2 X 5 mm channels on either side of the circular chamber with the cover slip in place. After 10 minutes, examine the sample under the high-dry objective and count at least 20 Whipple fields.

To calculate the concentration of organisms:

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

where:

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

Bacterial Counting Cells and Hemocytometers

The counting cells in this group are precisely-machined glass slides with a finely ruled grid on a counting plate and specially-fitted ground cover slip. The counting plate proper is separated from the cover slip mounts by parallel trenches on opposite sides. The grid is ruled such that squares as small as 1/20 mm (50 μ) to a side are formed within a larger 1-mm square. With the cover slip in place, the depth in a Petroff-Hausser cell is 1/50 mm (20μ) and in the hemocytometer 1/10 mm (100μ). An optical micrometer is not used.

With a pipet or medicine dropper, introduce a sample to the cell and at high magnification identify and count all the forms that fall within the gridded area of the cell.

To calculate the number of organisms per milliliter, multiply all the organisms found in the gridded area of the cell by the appropriate factor. For example, the multiplication factor for the Petroff-Hausser bacterial counting cell is based on the volume over the entire grid. The dimensions are 1 mm X 1 mm X 1/50 mm, which gives a volume of 1/50 mm^3 and a factor of 50,000.

Carefully follow the manufacturer's instructions that come with the chamber when purchased. Do not attempt routine counts until experienced in its use and the statistical validity of the results are satisfactory. The primary disadvantage of this type of counting cell is the extremely limited capacity, which results in a large multiplication factor. Densities as high as 50,000 cells/ml are seldom found in natural waters except during blooms. Such populations may be found in sewage stabilization ponds or in laboratory cultures.

For statistical purposes, a normal sample must be either concentrated or a large number of mounts per sample should be examined.

Membrane Filter

A special filtration apparatus and vacuum source are required, and a 1-inch, 0.45μ membrane filter is used.

Pass a known volume of the water sample through the membrane filter under a vacuum of 0.5 atmospheres. (Note: in coastal and marine waters, rinse with distilled water to remove salt.) Allow the filter to dry at room temperature for 5 minutes, and place it on top of two drops of immersion oil on a microscope slide. Place two drops of oil on top of the filter and allow it to dry clear (approximately 48 hours) at room temperature, cover with a cover slip, and enumerate the organisms. The occurrence of each species in 30 random fields is recorded.

Experience is required to determine the proper amount of water to be filtered. Significant amounts of suspended matter may obscure or crush the organisms.

Calculate the original concentration in the sample as a function of a conversion factor obtained from a prepared table, the number of quadrates or fields per filter, the amount of sample filtered, and the dilution factor. (See Standard Methods, 13th Edition.)

Inverted Microscope

This instrument differs from the conventional microscope in that the objectives are mounted below the stage and the illumination comes from above. This design allows cylindrical counting chambers (which may also be sedimentation tubes) with thin clear glass bottoms to be placed on the stage and sedimented plankton to be examined from below, and it permits the use of short focus, high-magnification objectives including oil immersion. A wide range of concentrations is automatically obtained by merely altering the height of the chamber. Chambers can be easily and inexpensively made: use tubular Plexiglas for large capacity chambers, and flat, plastic plates of various thicknesses, which have been carefully bored out to the desired dimension, for smaller chambers; then cement a No. 1 or No. 1-1/2 cover slip to form the cell bottom. Precision-made, all-glass counting chambers in a wide variety of dimensions are also available. The counting technique differs little from the S-R procedure, and either the strip or separate field counts can be used. The Whipple eyepiece micrometer is also used.

Transfer a sample into the desired counting chamber (pour with the large chambers, or pipet with 2-ml or smaller chambers), fill to the point of overflow, and apply a glass cover slip. Set the chamber aside and keep at room temperature until sedimentation is complete. On the average, allow 4 hours per 10 mm of height. After a suitable period of settling, place the chamber on the microscope stage and examine with the use of the 20X, 45X, or 100X oil immersion lens. Count at least two strips perpendicular to each other over the bottom of the chamber and average the values. Alternatively, random field counts can be made; the number depends on the density of organisms found. As a general rule, count a minimum of 100 of the most abundant species. At higher magnification, count more fields than under lower power.

When a 25.2 mm diameter counting chamber is used (the most convenient size), the conversion of counts to numbers per ml is quite simple:

$$\text{No. per ml (strip count)} = \frac{C \times 1000 \text{ mm}^3}{L \times W \times D \times S}$$

where:

C = number of organisms counted (tally)
L = length of a strip, mm
W = width of a strip (Whipple grid image width), mm
D = depth of chamber, mm
S = number of strips counted

$$\text{No. per ml (field count)} = \frac{C \times 1000 \text{ mm}^3}{A \times D \times F}$$

where:

C = number of organisms counted (tally)
A = area of a field (Whipple grid image area), mm^2
D = depth of chamber, mm
F = number of fields counted

Diatom Analysis

Study objectives often require specific identification of diatoms and information about the relative abundance of each species. Since the taxonomy of this group is based on frustule characteristics, low-power magnification is seldom sufficient, and permanent diatom mounts are prepared and examined under oil immersion.

To concentrate the diatoms, centrifuge 100 ml of thoroughly mixed sample for 20 minutes at 1000 X *g* and decant the supernatant with a suction tube. Pour the concentrated sample into a disposable vial, and allow to stand at least 24 hours before further processing. Remove the supernatant water from the vial with a suction tube. If the water contains more than 1 gm of dissolved solids per liter (as in the case of brackish water or marine samples), salt crystals form when the sample dries and obscure the diatoms on the finished slides. In this case, reduce the concentration of salts by refilling the vial with distilled water, resuspending the plankton, and allowing the vial to stand 24 hours before removing the supernatant liquid. Repeat the dilution several times if necessary.

If the plankton counts are less than 1000 per ml, concentrate the diatoms from a larger volume of sample (1 to 5 liters) by allowing them to settle out. Exercise caution in using this method, however, to ensure quantitative removal of cells smaller than 10 microns in diameter.

Thoroughly mix the plankton concentrate in a vial with a disposable pipet, and deliver several drops to a No. 1, circular 18-mm coverglass. Dry the samples on a hotplate at 95°C. (Caution: overheating may cause splattering and cross-contamination of samples.) When dry, examine the coverglasses to determine if there is sufficient material for a diatom count. If not, repeat the previous steps one or two more times, depending upon the density of the sedimented sample. Then heat the samples on a heavy-duty hotplate 30 minutes at approximately 570°C to drive off all organic matter. Remove grains of sand or other large objects on the cover glass with a dissection needle. The oil immersion objective has a very small working distance, and the slide may be unusable if this is not done.

Label the frosted end of a 25- X 75-mm microscope slide with the sample identification. Place the labelled slide on a moderately warm hotplate (157°C), put a drop of Hyrax or Aroclor 5442 (melt and use at about 138°C) mounting medium (Index of Refraction 1.66-1.82) at the center, and heat the slide until the solvent (xylene or toluene) has evaporated (the solvent is gone when the Hyrax becomes hard and brittle upon cooling).

While the coverglass and slide are still hot, grasp the coverglass with a tweezer, invert, and place on the drop of Hyrax on a slide. It may be necessary to add Hyrax at the margin of the coverglass. Some additional bubbles of solvent vapor may appear under the coverglass when it is placed on the slide. When the bubbling ceases, remove the slide from the hotplate and place on a firm, flat surface. Immediately apply slight pressure to the coverglass with a pencil eraser (or similar object), and maintain until the Hyrax cools and hardens (about 5 seconds). Spray a protective coating of clear lacquer on the frosted end of the slide, and scrape the excess Hyrax from around the coverglass.

Identify and count the diatoms at high magnification under oil. Examine random lateral strips the width of the Whipple grid, and identify and count all diatoms within the borders of the grid until 250 cells (500 halves) are tallied.

Ignore small cell fragments. If the slide has very few diatoms, limit the analysis to the number of cells encountered in 45 minutes of scanning.

When the count is completed, total the tallies and calculate the percentages of the individual species.

4.2 Zooplankton

4.2.1 Qualitative analysis of zooplankton

In the initial examination, remove excess preservative from the sample with the use of an aspirator bulb attached to a small piece of glass tubing whose orifice is covered with a piece of No. 20 mesh netting. Swirl the sample, and with a large-bore pipet, remove a portion of the suspension and place 2 ml into each section of a four-compartment glass culture dish (100 X 15 mm). Examine a total of 8 ml for adult Copepoda, Cladocera, and other large forms with the use of a binocular dissecting microscope at a magnification of 20 to 40X. Count and identify rotifers at a higher magnification (100X). All animals should be identified to species if possible. For qualitative analysis of relative frequency, the following classification is suggested:

Species in fields, %	Relative frequency
60 – 100	abundant
30 – 60	very common
5 – 30	common
1 – 5	occasional
<1	rare

The following taxonomic bench references are recommended:

Calman, W. T. 1912. The Crustacea of the order Cumacea in the collection of the United States National Museum. No. 1876-Proc. U. S. Natl. Mus. 41: 603-676.

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Ferguson, E., Jr. 1967. New ostracods from the Playa lakes of eastern New Mexico and western Texas. Trans. Amer. Microsc. Soc. 86(3):244-250.

Hyman, L. H. 1951. The Invertebrates: Acanthocephala, Aschelminthes, and Ectoprocta. The pseudocoelomate Bilateria. Vol. III. McGraw-Hill, New York, 572 pp.

Light, S. F. 1938. New subgenera and species of diaptomid copepods from the inland waters of California and Nevada. Univ. Calif. Publ. in Zool. 43(3): 67-78. Univ. Calif. Press, Berkeley.

Marsh, C. C. 1933. Synopsis of the calanoid crustaceans, exclusive of the Diaptomidae, found in fresh and brackish waters, chiefly of North America. No. 2959, Proc. U. S. Nat. Mus. 82 (Art. 18): 1-58.

Pennak, R. W. 1953. Fresh-water invertebrates of the United States. The Roland Press Co., New York. 369 pp.

Ruber, E. 1968. Description of a salt marsh copepod cyclops *(Apocyclops) spartinus* n. sp. and a comparison with closely related species. Trans. Amer. Microsc. Soc. 87(3):368-375.

Wilson, M. S. 1956. North American Harpacticoid copepods.

1. Comments on the known fresh water species of the Canthocamptidae.

2. *Canthocamptus oregonensis* n. sp. from Oregon and California. Trans. Amer. Microsc. Soc. 75 (3): 290-307.

Wilson, M. S. 1958. The copepod genus *Halicyclops* in North America, with a description of a new species from Lake Pontchartrain, Louisiana, and the Texas coast. Tulane Studies Zool. 6(4): 176-189.

Zimmer, C. 1936. California Crustacea of the order Cumacea. No. 2992. Proc. U. S. Natl. Mus. 83:423-439.

4.2.2 Quantitative analysis of zooplankton

Pipet Method

Remove excess liquid using a screened (No. 20 mesh net) suction device until a 125- to 250-ml sample volume remains. Pour the sample into a conical container graduated in milliliters, and allow the zooplankton to settle for 5 minutes. Read the settled volume of zooplankton; multiply the settled volume by a factor of five to obtain the total diluted volume; and add enough water to obtain this volume. Insert a 1-ml Stempel pipet into the water-plankton mixture, and stir rapidly with the pipet. While the mixture is still agitated, withdraw a 1-ml subsample from the center of the water mass. Transfer the subsample to a gridded culture dish (110 X 15mm) with 5-mm squares. Rinse the pipet with distilled water into a culture dish to remove any adherent organisms. Enumerate (about 200 zooplankters) and identify under a dissecting microscope.

To calculate the number of plankton with an unmetered collecting device:

$$\text{Total no.} = \frac{DV}{SV} \times TN$$

To calculate the number of plankton with a metered collecting device:

$$\text{No. per m}^3 \text{ of water} = \frac{TN \times \frac{DV}{SV}}{Q}$$

where:
DV = total diluted volume, ml
SV = total subsample volume, ml
TN = total no. zooplankters in sample
Q = quantity of water strained, m^3

Counting Chamber

Bring the entire concentrate (or an appropriate aliquot) to a volume of 8 ml, mix well, and transfer to a counting chamber 80 X 50 X 2 mm (8-ml capacity). To fill, use the technique previously described for the Sedgwick-Rafter cell. The proper degree of sample concentration can be determined only by experience.

Using a compound microscope equipped with an ocular Whipple grid, enumerate and identify the rotifers (to species if possible) in 10 strips scanned at a magnification of 100X (one-fifth of the chamber volume). Enumerate the nauplii also during the rotifer count. Count the adult microscrustacea under a binocular dissecting microscope at a magnification of 20 to 40X by scanning the entire chamber. Species identification of rotifers and microcrustacea often require dissection and examination under a compound microscope (see Pennak, 1953).

When calculating the number of plankton, determine the volume of the counting chamber from its inside dimensions. Convert the tallies to organisms per liter with the use of the following relationships:

$$\text{Rotifers per liter} = \frac{T \times C}{P \times V}$$

$$\text{Microcrustacea per liter} = \frac{T \times C}{S \times V}$$

where:
T = total tally
C = total volume of sample concentrate, ml
P = volume of 10 strips in the counting chamber, ml
V = volume of netted or grab sample, liters
S = volume of counting chamber, ml

5.0 BIOMASS DETERMINATION

Because natural plankton populations are composed of many types of organisms (i.e., plant, animal, and bacterial), it is difficult to obtain quantitative values for each of the component populations. Currently-used indices include dry and ash-free weight, cell volume, cell surface area, total carbon, total nitrogen, and chlorophyll content. The dry and ash-free weight methods yield data that include the particulate inorganic materials as well as the plankton. Cell volume and cell surface area determinations can be made on individual components of the population and thus yield data on the plant, the animal, or the bacterial volume, or surface area, or both. Chlorophyll determinations yield data on the phytoplankton.

5.1 Dry and Ash-Free Weight

To reduce the amount of contamination by dissolved solids, wash the sample with several volumes of distilled water by centrifugation or settling. After washing, concentrate the sample by centrifugation or settling. If possible, take sufficient sample to provide several aliquots each having at least 10 mg dry weight. Process at least two replicate aliquots for each sample. (Generally, 10 mg dry weight is equivalent to 100 mg wet weight.)

5.1.1 Dry weight

Place the aliquot of concentrated sample in a tared porcelain crucible, and dry to a constant weight at 105°C (24 hours is usually sufficient). Subtract the weight of crucible to obtain the dry weight.

5.1.2 Ash-free weight

After the dry weight is determined, place the crucible in a muffle furnace at 500°C for 1 hour. Cool, rewet the ash with distilled water, and bring to constant weight at 105°C. The ash is wetted to reintroduce the water of hydration of the clay and other minerals that, though not driven off at 105°C, is lost at 500°C. This water loss often amounts to 10 percent of the weight lost during ignition and, if not corrected for, will be interpreted as organic matter. Subtract the weight of crucible and ash from the dry weight to obtain ash-free weight.

5.2 Chlorophyll

All algae contain chlorophyll *a*, and measuring this pigment can yield some insight into the relative amount of algal standing crop. Certain algae also contain chlorophyll *b* and *c*. Since the chlorophyll concentration varies with species and with environmental and nutritional factors that do not necessarily affect the standing crop, biomass estimates based on chlorophyll measurements are relatively imprecise. Chlorophyll can be measured *in vivo* fluorometrically or in acetone extracts *(in vitro)* by fluorometry or spectrophotometry.

5.2.1 In vitro measurement

The algae differ considerably in the ease of pigment extraction. The diatoms extract easily, whereas the coccoid greens extract with difficulty. Complete extraction of pigments from all taxonomic groups, therefore, requires disruption of the cells with a tissue grinder or blender, or by freezing or drying. Generally, pigment is more difficult to extract from old cells than from young cells.

Concentrate the algae with a laboratory centrifuge, or collect on a membrane filter (0.45-μ porosity) or a glass fiber filter (0.45-μ effective pore size). If the analysis will be delayed, dry the concentrate and store frozen in a desiccator. Keep the stored samples in the dark to avoid photochemical breakdown of the chlorophyll.

Place the sample in a tissue grinder, cover with 2 to 3 milliliters of 90 percent aqueous acetone (use reagent grade acetone), add a small amount (0.2 ml) of saturated aqueous solution of magnesium carbonate and macerate.

Transfer the sample to a screw-capped centrifuge tube, add sufficient 90 percent aqueous acetone to bring the volume to 5 ml, and steep at 4°C for 24 hours in the dark. Use the solvent sparingly, avoiding unnecessary pigment dilution. Agitate midway during the extraction period and again before clarifying.

To clarify the extract, centrifuge 20 minutes at 500 *g*. Decant the supernatant into a clean, calibrated vessel (15-ml, screw-capped, calibrated centrifuge tube) and determine the volume. Minimize evaporation by keeping the tube capped.

Three procedures for analysis and concentration calculations are described.

<u>Trichromatic Method</u>

Determine the optical density (OD) of the extract at 750, 663, 645, and 630 nanometers (nm) using a 90 percent aqueous acetone blank. Dilute the extract or shorten the light path if necessary, to bring the OD_{663} between 0.20 and 0.50. The 750 nm reading is used to correct for turbidity. Spectrophotometers having a resolution of 1 nm or less are preferred. Stopper the cuvettes to minimize evaporation during the time the readings are being made.

The chlorophyll concentrations in the extract are determined by inserting the corrected 1-cm OD's in the following equations. (UNESCO 1966).

$$C_a = 11.64D_{663} - 2.16D_{645} + 0.10D_{630}$$
$$C_b = -3.94D_{663} + 20.97D_{645} - 3.66D_{630}$$
$$C_c = -5.53D_{663} - 14.81D_{645} + 54.22D_{630}$$

where C_a, C_b, C_c are the concentrations, in milligrams per liter, of chlorophyll *a*, *b*, and *c*, respectively, in the extract; and D_{663}, D_{645}, and D_{630} are the 1-cm OD's at the respective wavelengths, after subtracting the 750-nm blank.

The concentration of pigment in the phytoplankton grab sample is expressed as mg/m^3 or $\mu g/m^3$ or μg/liter and is calculated as follows:

$$\text{mg chlorophyll } a/\text{m}^3 = \frac{C_a \times \text{volume of extract (liters)}}{\text{volume of grab sample (m}^3)}$$

Fluorometric (for chlorophyll *a*)

The fluorometric method is much more sensitive than the photometric method and permits accurate determination of much lower concentrations of pigment and the use of smaller sample volumes. Optimum sensitivity is obtained at excitation and emission wavelengths of 430 and 663 nm, respectively, using a R-136 photomultiplier tube. Fluorometers employing filters should be equipped with Corning CS-5-60 excitation and CS-2-64 emission filters, or their equivalents. Calibrate the fluorometer with a chlorophyll solution of known concentration.

Prepare a chlorophyll extract and determine the concentration of chlorophyll *a* by the spectrophotometric method as previously described.

Prepare serial dilutions of the extract to provide concentrations of approximately 0.002, 0.006, 0.02 and 0.06 mg chlorophyll *a* per liter of extract, so that a minimum of two readings are obtained in each sensitivity range of the fluorometer (1/3 and 2/3 of full scale). With the use of these values, derive factors to convert the fluorometer readings in each sensitivity range to milligrams of chlorophyll *a* per liter of extract.

$$F_s = \frac{\text{Conc. chlorophyll } a \text{ (mg/l)}}{\text{fluorometer reading}}$$

where F_s is the fluorometric conversion factor and s is the sensitivity range (door).

5.2.2 In vivo measurement

Using fluorescence to determine chlorophyll *a in vivo* is much less cumbersome than methods involving extraction; however, it is reportedly considerably less efficient than the extraction method and yields about one-tenth as much fluorescence per unit weight as the same amount in solution. The fluorometer should be calibrated with a chlorophyll extract that has been analyzed with a spectrofluorometer.

To determine the chlorophyll *a*, zero the fluorometer with a distilled water blank before taking the first sample reading at each sensitivity level.

Mix the phytoplankton sample thoroughly to ensure a homogenous suspension of algal cells. Pour an aliquot of the well-mixed sample into a cuvette, and read the fluorescence. If the reading (scale deflection) is over 90 units, use a lower sensitivity setting, e.g., $30\times > 10\times > 3\times > 1\times$. Conversely, if the reading is less than 15 units, increase the sensitivity setting. If the samples fail to fall in range, dilute accordingly. Record the fluorescent units based on a common sensitivity factor, e.g., a reading 50 at 1X equals 1500 at 30X.

5.2.3 Pheophytin Correction

Pheophytin is a natural degradation product of chlorophll and often occurs in significant quantities in phytoplankton. Pheophytin *a*, although physiologically inactive, has an absorption peak in the same region of the visible spectrum as chlorophyll *a* and can be a source of error in chlorophyll determinations. In nature, chlorophyll is converted to pheophytin upon the loss of magnesium from the porphyrin ring. This conversion can be accomplished in the laboratory by adding acid to the pigment extract. The amount of pheophytin *a* in the extract can be determined by reading the OD_{663} before and after acidification. Acidification of a solution of pure chlorophyll *a* results in a 40 percent reduction in the OD_{663}, yielding a "before:after" OD ratio ($663_b/663_a$) of 1.70. Samples with $663_b/663_a$ ratios of 1.70 are considered free of pheophytin *a*, and contain algal populations consisting mostly of intact, nondecaying organisms.

Conversely, samples containing pheophytin *a* but not chlorophyll *a* show no reduction in OD_{663} upon acidification, and have a $663_b/663_a$ ratio of 1.0. Samples containing both pigments will have ratios between 1.0 and 1.7.

To determine the concentration of pheophytin *a*, prepare the extract as previously described and determine the OD_{663}. Add one drop of 1 N HCl to the cuvette, mix well, and reread the OD_{750} and OD_{663} after 30 seconds.

Calculate the chlorophyll *a* and pheophytin *a* as follows:

$$\text{Chlorophyll } a \text{ (mg/m}^3\text{)} = \frac{26.7\,(663_b - 663_a) \times E}{V \times L}$$

$$\text{Pheophytin } a \text{ (mg/m}^3\text{)} = \frac{26.7\,(1.7 \times 663_a - 663_b) \times E}{V \times L}$$

where 663_b is the 1-cm corrected OD_{663} before acidification; 663_a is the OD_{663} after acidification; E the volume of acetone used for the extraction (ml); V the volume of water filtered (liters); and L the path length of the cuvette (cm).

5.3 Cell Volume

5.3.1 Microscopic (algae and bacteria)

Concentrate an aliquot of sample by settling or centrifugation, and examine wet at a 1000X magnification with a microscope equipped with a calibrated ocular micrometer. Higher magnification may be necessary for small algae and the bacteria. Make optical measurements and determine the volume of 20 representative individuals of each major species. Determine the average volume (cubic microns), and multiply by number of organisms per milliliter.

5.3.2 Displacement (zooplankton)

Separate sample from preservative by pouring through a piece of No. 20 mesh nylon bolting cloth placed in the bottom of a small glass funnel. To hasten evaporation, wash sample with a small amount of 50 percent ethanol to remove excess interstitial fluid and place on a piece of filter or blotting paper. Place the drained plankton in a 25-, 50-, or 100-ml (depending on sample size) graduated cylinder, and add a known volume of water from a burette. Read the water level in the graduated cylinder. The difference between the volume of the zooplankton plus the added water and the volume of the water alone is the displacement volume and, therefore, the volume of the total amount of zooplankton in the sample.

5.4 Cell Surface Area of Phytoplankton

Measure the dimensions of several representative individuals of each major species with a microscope. Assume the cells to be spherical cylindrical, rectangular, etc., and from the linear dimensions, compute the average surface area (μ^2) per species. Multiply by the number of organisms per milliliter (Welch, 1948, lists mathematical formulas for computing surface area).

6.0 PHYTOPLANKTON PRODUCTIVITY

Phytoplankton productivity measurements indicate the rate of uptake of inorganic carbon by phytoplankton during photosynthesis and are useful in determining the effects of pollutants and nutrients on the aquatic community.

Several different methods have been used to measure phytoplankton productivity. Diurnal curve techniques, involving pH and dissolved oxygen measurements, have been used in natural aquatic communities by a number of investigators. Westlake, Owens, and Talling (1969) present an excellent discussion concerning the limitations, advantages, and disadvantages of diurnal curve techniques as applied to non-isolated natural communities. The oxygen method of Gaarder and Gran (1927) and the carbon-14 method of Steeman-Neilson (1952) are techniques for measuring *in situ* phytoplankton productivity. Talling and Fogg (1959) discussed the relationship between the oxygen and carbon-14 methods, and the limitations of both methods. A number of physiological factors must be considered in the interpretation of the carbon-14 method for measurement of phytoplankton productivity. Specialized applications of the carbon-14 method include bioassay of nutrient limiting factors and measurement of the potential for algal growth.

The carbon-14 method and the oxygen method have the widest use, and the following procedures are presented for the *in situ* field measurement of inorganic carbon uptake by these methods.

6.1 Oxygen Method

General directions for the oxygen method are found in: Standard Methods for the Examination of Water and Wastewater, 13th Edition, pp. 738-739 and 750-751.

Specific modifications and additions for apparatus, procedures, and calculations are:

Apparatus – Rinse the acid-cleaned sample bottles with the water being tested prior to use.

Procedure – Obtain a profile of the input of solar radiation for the photoperiod with a pyroheliometer. Incubate the samples at least 2 hours, but never longer than to that point where oxygen-gas bubbles are formed in the clear bottles or dissolved oxygen is depleted in the dark bottles.

Calculations – Using solar radiation profile and photosynthetic rate during the incubation period, adjust the data to represent phytoplankton productivity for the entire photoperiod.

6.2 Carbon-14 Method

General directions for the carbon-14 method are found in Standard Methods for the Examination of Water and Wastewater, 13th Edition, pp. 739-741 and 751-752.

Specific modifications and additions for apparatus, procedures, and calculations are listed below:

Apparatus – A fuming chamber is not required. Use the methods of Strickland and Parsons (1968) to prepare ampoules containing a carbonate solution of the activity desired.

Procedure – The carbon-14 concentration in the filtered sample should yield the number of counts required for statistical significance; Strickland and Parsons suggest a minimum of 1,000 counts per minute. Obtain a profile of the input of solar radiation for the photoperiod with a pyroheliometer. Incubate up to 4 hours; if measurements are required for the entire photoperiod, overlap 4-hour periods from dawn until dusk (e.g., 0600-1000, 0800-1200, ..., 1400-1800, 1600-2000). A 4-hour incubation period may be sufficient, however, provided energy input is used as the basis for integrating the incubation period into the entire photoperiod. To dry and store the filters, place the membranes in a desiccator for 12 hours following filtration. Fuming with HCl is not required, and dried filters may be stored indefinitely.

Calculations – Using solar radiation profile and photosynthetic rates during the incubation period, adjust data to represent phytoplankton productivity for the entire photoperiod.

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PERIPHYTON

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1.0 INTRODUCTION

Periphyton is an assemblage of a wide variety of organisms that grow on underwater substrates and includes but is not limited to, bacteria, yeasts and molds, algae, protozoa, and forms that may develop large colonies such as sponges and corals. All organisms within the community are not necessarily attached but some may burrow or live within the community structure of the attached forms.

Literally translated, periphyton means "around plants," such as organisms overgrowing pond weeds, but through widespread usage, the term has become associated with communities of microorganisms growing on substrates of any nature. *Aufwuchs* (Seligo, 1905), the German noun for this community, does not have an equivalent English translation, but essentially means growing on and around things. Other terms that are essentially synonymous with periphyton or describe important or predominant components of the periphytic community are: nereiden, bewuchs, laison, belag, besatz, attached, sessile, sessile-attached, sedentary, seeded-on, attached materials, slimes, slime growths, and coatings. Some of these terms are rarely encountered in the literature. Terminology based on the nature of the substrate is as follows:

Substrate	*Adjective*
various	epiholitic, nereiditic, sessile
plants	epiphytic
animals	epizooic
wood	epidendritic, epixylonic
rock	epilithic

Most above-listed Latin-root adjectives are derivatives of nouns such as epihola, epiphyton, epizoa, etc. (After Srameck-Husek, 1946 and Sladeckova, 1962).

Periphyton was recognized as an important component of aquatic communities before the beginning of the 20th century, and the study of periphyton was initiated in Europe in the early 1900's. Kolkwitz and Marsson in two articles (1908 and 1909) made wide use of components in this community in the development of the saprobic system of water quality classification. This system has been continued and developed in Middle and Eastern Europe (Srameck-Husek, 1946; Butcher, 1932, 1940, 1946; Sladeckova, 1962; Sladecek and Sladeckova, 1964; Fjerdingstad, 1950, 1964, 1965).

The study of periphyton was introduced in the United States in the 1920's and expanded in the 1930's. The use of the community has grown steadily and rapidly in water quality investigations (Blum, 1956; Cooke, 1956; Patrick, 1957; Cairns, et al., 1968).

The periphyton and plankton are the principal primary producers in waterways – they convert nutrients to organic living materials and store light energy through the processes of photosynthesis. In extensive deep waters, the plankton are probably the predominant primary producers. In shallow lakes, ponds, and rivers, the periphyton are the predominant primary producers.

Periphyton is the basis of the trickling filter system form of secondary sewage treatment. It is the film of growths covering the substrate in the filter that consumes nutrients, micro-solids, and bacteria from the primary treated sewage passing through the filter. As these growths accumulate, they eventually slough from the substrate, pass through the filter, and are captured in the final clarifier; thus, they change chemical and biological materials to a solid that can be removed with the physical process of settling. Excellent studies and reports on this process have been published by Wisniewski (1948), Cooke (1959), and Holtje (1943).

The periphyton community is an excellent indicator of water quality. Changes may range from subtle alteration of species composition to extremely dramatic results, such as when the addition of organic wastes to waters supporting a community of predominately diatom growths result in their replacement by extensive slime colonies composed predominately of bacteria such as *Sphaerotilus* or *Leptomitus* and vorticellid protozoans.

Excessive growth stimulated by increased nutrients can result in large, filamentous streamers that are esthetically unpleasing and interfere with such water uses as swimming, wading, fishing, and boating, and can also affect the quality of the overlying water. Photosynthesis and respiration can affect alkalinity (U. S. FWPCA, 1967) and dissolved oxygen concentrations (O'Connell and Thomas, 1965) of lakes and streams. Metabolic byproducts released to the overlying water may impart tastes and odors to drinking waters drawn from the stream or lake, a widespread problem throughout the United States (Lackey, 1950; Silvey, 1966; Safferman, et al., 1967). Large clumps of growth may break from the site of attachment and eventually settle to form accumulations of decomposing, organic, sludge-like materials.

Periphyton have proven useful in reconnaissance surveys, water quality monitoring studies, short-term investigations, research and development, and enforcement studies. The investigation objectives dictate the nature, approach, and methodology of sampling the periphyton community. Factors to be considered are the time and duration of the study and the characteristics of the waterway.

Sladeckova (1962) published an extensive review of methodology used in investigating this community.

2.0 SAMPLE COLLECTION AND PRESERVATION

2.1 Qualitative Sampling

Time limitations often prohibit the use of artificial substrate samplers for quantitative collection, and thus necessitate qualitative sampling from natural substrates. Periphyton usually appear as brown, brownish-green, or green growths on the substrate. In standing or flowing water, periphyton may be qualitatively collected by scraping the surfaces of several different rocks and logs with a pocket knife or some other sharp object. This manner of collecting may also be used as a quantitative method if accurate measurements are made of the sampled areas. When sampling this way, limit collections to littoral areas in lakes and shallow or riffle areas in flowing water where the greatest number and variety of organisms are found. Combine the scrapings to a volume of 5 to 10 ml for a sufficient sample. In lakes and streams where long strands of filamentous algae occur, weigh the sample.

After scraping has been completed, store the material in bottles containing 5 percent formalin. If the material is for chlorophyll analysis, do not preserve. Store at 4°C in the dark in 100 ml of 90 percent aqueous acetone. Use bottle caps with a cone-shaped polyethylene seal to prevent evaporation.

2.2 Quantitative Sampling

The standard (plain, 25 X 75 mm) glass microscope slide is the most suitable artificial substrate for quantitative sampling. If less fragile material is preferred, strips of Plexiglas may be used in place of glass slides.

Devices for exposing the substrates can be modified to suit a particular situation, keeping in mind that the depth of exposure must be consistant for all sampling sites. In large rivers or lakes, a floating sampler (APHA, 1971) is advantageous when turbidities are high and the substrates must be exposed near the surface. In small, shallow streams or littoral areas of lakes where turbidity is not a critical factor, substrates may be exposed in several ways. Two possible methods are: (a) attach the substrates with PLASTIC TAK adhesive to bricks or flat rocks in the stream bed, or (b) anchor Plexiglas racks to the bottom to hold the substrates. In areas where siltation is a problem, hold the substrates in a vertical position to avoid a covering of silt. If desired, another set of horizontally-exposed substrates could be used to demonstrate the effects of siltation on the periphyton community.

The number of substrates to be exposed at each sampling site depends on the type and number of analyses to be performed. Because of unexpected fluctuations in water levels, currents, wave action, and the threat of vandalism, duplicate samplers should be used. A minimum of four replicate substrates should be taken for each type of analysis.

The length of exposure depends upon many factors, including the survey time schedule, growth patterns, which are seasonal, and prevailing hydrologic conditions. On the assumption that periphyton growth rate on clean substrates proceeds exponentially for 1 or 2 weeks and then gradually declines, the optimum exposure period is 2 to 4 weeks.

3.0 SAMPLE PREPARATION AND ANALYSIS

3.1 Sample Preparation

Sample preparation varies according to the method of analysis; see the 13th edition of Standard Methods, Section 602-3 (APHA, 1971).

3.2 Sample Analysis

3.2.1 Identification

In addition to the taxonomic references listed in the Plankton Section, the following bench references are essential for day-to-day periphyton identification.

Algae

Desikachary, T. W. 1956. Cyanophyta. Indian Counc. Agric. Res., New Delhi.

Fairdi, M. 1961. A monograph of the fresh water species of *Cladophora* and *Rhizoclonium*. Ph.D. Thesis, Univ. Kansas (available in Xerox from University Microfilms, Ann Arbor).

Islan, A. K., and M. Nurul. 1963. Revision of the genus *Stigeoclonium*. Nova Hedwigia, Suppl. 10. J. Cramer, Weinheim, Germany.

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Randhawa, M. S. 1959. Zygnemaceae. Indian Counc. Agric. Res., New Delhi.

Tiffany, L. H. 1937. Oedogoniales, Oedogoniaceae. In: North American Flora, 11(1):1-102. N. Y. Bot. Garden, Hafner Publ. New York.

Fungi

Cooke, W. Bridge. 1963. A laboratory guide to fungi in polluted waters, sewage, and sewage treatment systems. USDHEW, USPHS, DWSPC, Cincinnati.

Protozoa

Bick, H. 1967-69. An illustrated guide to ciliated protozoa (used as biological indicators in freshwater ecology). Parts 1-9. World Hlth. Organ., Geneva, Switzerland.

Kudo, R. R. 1963. Protozoology. Charles Thomas, Publ., Springfield, Ill..

Rotifers

Donner, J. 1966. Rotifers. Butler and Tanner, Ltd., London.

Edmundson, W. T. 1959. Freshwater biology. John Wiley and Sons, New York.

Pennak, R. W. 1953. Freshwater invertebrates of the United States. Ronald Press, New York.

Microcrustacea

Edmondson, W. T. (see above).

Pennak, R. W. (see above).

3.2.2 Counts and enumeration

Sedgwick-Rafter Method

Shake vigorously to mix the sample, transfer 1 ml to a Sedgwick-Rafter cell, and make strip counts, as described in the Plankton Section, except that a *cell count* is made of *all* organisms. If the material is too concentrated for a direct count, dilute a 1-ml aliquot with 4 ml of distilled water; further dilution may be necessary. Even after vigorous shaking, the scrapings may contain large clumps of cells. These clumps can result in an uneven distribution of material in the counting chamber that could seriously affect the accuracy of the count. Should this condition occur, stir 50 ml of the sample (or a proper dilution) in a blender for 1 minute and reexamine. Repeat if necessary. *Caution*: Some colonial organisms cannot be identified in a fragmented condition. Therefore, the sample must be examined before being blended.

The quantitative determination of organisms on a substrate can then be expressed as:

$$\text{No. cells/mm}^2 = \frac{C \times 1000\ \text{mm}^3 \times V \times DF}{L \times W \times D \times S \times A}$$

where:

C = number of cells counted (tally)

V = sample volume, ml

DF = dilution factor

L = length of a strip, mm

W = width of a strip (Whipple grid image width), mm

D = depth of a strip (S-R cell depth), mm

S = number of strips counted

A = area of substrate scraped, mm^2

Diatom Species Proportional Count

Before preparing the diatom slides, use an oxidizing agent to digest the gelatinous stalks and other extracellular organic materials causing cell clumping. Before the oxidant is added, however, centrifuge or settle the sample to remove the formalin.

If centrifugation is preferred, transfer the sample to a conical tube and centrifuge 10 minutes at 1000 X G. Decant the formalin, resuspend the sample in 10 ml of distilled water, and recentrifuge. Decant, take up the sample in 8 ml of 5 percent potassium (or ammonium) persulfate, and transfer back to the (rinsed) sample vial.

If the settling method is preferred, follow the instructions given in the Plankton Section for removing salt from the diatom concentrate, but add persulfate or hydrogen peroxide instead of distilled water. After the formalin is replaced by the oxidant, heat the sample to 95°C for 30 minutes (do not boil). Cool, remove the oxidant by centrifugation or settling, and take up the diatoms in 2 to 3 ml of distilled water. Proceed with the preparation of the permanent diatom mount as described in the Plankton Section. Label the slide with the station location and inclusive sample dates. Carry out the diatom strip count as described in the Plankton Section, except that separated, individual valves (half cell walls) are tallied as such, and the tally is divided by two to obtain cell numbers.

3.2.3 Biomass

Cell Volume

See the Plankton Section.

Dry and Ash-free Weight

See the Plankton Section.

Centrifugation, Sedimentation and Displacement

Centrifugation. Place sample in graduated centrifuge tube and centrifuge for 20 minutes at 1000 X G. Relate the volume in milliliters to the area sampled.

Sedimentation. Place sample in *graduated cylinder* and allow sample to settle at least 24 hours. Relate the volume in milliliters to the area sampled.

Displacement. Use displacement for large growths of periphyton when excess water can be readily removed. Once the excess water is removed, proceed as per Plankton Section; however, do not pour sample through a No. 20 mesh, nylon bolting cloth.

Chlorophyll

The chlorophyll content of the periphyton is used to estimate the algal biomass and as an indicator of the nutrient content (or trophic status) or toxicity of the water and the taxonomic composition of the community. Periphyton growing in surface water relatively free of organic pollution consists largely of algae, which contain approximately 1 to 2 percent chlorophyll *a* by dry weight. If dissolved or particulate organic matter is present in high concentrations, large populations of filamentous bacteria, stalked protozoa, and other nonchlorophyll bearing microorganisms develop and the percentage of chlorophyll *a* is then reduced. If the biomass–chlorophyll *a* relationship is expressed as a ratio (the autotrophic index), values greater than 100 may result from organic pollution (Weber and McFarland, 1969; Weber, 1973).

$$\text{Autotrophic Index} = \frac{\text{Ash-free Wgt (mg/m}^2\text{)}}{\text{Chlorophyll a (mg/m}^2\text{)}}$$

To obtain information on the physiological condition (or health) of the algal periphyton, measure the amount of pheophytin *a*, a physiologically inactive degradation product of chlorophyll *a*. This degradation product has an absorption peak at nearly the same wavelength as chlorophyll *a* and, under severe environmental conditions, may be responsible for most if not all of the OD_{663} in the acetone extract. The presence of relatively large amounts of pheophytin *a* is an abnormal condition indicating water quality degradation. (See the Plankton Section.)

To extract chlorophyll, grind and steep the periphyton in 90 percent aqueous acetone (see Plankton Section). Because of the normal seasonal succession of the algae, the taxonomic composition and the efficiency of extraction by steeping change continually during the year. Although mechanical or other cell disruption may not increase the recovery of pigment from every sample, routine grinding will significantly increase (10 percent or more) the average recovery of chlorophyll from samples collected over a period of several months. Where glass slides are used as substrates, place the individual slides bearing the periphyton directly in separate small bottles (containing 100 ml) of acetone when removed from the sampler. Similarly, place periphyton removed from other artificial or natural substrates in the field immediately in 90 percent aqueous acetone. (Samples should be macerated, however, when returned to the lab.)

Acetone solutions of chlorophyll are extremely sensitive to photodecomposition and lose more than 50 percent of their optical activity if exposed to direct sunlight for only 5 minutes. Therefore, samples placed in acetone in the field must be protected from more than momentary exposure to direct sunlight and should be placed immediately in the dark. Samples not placed in acetone in the field should be iced until processed. If samples are not to be processed on the day collected, however, they should be frozen and held at -20°C.

For the chlorophyll analysis, see the Plankton Section.

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MACROPHYTON

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1.0 INTRODUCTION

Macrophytes are all aquatic plants possessing a multi-cellular structure with cells differentiated into specialized tissues. Included are the mosses, liverworts, and flowering plants. Their sizes range from the near microscopic watermeal to massive cypress trees. The most commonly dealt with forms are the herbaceous water plants.

Macrophyton may be conveniently divided into three major growth types:

Floating. These plants have true leaves and roots and float on the water surface (duckweed, watermeal, water hyacinth).

Submerged. These plants are anchored to the substratum by roots and may be entirely submersed or have floating leaves and aerial reproductive structures (water milfoil, eel grass, pondweeds, bladderwort).

Emersed. These plants are rooted in shallow water and some species occur along moist shore lines. The two major groups are:

Floating leafed plants (water lilies and water shields).

Plants with upright shoots (cattails, sedges, woody shrubs, rice and trees).

The use of macrophytes in water quality investigations has been sorely neglected. Kolkwitz and Marsson (1908) used some species in their saprobic system of water quality classification, but they are rarely mentioned in most literature. A number of pollutants have dramatic effects on macrophyte growth:

Turbidity restricting light penetration can prevent the growth of submerged weeds.

Nutrients can stimulate overproduction of macrophytes in numbers sufficient to create nuisances or can stimulate excessive plankton growths that effect an increase in turbidities, thus eliminating macrophyte growths.

Herbicidal compounds, if present at sublethal concentrations, can stimulate excessive growths or they can, at higher concentrations, destroy plant growths.

Organic or inorganic nutrients, or both, can support periphytic *algal* and *slime growths* sufficient to smother and thus destroy submersed forms.

Sludge deposits, especially those undergoing rapid decomposition, usually are too unstable or toxic to permit the growth of rooted plants.

The rampant growth of some macrophytes has caused concern over recent years (Holm et al. 1969). Millions of dollars are spent each year in controlling macrophytes that interfere with irrigation operation, navigation, and related recreational uses. Mechanical cutting, application of herbicides, and habitat alteration are the primary control methods. Mackenthun and Ingram (1967) and Mackenthun (1969) have reviewed and summarized control techniques.

Yount and Crossman (1970) and Boyd (1970) discussed schemes for using macrophytes to remove nutrients from effluents and natural waters.

Aquatic macrophytes are a natural component of most aquatic ecosystems, and are present in those areas suitable for macrophyte growth, unless the habitat is altered. Furthermore, the proper proportions of macrophytes are ecologically desirable (Wilson, 1939; Hotchkiss, 1941; Penfound, 1956; Boyd, 1971). Boyd (1970, 1971) introduced concepts of macrophyte management opposed to the current idea of eradicating aquatic macrophytes from many aquatic ecosystems. Much additional research is needed on the role of macrophytes in aquatic ecosystems.

The objective of an investigation dictates the nature and methodology of sampling macrophytes. Critical factors are the time available, how critical the information is, expertise available, duration of the study, and characteristics of the waterway.

Techniques are few, and the investigator's best asset is his capability for innovating sound procedures.

2.0 SAMPLE COLLECTION AND ANALYSIS

Collecting representative genera from the macrophyton community is generally not difficult because of their large size and littoral habitats. Macrophytes may be readily identified to genera and some to species in the field, or they may be dried in a plant press and mounted for further identification. Small, delicate species may be preserved in buffered 4 percent formalin solution. Some of the more useful taxonomic works for identification are Muenscher (1944), Eyles and Robertson (1944), Fassett (1960) and Winterringer and Lopinot (1966).

2.1 Qualitative Sampling

Qualitative sampling includes visual observation and collection of representative types from the study area. Report the extent of growth as *dense* when coverage is continuous, *moderate* when growths are common, and *sparse* when the growth is rarely encountered. The crop of plants may be comprised of just one genus or may be a mixture; if a mixture, estimate the percentage of individual types.

Sampling gear is varied and the choice of tools usually depends on water depth. In shallow water, a garden rake or similar device is very effective for collecting macrophytes. In deeper water, employ grabs, such as the Ekman, to collect submersed types. In recent years, scuba diving has gained popularity with many investigators in extensive plant surveys. Phillips (1959) provides detailed information on qualitative sampling.

2.2 Quantitative Sampling

Quantitative sampling for macrophytes is usually to determine the extent or rate of growth or weight of growth per unit of area. The study objectives determine whether measurements will involve a single species or several.

Before beginning a quantitative investigation, develop a statistical design to assist in determining the best sampling procedure, sampling area size, and number of samples. Often procedures adapted from terrestrial plant surveys are applicable in the aquatic environment. The following references will be helpful in adopting a suitable technique: Penfound, 1956; Westlake, 1966; Boyd, 1969; Forsberg, 1959, 1960; Edwards and Owens, 1960; Jervis, 1969; Blackburn, et al., 1968.

Standing crop. Sampling should be limited to small, defined subareas (quadrates) with conspicuous borders. Use a square framework with the poles anchored on the bottom and floating line for the sides. Collect the plants from within the frame by hand or by using a long-handled garden rake. Forsberg (1959) has described other methods such as laying out long, narrow transects.

Obtain the wet weight of material after the plants have drained for a standard period of time, determined by the investigator. Dry the samples (or subsamples for large species) for 24 hours at 105°C and reweigh. Calculate the dry weight of vegetation per unit area.

Planimeter accurate maps to determine the total area of investigation. If additional boat or air reconnaissance (using photographs) is done to determine type and extent of coverage, data collected from the subareas can then be expanded for the total study area. Boyd (1969) describes a technique for obtaining surface coverage by macrophytes in a small body of water.

Productivity. Estimate standing crops at predetermined intervals to relate growth rates to pollution, such as nutrient stimulation, retardation, or toxicity from heavy metals and thermal effects. Wetzel (1964) and Davies (1970) describe a more accurate method with the use of a carbon-14 procedure to estimate daily productivity rates of macrophytes.

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MACROINVERTEBRATES

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1.0 INTRODUCTION

The aquatic macroinvertebrates, as discussed in this section, are animals that are large enough to be seen by the unaided eye and can be retained by a U. S. Standard No. 30 sieve (28 meshes per inch, 0.595 mm openings) and live at least part of their life cycles within or upon available substrates in a body of water or water transport system.

Any available substrate may provide suitable habitat including bottom sediments, submerged logs, debris, pilings, pipes, conduits, vascular aquatic plants, filamentous algae, etc.

The major taxonomic groups included in fresh water are the insects, annelids, molluscs, flatworms, roundworms, and crustaceans. The major groups in salt water are the molluscs, annelids, crustaceans, coelenterates, porifera, and bryozoans.

Benthic macroinvertebrates can be defined by location and size but not by position in the trophic structure since they occupy virtually all levels. They may be omnivores, carnivores, or herbivores; and in a well-balanced system, all three types will likely be present. They include deposit and detritus feeders, parasites, scavengers, grazers, and predators.

Species present, distribution, and abundance of aquatic macroinvertebrates may be subject to wide seasonal variations. Thus, when conducting comparative studies, the investigator must be quite careful to avoid the confounding effects of these seasonal changes. Seasonal variations are particularly important in fresh-water habitats dominated by aquatic insects having several life stages, not all of which are aquatic.

The macroinvertebrates are important members of the food web, and their well-being is reflected in the well-being of the higher forms such as fish. Many invertebrates, such as the marine and fresh-water shellfish, are important commercial and recreational species. Some, such as mosquitos, black flies, biting midges, and Asiatic clams, are of considerable public health significance or are simple pests; and many forms are important for digesting organic material and recycling nutrients.

A community of macroinvertebrates in an aquatic ecosystem is very sensitive to stress, and thus its characteristics serve as a useful tool for detecting environmental perturbations resulting from introduced contaminants. Because of the limited mobility of benthic organisms and their relatively long life span, their characteristics are a function of conditions during the recent past, including reactions to infrequently discharged wastes that would be difficult to detect by periodic chemical sampling.

Also, because of the phenomenon of "biological magnification" and relatively long-term retention of contaminants by benthic organisms, contaminants such as pesticides, radioactive materials, and metals, which are only periodically discharged or which are present at undetectable levels in the water, may be detected by chemical analyses of selected components of the macroinvertebrate fauna.

In pollution-oriented studies of macroinvertebrate communities, there are basically two approaches—quantitative and qualitative—that may be utilized singly or in combination. Because of the basic nature of this decision, the section of this manual relating to sampling methods and data evaluation of macroinvertebrates is arranged on the basis of whether a quantitative or qualitative approach is used.

Ideally, the design of macroinvertebrate studies should be based upon study goals or objectives; however, the ideal must frequently be tempered by the realities of available resources, time limitations imposed on the study, and the characteristics of the habitat to be studied. To aid in selecting the most advantageous sampling method, sample sites, and data evaluation, the reader of this section should be familiar with the material in the "Introduction" of this manual, particularly those portions outlining and discussing requirements of the various types of field studies in which an investigator may become involved.

To supplement the material contained in this manual, a number of basic references should be available to investigators of the benthic community, particularly to those engaged in water pollution studies. These include Standard Methods (2), Welch (57), Mackenthun (37), Kittrell (29), Hynes (26), and Buchanan and Sommers (9).

2.0 SELECTION OF SAMPLE SITES

As discussed and defined more fully in the section on biometrics, sample sites may be selected systematically or by various randomization procedures.

2.1 Systematic Sampling

Unless the data are to be utilized for quantitative evaluations, some type of systematic sampling is generally employed for synoptic surveys and reconnaissance studies. Line transects established at discrete intervals across a river or stream and sampled at quarter points or more frequent intervals are a form of systematic sampling and serve as an excellent means of delimiting and mapping the habitat types. In lakes, reservoirs, and estuaries, transects may be established along the short or long axis or may radiate out from a pollution source. If a random start point is used for selecting sampling sites along the transects, the data may be amenable to quantitative evaluation (see Biometrics Section). As will be discussed, however, the confounding effects of changes in physical characteristics of the environment along the transect must be fully recognized and accounted for.

In another form of systematic sampling, the investigator, using a variety of gear, consciously selects and intensively samples all recognizable habitat types. As previously mentioned, this form of sample site selection is useful for synoptic surveys and for comparative studies where qualitative comparisons are being made.

2.2 Random Sampling

For conducting quantitative studies, where a measure of precision must be obtained, some type of randomization procedure must be employed in selecting sampling sites. This selection may be carried out on the whole of the area under study (simple random sampling), or the randomization procedure may be conducted independently on selected strata (stratified random sampling). Because the characteristics of macroinvertebrate communities are so closely related to physical factors such as substrate type, current velocity, depth, and salinity, a design using simple random sampling is seldom meaningful. Therefore, the investigator should stratify the habitat on the basis of known physical habitat differences and collect samples by the random grid technique within each habitat type.

As alluded to above, and regardless of the method of sample site selection, the biologist must consider and account for those natural environmental variations that may affect the distribution of organisms. Among the more important natural environmental variables in fresh-water habitats are substrate type, current velocity, and depth. In estuaries, the salinity gradient is an additional variable that must be accounted for.

2.3 Measurement of Abiotic Factors

2.3.1 Substrate

Substrate is one of the most important factors controlling the characteristics of the community of aquatic macroinvertebrates found at a given location in a body of water (49). Over a period of time, the natural substrates may be greatly altered by the discharge of particulate mineral or organic matter, and the location and expanse of various substrate types (silt, sand, gravel, etc.) may change because of normal variations in hydrolic factors such as current velocity and stream flow. The biologist, therefore, must be cognizant of changes in the nature and properties of the substrate which may provide clues on the quality and quantity of pollutants and consider factors which affect the normal distribution of the benthic fauna.

Where the pollutant has a direct effect on the characteristics of the substrate, the effects of changes in water quality may be inseparable from the effects of changes in the substrate. In cases where substrate deterioration has occurred, faunal effects may be so obvious that extensive sampling may not be required, and special attention should be given to the physical and/or chemical characterization of the deposits.

In conducting synoptic surveys or other types of qualitative studies and taking into account the limitations of available sampling devices, sampling sites should be selected to include all available substrates. If these qualitative samples are to be used for determining the effects of pollutants where the pollutant does not have a direct affect on the substrate, the investigator must bear in mind that only the fauna from sites having similar substrates (in terms of organic content, particle size, vegetative cover, and detritus) will provide valid data for comparison.

For quantitative studies, it is sometimes necessary in the interest of economy and efficiency and within the limitations of the available gear, to sample primarily at sites having substrates which normally support the most abundant and varied fauna, and devote a minimum effort to those substrates supporting little or no life. For instance, in many large, swiftly-flowing rivers of the Midwest and Southeast, the areas of "scour" with a substrate of shifting sand or hardpan may be almost devoid of macroinvertebrates; sampling effort may be reduced there in favor of the more productive areas of "deposition" on the inside of bends or in the vicinity of obstructions. Just the opposite situation may occur in many of the swiftly-flowing upland streams, where most of the effort may be devoted to sampling the productive rubble and gravel riffle areas instead of the pools.

Because of the importance of substrate (in terms of both organic content and particle size) in macroinvertebrate studies, it is suggested that sufficient samples be collected to conduct the following minimal analyses and evaluations:

- In the field, classify and record, on suitable forms, the mineral and organic matter content of the stream, lake, or estuary bottom at each sample site on a percentage basis with the use of the categories shown in Table 1. Although the categories given in Table 1 may not apply universally, they should be applicable to most situations with only slight modification.

TABLE 1. CATEGORIES FOR FIELD EVALUATION OF SOIL CHARACTERISTICS*

Type	Size or characteristic
Inorganic Components	
Bed rock or solid rock	
Boulders	>256 mm (10 in.) in diameter
Rubble	64 to 256 mm (2½ to 10 in.) in diameter
Gravel	2 to 64 mm (1/12 to 2½ in.) in diameter
Sand	0.06 to 2.0 mm in diameter; gritty texture when rubbed between fingers.
Silt	0.004 to 0.06 mm in diameter
Clay	<0.004 mm in diameter; smooth, slick feeling when rubbed between fingers
Marl	Calcium carbonate; usually gray; often contains fragments of mollusc shells or *Chara;* effervesces freely with hydrochloric acid
Organic Components	
Detritus	Accumulated wood, sticks, and other undecayed coarse plant materials
Fibrous peat	Partially decomposed plant remains; parts of plants readily distinguishable
Pulpy peat	Very finely divided plant remains; parts of plants not distinguishable; varies in color from green to brown; varies greatly in consistence–often being semi-fluid
Muck	Black, finely divided organic matter; completely decomposed

*Modified from Roelofs, E. W. 1944. Water soils in relation to lake productivity. Tech. Bull. 190. Agr. Exp. Sta., State College, Lansing, Mich.

- In the laboratory, evaluate the inorganic components by conducting a wet and dry particle size analysis on one or more samples and preferably on replicate samples from each sampling site with the use of standard sieves and following the modified Wentworth classification shown in Table 2. Detailed procedures for sediment analysis are found in IBP handbook No. 16.*

TABLE 2. SOIL PARTICLE SIZE CLASSIFICATION*

Name	Particle size (mm)	U.S. standard sieve series #
Boulder	>256	
Rubble	64-256	
Coarse gravel	32-64	
Medium gravel	8-32	†
Fine gravel	2-8	10
Coarse sand	0.5-2	35
Medium sand	0.25-0.5	120
Fine sand	0.125-0.25	230
Very fine sand	0.0625-0.125	
Silt	0.0039-0.0625	Centrifuge (750 rpm, 3 min)‡
Clay	<0.0039	Evaporate and weigh residue

*Modified from Wentworth (58); see Cummins, K. A. 1962. An evaluation of some techniques for the collection and analysis of benthic samples with special emphasis on lotic waters. Amer. Midl. Nat. 67:477-504.

†Standard sieves with 8-mm diameter openings are commonly available.

‡Jackson, M. L. 1956. Soil chemical analysis. Univ. Wisconsin Press, Madison.

- Determine the organic content by drying and ashing a representative sample of the sediments; use the methods outlined in the Plankton Section.

2.3.2 Depth

Depth indirectly affects the distribution of aquatic macroinvertebrates as a result of its influence on the availability of light for plant growth, on water temperature, on the zonation of bottom deposits, on the water chemistry (particularly oxygen), and phototactic responses of organisms. In regard to the selection of sampling sites for both qualitative and studies, depth must be measured and included as an independent variable in the study design.

*Holme, N. A., and A. D. MacIntyre. 1971. Methods for the study of marine benthas. International Biological Program, Davis Company, Philadelphia. 346 pp.

2.3.3 Current velocity

Current velocity affects the distribution of organisms in lotic environments and along the windswept shores of lentic environments, both directly (because of differing species requirements) and indirectly (sorting of bottom sediments). Therefore, it is of critical importance that velocity be considered when sampling sites are selected, and when data are analyzed. Only sites with comparable velocity should be compared. At the actual time of sampling, determine velocity at each sample site by using a suitable current measuring device. The TSK flow meter listed in the appendix is suitable if modified by the addition of a stabilizing fin and propeller lock.

- At depths greater than 3 feet, use the two-point method (1); take readings at 0.2 and 0.8 of the depth below the surface. The average of these two observations is taken as the mean velocity.
- At depths less than 3 feet, the 0.6-depth method (1) is used; take readings at 0.6 of the depth below the surface.
- Where artificial substrate samplers are being utilized, take the reading directly upstream of the sampler and at the same depth.

2.3.4 Salinity

Salinity is an important factor in marine and estuarine environments. The salinity of sea water is approximately 35 parts per thousand; salinity of fresh water is generally a few parts per million. In estuaries, where sea water and fresh water meet, there may be wide fluctuations of salinity with tides and river discharge. This area may be inhabited to some extent by both fresh- and salt-water forms, but the number of species is usually less than that that occurs under more stable conditions of salinity (35). Since movement, as well as general location of many species, is governed by tides and salinity, these must be taken into account in determining sampling time and location.

Because of the extreme spatial and temporal fluctuations of salinity in estuaries, simple, rapid instrumental methods of measurement are more desirable than slower, more precise chemical methods (38).

Wide-range, temperature-compensated conductivity salinometers are recommended for determining both horizontal and vertical salinity profiles at high-slack and low-slack tide levels in the area of estuary or reach of river being studied.

3.0 SAMPLING METHODS

3.1 QUANTITATIVE

3.1.1 Definitions and purpose

Although the data may be evaluated in various ways, a quantitative method essentially involves an estimation of the numbers or biomass (standing crop) of the various components of the macroinvertebrate community per unit area in all or a portion of the available habitats (including artificially introduced habitats) in the ecosystem being studied, and provides information on the species composition, richness of species and distribution of individuals among the species.

3.1.2 Requirements

Obtain quantitative estimates by using devices that sample a unit area or volume of habitat, such as a Surber square-foot sampler, which in use presumably collects all organisms enclosed within the frame of the sampler, or an artificial substrate sampler having a fixed volume or exposing a fixed amount of surface.

In the study of macroinvertebrate populations, the sampling precision is affected by a number of factors, including: size, weight, and construction of the sampling device, the type of substrate, and the distribution of organisms in and on the substrate. For example, it is expected that the estimates of standing crop drawn from a series of samples will be more precise (have a lower coefficient of variation) when the community consists of a few species represented by a large number of individuals, evenly distributed in the substrate. Conversely, a large coefficient of variation would be expected if the fauna consists of a large number of species with a patchy distribution of individuals. To obtain the same level of precision at a given level of probability, a larger number of replicates would be required in the latter case than in the former. In general, the smaller the surface area encompassed by a sampling device, the larger the number of samples required to obtain a desired level of precision. Thus, precision can be increased by collecting larger samples, or by increasing the numbers of samples collected.

An objective, quantitative approach necessitates that a measure of the precision of the estimates be obtained – thus, replicate sampling in each habitat or stratum selected for study is an absolute requirement. For measurement of precision, three replicates are an absolute minimum. (A series of single samples taken at discrete points along a transect do not represent replicate samples of benthic organisms unless it can be demonstrated that the physical characteristics of the habitat do not change along the transect.)

It is preferable, if data are available (or can be obtained by reconniassance or exploratory studies), to determine the number of replicates on the basis of the desired level of precision as discussed in the Biometrics Section.

3.1.3 Advantages

In addition to providing the same data obtained from a qualitative study, the standing crop data generated by a quantitative study provide a means of comparing the productivity of different environments; and if a measure of turnover is available, the actual production can be computed.

The use of quantitative sampling devices in carefully chosen habitats is recommended because they reduce sampling bias resulting from differences in expertise of the sample collector.

The data from properly designed quantitative studies are amenable to the use of simple but powerful statistical tools that aid in maintaining the objectivity of the data evaluation process. The measures of precision and probability statements that can be attached to quantitative data reduce the possibilities of bias in the data evaluation process and make the results of different investigators more readily comparable.

The advantages, then, of quantitative methods are:

- They provide a measure of productivity.
- The investigator can measure precision of estimates and attach probability statements, thus providing objective comparisons.
- The data of different investigators may be compared.

3.1.4 Limitations

Presently, no sampling devices are adequate to sample all types of habitat; so when quantitative devices are used, only selected portions of the environment may be sampled.

Sampling precision is frequently so low that prohibitive numbers of replicate samples may be required to obtain meaningful estimates. Sample processing and analysis are slow and time-consuming. In some cases, therefore, time limitations placed on a study may prohibit the use of quantitative techniques.

3.2 Qualitative

3.2.1 Definitions and purpose

The objective of qualitative studies is to determine the presence or absence of forms having varying degrees of tolerance to contaminants and to obtain information on "richness of species." Samples are obtained with the use of a wide variety of collecting methods and gear, many of which are not amenable to quantitation on a unit-area basis. When conducting qualitative studies, an attempt is usually made to collect all species present by exhaustive sampling in all available habitat types.

3.2.2 Requirements

Recognizing and locating various types of habitats where qualitative samples can be collected and selecting suitable collecting techniques require experience and a high level of expertise.

When conducting comparative studies of the macrobenthos, a major pitfall is the confounding effect of the differences in physical habitat among the different stations being studied. This danger is particularly inherent in qualitative studies when an attempt is made to systematically collect representative specimens of all species present at the sampling stations or reaches of river being compared. Unfortunately, differences in habitat unrelated to the effects of introduced contaminants may render such comparisons meaningless. Minimize this pitfall by carefully recording, in the field, the habitats from which specimens are collected and then basing comparisons only on stations with like habitats in which the same amount of collecting effort has been expended.

3.2.3 Advantages

Because of wide latitude in collecting techniques, the types of habitat that can be sampled are relatively unrestricted. Assuming taxonomic expertise is available, the processing of qualitative samples is often considerably faster than that required for quantitative samples.

3.2.4 Limitations

Collecting techniques are subjective and depend on the skill and experience of the individual who makes the field collections. Therefore, results of one investigator are difficult to compare with those of another.

As discussed elsewhere, the drift of organisms into the sample area may bias the evaluation of qualitative data and render comparisons meaningless.

No information on standing crop or production can be generated from a qualitative study.

3.3 Devices

3.3.1 Grabs

Grabs are devices designed to penetrate the substrate by virtue of their own weight and leverage, and have spring- or gravity-activated closing mechanisms. In shallow waters, some of these devices may be rigged on poles or rods and physically pushed into the substrate to a predetermined depth. Grabs with spring-activated closing devices include the Ekman, Shipek, and Smith-McIntyre; gravity-closing grabs include the Petersen,* Ponar, and Orange Peel. Excellent descriptions of these devices are given in Standard Methods (2) Welch (57). Grabs are useful for sampling at all depths in lakes, estuaries, and rivers in substrates ranging from soft muds through gravel.

In addition to the previously discussed problems related to the patchy distribution of organisms in nature, the number and kinds of organisms collected by a particular grab may be affected by:

- depth of penetration
- angle of closure
- completeness of closure of the jaws and loss of sample material during retrieval
- creation of a "shock" wave and consequent "wash-out" of near-surface organisms
- stability of sampler at the high-flow velocities often encountered in rivers.

Depth of penetration is a very serious problem and depends on the weight of sampler as opposed to the particle size and degree of compaction of the bottom sediments. The Ekman grab is light in weight and most useful for sampling soft, finely divided substrates composed of varying proportions of fine sand, clay, slit, pulpy peat, and muck. For clay hardpan and coarse substrates, such as coarse sands and gravels, the heavier grabs such as the orange peel or clam shell types (Ponar, Petersen, Smith-McIntyre) are more satisfactory. Auxiliary weights may be added to aid penetration of the substrate and to add stability in heavy currents and rough waters.

Because of differences in the depth of penetration and the angle of "bite" upon closure, data from the different grabs are not comparable. The Ekman essentially encloses a square, which is equal in area from the surface to maximum depth of penetration before closure. In soft substrates, for which this grab is best suited, the penetration is quite deep and the angular closure of the spring-loaded jaws has very little effect on the volume of sample collected. In essence this means that if the depth of penetration is 15 cm, the organisms lying at that depth have the same opportunity to be sampled as those lying near the surface.

In clam-shell type grabs, such as the Petersen, Ponar, Shipek, and Smith-McIntyre, the original penetration is often quite shallow: because of the sharp angle of "bite" upon closure, the area enclosed by the jaws decreases at increasing depths of substrate penetration. Therefore, within the enclosed area, organisms found at greater depths do not have an equal opportunity to be sampled as in the case of the Ekman grab and other sampling methods described in the next section. This problem is particularly true of the Shipek sampler – the jaws do not penetrate the substrate before closure and, in profile, the sample is essentially one-half of a cylinder.

Probably one of the most frustrating aspects of sampling macroinvertebrates with various types of grabs relates to the problem of incomplete closure of the jaws. Any object – such as clumps of vegetation, woody debris, and gravel – that cannot be sheared by the closing action of the jaws often prevents complete closure. In the order of their decreasing ability to shear obstructing materials, the common grabs may be ranked: Shipek, Smith-McIntyre, Orange Peel, Ponar, Petersen, and Ekman. If the Ekman is filled to within more than 5 cm of the top, there may be loss of substrate material on retrieval (16). An advantage of the Ekman grab is that the surface of the sediment can be examined upon retrieval, and only those samples in which the sediment surface is undisturbed should be retained.

All grabs and corers produce a "shock" wave as they descend. This disturbance can affect the efficiency of a sampler by causing an outward wash (blow-out) of flocculent materials near the mud–water interface that may result in

*Forest Modification of the Petersen grab described in Welch (57).

inadequate sampling of near-surface organisms such as phantom midge larvae, and some chironomid midges. The shock wave of the Ekman grab is minimized by the use of hinged, freely-opening top flaps. The Ponar grab is a modified Petersen with side curtains and a screen on the top. The screen allows water to pass and undoubtedly reduces the shock wave; however, divers have observed blow-out with this device (16).

Grab-collected samples provide a very imprecise estimate of the numbers of individuals and numbers of taxa of aquatic macroinvertebrates. A summary of data from various sources shows that the mean coefficient of variation (C) for numbers of individuals collected by Ponar, Petersen, and Ekman grabs was 46, 48, and 50 percent, respectively (Table 3). In most of the studies on which the calculations in Table 3 are based, the level of replication ranged from three to six samples. Estimations of number of taxa are more precise: for Ponar, Petersen, and Ekman grabs, the mean calculated C was 28, 36, and 46 percent respectively (Table 3).

On the basis of the calculations in Table 4, there appear to be no consistent differences in the precision of estimates collected by Ekman, Ponar, and Petersen grabs in mud or sand substrates. The poor closure ability of the Ekman in coarse substrates such as gravel is demonstrated by the large C values for the Ekman as compared with values for the Petersen and Ponar in gravel substrates.

Another way of demonstrating the reliability of grab sample estimates of macrobenthos standing crop is to calculate, at a given probability level, the range of values around the sample mean in which the true mean should lie if a given number of replicate samples were collected. From the data shown in Table 3 for the Petersen, Ponar, and Ekman grabs in various types of substrate, coefficients of variation near 50 percent for numbers of individuals and 35 percent for numbers of taxa should be expected with 3 to 6 replicates. With the use of these expected values, the true mean for numbers of individuals and number of taxa of macroinvertebrates should lie within plus or minus 36 percent

TABLE 3. MEAN AND MODAL VALUES FOR COEFFICIENTS OF VARIATION* (EXPRESSED AS PERCENTAGE) FOR NUMBERS OF INDIVIDUALS AND NUMBERS OF TAXA OF MACROINVERTEBRATES COLLECTED BY VARIOUS DEVICES

Sampling device	Individuals		Taxa		Remarks
	Mean	Mode†	Mean	Mode‡	
Rock-filled barbeque basket	32	21-30	20	11-20	22 sets of samples with 4-6 reps. per set (52) and 2 sets of samples having 15 and 16 reps. (13).
Ponar	46	41-50	28	11-20	12 sets of samples with 3-12 reps. per set (16, 31).
Petersen	48	51-60	36	21-30	21 sets of samples with 3-6 reps. per set (31, 53, 54).
Ekman	50	41-50	46	31-40	27 sets of samples with 3-12 reps. per set (8, 16, 31, 45, 53).
Surber	50	41-50			60 sets of samples having 6 reps. per set (20).
Corer†	50				7 sets of samples having 10 reps. per set (8).
Stovepipe	56	31-40	38	21-30	32 sets of samples having 3-4 reps. per set (53).

*Coefficient of variation = (standard deviation x 100)/mean.
†Frequency distribution based on 10% increments.
‡Oligochaetes only.

and 25 percent, respectively, of the sample mean at a 95 percent probability level, if 10 replicates were collected. (See Biometrics Section.)

Precision would, of course, be increased if additional samples were collected, or if the sampling method were more precise.

Since the assumptions necessary for the statistical calculations shown in Tables 3 and 4 are not likely met in the data of different investigators collected from different habitats, the above calculations only provide a gross approximation of the precision to be expected. They do, however, serve to emphasize the very imprecise nature of grab sample data and the resultant need for careful stratification of the type of the habitat sampled and sample replication.

TABLE 4. MEAN COEFFICIENTS OF VARIATION (EXPRESSED AS PERCENTAGE) FOR NUMBERS OF INDIVIDUALS AND NUMBERS OF TAXA OF MACROINVERTEBRATES COLLECTED IN DIFFERENT SUBSTRATES BY GRAB-TYPE DEVICES AND A CORER DEVICE*

Sampling device	Substrate					
	Mud		Sand		Gravel	
	Ind.	Taxa	Ind.	Taxa	Ind.	Taxa
Ekman	49	40	41	21	106	74
Petersen	41	29	50	41	49	20
Ponar	46	25	38	33	48	19
Corer†	50					

*Calculated from data in references (8, 16, 31, 45, 53, 54).
†Oligochaetes only.

3.3.2 Sieving devices

For quantitative sampling, the well-known Surber square-foot sampler (2, 57) is the most commonly used sieving device. This device can be used only in flowing water having depths not greater than 18 inches and preferably less than 12 inches. It is commonly used for sampling the rubble and gravel riffles of small streams and may be used in pools where the water depth is not too great.

When using a sieving-type device for quantitative estimates, reliability may be affected by:

- adequacy of seating of the frame on the substrate
- backwash resulting from resistance of the net to water flow – at high velocity of flow this may be significant
- care used in recovering the organisms from the substrate materials
- depth to which the substrate is worked
- drift of organisms from areas upstream of the sample site

To reduce the possibility of bias resulting from upstream disturbance of the substrate, always stand on the downstream side of a sieving device and take replicates in an upstream or lateral direction. Never start in the upstream portion of a pool or riffle and work in a downstream direction.

The precision of estimates of standing crops of macrobenthos obtained with Surber-type sieving devices varies widely and depends on a number of factors including the uniformity of substrate and distribution of organisms therein, the care used in collecting samples, and level of sample replication.

For a large series of Surber samples from southeastern U. S. trout streams, the coefficient of variation (C) ranged from 11 percent to greater than 100 percent (Table 3). The mean value of C was near 50 percent, and more than one-half of the C values fell between 30 and 50 percent. These values are similar to the 20 to 50 percent reported by Allen (1) and for those discussed above for grab sample data.

3.3.3 Coring devices

Included in this category are single- and multiple-head coring devices, tubular inverting devices, and open-ended stovepipe-type devices.

Coring devices are described in Standard Methods (2) and Welch (57). Corers can be used at various depths in any substrate that is sufficiently compacted so that the sample is retained; however, they are best suited for sampling the relatively homogeneous soft sediments of the deeper portions of lakes.

Because of the small area sampled, data from coring devices are likely to provide very imprecise estimates of the standing crop of macrobenthos. As the data in Table 3 illustrate, the variability in numbers of oligochaetes (a dominant component of the fauna studied) collected in corers is similar to that for grab-type devices; however, the corer data were calculated from two to three times as many replicate samples and were collected from a relatively homogeneous substrate.

Such additional replication with corers is feasible because of the small amount of material per sample that must be handled in the laboratory. Multiple-head corers have been used in an attempt to reduce the field sampling effort that must be expended to collect large series of core samples (19).

The Dendy inverting sampler (57) is a highly efficient coring-type device used for sampling at depths to 2 or 3 meters in nonvegetated substrates ranging from soft muds through coarse sand. Because of the small surface area sampled, data obtained by this sampler suffer from the same lack of precision (51) as the coring devices described above. Since the per-sample processing time is reduced, as with the corers, large series of replicates can be collected. The Dendy sampler is highly recommended for use in habitats for which it is suitable.

Stovepipe-type devices include the Wilding sampler (2, 57) and any tubular material such as 60 to 75 cm sections of standard 17-cm-diameter stovepipe (51) or 75 cm sections of 30-cm-diameter aluminum irrigation pipe fitted with handles. In use, the irrigation pipe or commercial stovepipe is manually forced into the substrate, after which the contained vegetation and coarse substrate materials are removed by hand. The remaining materials are repeatedly stirred into suspension, removed with a long-handled dipper, and poured through a wooden-framed floating sieve. Because of the laborious and repetitive process of stirring, dipping, and sieving large volumes of material, the collection of a sample often requires 20 to 30 minutes.

The use of stovepipe samplers is limited to standing or slowly moving waters having a maximum depth of less than 60 cm. Since problems relating to depth of sediment penetration, changes in cross-sectional area with depth of penetration, and escapement of organisms are circumvented by stovepipe samplers, they are recommended for quantitative sampling in all shallow water benthic habitats. They probably represent the only quantitative device suitable for sampling shallow-water habitats containing stands of rooted vascular plants and will collect organisms inhabiting the vegetative substrates as well as those living in sediments. The coefficients of variation for the stovepipe samples in Table 3 are comparable to the coefficients for grab samples, although the stovepipe samples were collected in heavily vegetated and consequently highly variable habitats.

3.3.4 Artificial substrates

The basic multiple-plate sampler (23) and rock-filled basket sampler (21) have been modified by numerous workers (17, 40) and are widely used for investigating the macroinvertebrate community. Both samplers may be suspended from a surface float or may be modified for use in shallow streams by placing them on a rod that is driven into the stream bottom or anchored in a piece of concrete (24).

A multiple-plate sampler similar to that described by Fullner (17), except with circular plates and spacers, is recommended for use by EPA biologists. This sampler is constructed of 0.3-cm tempered hardboard cut into 7.5-cm diameter circular plates and 2.5-cm circular spacers. A total of 14 plates and 24 spacers are required for each sampler. The hardboard plates and spacers are placed on a ¼-inch (0.625 cm) eyebolt so that there are eight single spaces, one double space, two triple spaces, and two quadruple spaces between the plates. This sampler has an effective surface area (excluding the bolt) of 0.13 square meter and conveniently fits into a wide-mouth glass or plastic jar for shipment and storage. Caution should be exercised in the reuse of samplers that may have been subjected to contamination by toxicants, oils, etc.

The rock basket sampler is a highly effective device for studying the macroinvertebrate community. A cylindrical, chromeplated basket

(2) or comparable enclosure filled with 30, 5 to 8-cm-diameter rocks or rock-like material is recommended for use by EPA biologists.

To reduce the number of organisms that escape when the samplers are retrieved, the multiple-plate sampler and the rock-filled basket sampler should be enclosed by a dip net constructed of 30-mesh or finer grit bolting cloth.

Artificial substrate samplers, to a great extent, depend on chance colonization by drifting or swimming organisms; and, thus, the time of exposure may be critical to the development of a relatively abundant and diverse community of organisms. Adequate data are currently unavailable to determine the optimum exposure period, which is likely to differ in different bodies of water and at different times of the year. Until more data become available, adoption of a 6-week exposure period (2) is provisionally recommended as standard. If study time limitations reduce this period, the data must be evaluated with caution and, in no case, should data be compared from samplers exposed for different time periods (43).

In deeper waters, artificial substrate samplers should be suspended from floats and should be well up in the photic zone so that periphytic growths can develop and provide food for grazing forms of macroinvertebrates. Unless the water is exceptionally turbid, a 1.2-meter (4-foot) depth is recommended as standard. If the water is less than 2.5 meters deep, the sampler should be suspended from a float halfway between the water surface and the stream bed.

In some situations, artificial substrate methods are the best means of conducting quantitative studies of the ability of an aquatic environment to support a diverse assemblage of macroinvertebrate organisms. Advantages of the method are:

- The confounding effects of substrate differences are reduced.
- A higher level of precision is obtained than with other sampling devices (Table 3).
- Quantitatively comparable data can be obtained in environments from which it is virtually impossible to obtain samples with conventional devices.
- Samples usually contain negligible amounts of extraneous material, permitting quick laboratory processing.

Limitations of artificial substrate samplers are:

- The need for a long exposure period makes the samplers unsuited for short-term survey studies.
- Samplers and floats are sometimes difficult to anchor in place and may present a navigation hazard.
- Samplers are vulnerable to vandalism and are often lost.
- Samplers provide no measure of the condition of the natural substrate at a station or of the effect of pollution on that substrate, including settled solids.
- Samplers only record the community that develops during the sampling period, thus reducing the value of the collected fauna as indicators of prior conditions.

Two other objections often made to the use of artificial substrate samplers are that they are selective to certain types of fauna and the data obtained do not provide a valid measure of the productivity of a particular environment. The validity of the latter objection depends on study objectives and may be of minor consequence in many pollution-oriented studies. The selectivity of artificial substrate samplers is a trival objection, since all currently available devices are selective. The selectivity of conventional sampling devices other than artificial substrates is directed toward those organisms that inhabit the types of substrate or substrates for which a particular type of sampler is designed.

3.3.5 Drift nets

Nets having a 15 by 30-cm upstream opening and a bag length of 1.3 m (No. 40 mesh netting) are recommended for small, swift streams. In large, deep rivers with a current of approximately 0.03 meters per second (mps), nets having an opening of 0.093 m^2 are recommended (2). Anchor the nets in flowing water (current not less than 0.015 mps) for from 1 to 24 hours, depending on the density of bottom fauna and hydrologic conditions. Place the top of the nets just below the surface of the water to permit calculation of the flow through the nets and to lessen the chance for collection of floating terrestrial insects. Do not permit the nets to touch bottom. In large rivers, maximum catches are obtained 0.3 to 0.6 meter above the bottom in the shoreline zone at depths not exceeding 3 meters.

Drift nets are useful for collecting macroinvertebrates that migrate or are dislodged from the substrate; they are particularly well-suited for synoptic surveys because they are lightweight and easily transported. Thousands of organisms – including larvae of stoneflies, mayflies, caddisflies, and midges and other Diptera, may be collected in a sampling period of only a few hours. Maximum drift intensity occurs between sunset and midnight (55). Elliot (14) presents an excellent synopsis of drift net methodology.

3.3.6 Photography

The use of photography is mainly limited to environments that have suitably clear water and are inhabited by sessile animals and rooted plants. Many estuarine habitats, such as those containing corals, sponges, and attached algal forms, fall in this category and can be photographed before, during, and after the introduction of stress. The technique has been used with success in south Florida to evaluate changes brought about by the introduction of heated effluents.

The technique for horizontal underwater photos using scuba gear involves placing a photographically identifiable marker in the habitat to be photographed and an additional nearby marker on which the camera is placed each time a photograph is taken. By this means, identical areas can be photographed repeatedly over a period of time to evaluate on-site changes in sessile forms at both affected and control stations. Vertical, overhead photos may also be taken under suitable conditions.

3.3.7 Qualitative devices

The investigator has an unlimited choice of gear for collecting qualitative samples. Any of the qualitative devices discussed previously, plus hand-held screens, dip nets, rakes, tongs, post hole diggers, bare hands, and forceps can be used. For deep-water collecting, some of the conventional grabs described earlier are normally required. In water less than 2 meters deep, a variety of gear may be used for sampling the sediments including long-handled dip nets and post-hole diggers. Collections from vascular plants and filamentous algae may be made with a dip net, common garden rake, potato fork, or oyster tongs. Collections from floating debris and rocks may be made by hand, using forceps to catch the smaller organisms.

In shallow streams, short sections of common window screen may be fastened between two poles and held in place at right angles to the water flow to collect organisms dislodged from upstream materials that have been agitated.

4.0 SAMPLE PROCESSING

4.1 Sieving

Samples collected with grabs, tubular devices, and artificial substrates contain varying amounts of finely divided materials such as completely decomposed organic material, silts, clays, and fine sand. To reduce sample volume and expedite sample processing in the laboratory, these fines should be removed by passing the sample through a U. S. Standard No. 30 sieve. Sieves may range from commercially constructed models to homemade sieves framed with wood or metal. Floating sieves with wooden frames reduce the danger of accidental loss of both sieve and sample when working over the side of a boat in deeper waters. A good sieve contains no cracks or crevices in which small organisms can become lodged.

If at all possible, sieving should be done in the field immediately after sample collection and while the captured organisms are alive. Once preserved, many organisms become quite fragile and if subjected to sieving will be broken up and lost or rendered unidentifiable.

Sieving may be accomplished by one of several techniques depending upon the reference of the individual biologist. In one technique, the sample is placed directly into a sieve and the sieve is then partially submerged in water and agitated until all fine materials have passed through. The sieve is agitated preferably in a tub of water.

A variation of this technique is to place the original sample in a bucket or tub, add screened water, stir, and pour the slurry through a U. S. Standard No. 30 sieve. Only a moderate amount of agitation is then required to completely clean the sample. Since this method requires considerably less effort, most biologists probably prefer it.

In both of the above methods, remove all the larger pieces of debris and rocks from samples collected, clean carefuly, and discard before the sample is stirred or agitated.

The artificial substrate samplers are placed in a bucket or tub of screened water and are dismantled. Each individual piece of substrate material is shaken and then cleaned gently under water with a soft brush (a soft grade of toothbrush is excellent), examined visually, and laid aside. The water in the bucket or tub is then poured through a U. S. Standard No. 30 sieve to remove the fines.

4.2 Preservation

Fill sample containers no more than one-half full of sample material (exclusive of the preservative). Supplemental sample containers are used for samples with large volumes of material. Obtain ample numbers and kinds of sample containers before the collection trip: allow two or three 1-liter containers per grab sample, a 1-liter container for most artificial substrate samples, and 16-dram screw-cap vials for miscellaneous collections.

Preserve the sample in 70 percent ethanol. A 70 percent ethanol solution is approximated by filling the one-half-full bottle, containing the sample and a small amount of rinse water, with 95 percent ethanol. Do not use formalin.

4.3 Labelling

Make sample labels of water-resistant paper and place inside the sample container. Write all information on the label with a soft-lead pencil. Where the volume of sample is so great that several containers are needed, additional external labels with the log number and notations such as 1 of 2, 2 of 2, are helpful for identifying sample containers in the laboratory.

Minimum information required on the sample label is a sample identification (log) number. The log number identifies the sample in a bound ledger where the name of water body, station number, date, sampling device used, name of sample collector, substrate characteristics, depth, and other environmental information are placed.

4.4 Sorting and Subsampling

For quantitative studies, sort and pick all samples by hand in the laboratory using a low-power scanning lens. To pick organisms efficiently and accurately, add only very small amounts of detritus (no more than a heaping tablespoon full) to standard-sized (25 X 40 X 5 cm), white enamel pans filled approximately one-third full of water. Small insects and worms will float free of most debris when ethanol-preserved samples are transferred to the water-filled pan.

Analysis time for samples containing excessively large numbers of organisms can be substantially reduced if the samples are subdivided before sorting. The sample is thoroughly mixed and distributed evenly over the bottom of a shallow tray. A divider, delineating one-quarter sections, is placed in a tray, and two opposite quarters are sorted. The two remaining quarters are combined and sorted for future reference or discarded (57). The aliquot to be sorted must be no smaller than one-quarter of the original sample; otherwise considerable error may result in estimating the total numbers of oligochaetes or other organisms that tend to clump. The same procedure may be followed for individual taxonomic groups, such as midges and worms, that may be present in large numbers.

Numerous techniques other than hand-picking have been proposed to recover organisms from the sample, including sugar solutions, salt solutions, stains, electricity for unpreserved samples in the field, bubbling air through sample in a tube, etc. The efficacy of these techniques is affected both by the characteristics of the substrate material and the types of organisms. No technique, or combination of techniques, will completely sort out or make more readily discernible all types of organisms from all types of substrate material. In the end, the total sample must be examined. If technicians are routinely conducting the picking operation, these techniques may lead to overconfidence and careless examination of the remainder of the sample. If used with proper care, such aids are not objectionable; however, they are not recommended as standard techniques.

As organisms are picked from the debris, they should be sorted into major categories (i.e., insect orders, molluscs, worms, etc.) and placed into vials containing 70 percent ethanol. All vials from a sample should be labeled internally with the picker's name and the lot number and kept as a unit in a suitable container until the organisms are identified and enumerated, and the data are recorded on the bench sheets. A typical laboratory bench sheet for fresh-water samples is shown in the Appendix.

4.5 Identification

The taxonomic level to which animals are identified depends on the needs, experience, and available resources. However, the taxonomic level to which identifications are carried in each major group should be constant throughout a given study. The accuracy of identification will depend greatly on the availability of taxonomic literature. A laboratory library of basic taxonomic references is essential. Many of the basic references that should be available in a tenthos laboratory are listed at the end of the chapter.

For comparative purposes and quality control checks, store identified specimens in a reference collection. Most identifications to order and family can be made under a stereoscopic microscope (up to 50X magnification). Identification to genus and species often requires a compound microscope, preferably equipped with phase contrast (10, 45, and 100X objectives) or Nomarski (interference phase) optics.

To make species identifications, it is often necessary to mount the entire organism or parts thereof on glass slides for examination at high magnification. Small whole insects or parts thereof may be slide-mounted directly from water or 70 percent ethanol preservative if CMC mounting media is used. Label the slides immediately with the sample log number and the name of the structure mounted. Euparol mounting medium may be preferable to CMC for mounts to be kept in a reference collection. Place specimens to be mounted in Euparol in 95 percent ethanol before mounting.

To clear opaque tissue, heat (do not boil) in a small crucible (5-ml capacity) containing 5 to 10 percent KOH solution (by weight) until it becomes transparent. The tissue can be checked periodically under a stereoscopic microscope to determine if it is sufficiently cleared. Then transfer the tissue stepwise to distilled water and 95 percent ethanol for 1 minute each and mount with CMC or Euparol. Several different structures can be heated simultaneously, but do not reuse the KOH solution.

The above methods work well for clearing and mounting midges, parts of caddisflies, mayflies, stoneflies, other insects, crustaceans, and molluscs; however, worms, leeches, and turbellarians require more specialized treatment before mounting (10, 47).

Larval insects often comprise the majority of macroinvertebrates collected in artificial substrate samplers and bottom samples. In certain cases, identifications are facilitated if exuviae, pupae, and adults are available. Collect exuviae of insects with drift nets or by skimming the water's surface with a small dip net near the shore. Obtain adults with sweep nets and tent traps in the field or rear larvae to maturity in the laboratory.

The life history stages of an insect can be positively associated only if specimens are reared individually. Rear small larvae individually in 6- to 12-dram vials half filled with stream water and aerated with the use of a fine-drawn glass tubing. Mass rearing can be carried out by placing the larvae with sticks and rocks in an aerated aquarium. Use a magnetic stirrer inside of the aquarium (41) to provide a current.

4.6 Biomass

Macroinvertebrate biomass (weight of organisms per unit area) is a useful quantitative estimation of standing crop. To determine wet weights, soak the organisms in distilled water for 30 minutes, centrifuge for 1 minute at 140 g in wire mesh cones, and weigh to the nearest 0.1 mg. Wet weight, however, is not recommended as a useful parameter unless, by a determination of suitable conversion factors, it can be equated to dry weight.

To obtain dry weight, oven dry the organisms to a constant weight at 105°C for 4 hours or vacuum dry at 105°C for 15 to 30 minutes at 1/2 atmosphere. Cool to room temperature in a desiccator and weigh. Freeze drying (-55°C, 10 to 30 microns pressure) has advantages over oven drying because the organisms remain intact for further identification and reference, preservatives are not needed, and cooling the material in desiccators after drying is not required. The main disadvantage of freeze drying is the length of time (usually 24 hours) required for drying to a constant weight.

To completely incinerate the organic material, ash at 550°C for 1 hour. Cool the ash to ambient temperature in a desiccator and weigh. Express the biomass as ash-free dry weight.

5.0 DATA EVALUATION

5.1 Quantitative Data

5.1.1 Reporting units

Data from quantitative samples may be used to obtain:

- total standing crop of individuals, or biomass, or both per unit area or unit volume or sample unit, and
- numbers or biomass, or both, of individual taxa per unit area or unit volume or sample unit.

Data from quantitative samples may also be evaluated in the same manner as discussed for qualitative s mples in part 5.2.

For purposes of comparison and to provide data useful for determining production, a uniform convention must be established for the units of data reported. For this purpose, EPA biologists should adhere to the following units:

- Data from devices sampling a unit area of bottom will be reported in grams dry weight or ash-free dry weight per square meter (gm/m^2), or numbers of individuals per square meter, or both.
- Data from multiplate samplers will be reported in terms of the total surface area of the plates in grams dry weight or ash-free dry weight or numbers of individuals per square meter, or both.
- Data from rock-filled basket samplers will be reported as grams dry weight or numbers of individuals per sampler, or both.

5.1.2 Standing crop and taxonomic composition

Standing crop and numbers of taxa in a community are highly sensitive to environmental perturbations resulting from the introduction of contaminants. These parameters, particularly standing crop, may vary considerably in unpolluted habitats, where they may range from the typically high standing crop of littoral zones of glacial lakes to the sparse fauna of torrential soft-water streams. Thus, it is important that comparisons are made only between truly comparable environments. Typical responses of standing crop or taxa to various types of stress are:

Stress	Standing crop (numbers or biomass)	Number of taxa
Toxic substance	Reduce	Reduce
Severe temperature alterations	Variable	Reduce
Silt	Reduce	Reduce
Inorganic nutrients	Increase	Variable – often no detectable change
Organic nutrients (high O_2 demand)	Increase	Reduce
Sludge deposits (non-toxic)	Increase	Reduce

Organic nutrients and sludge deposits are frequently associated. The responses shown are by no means simple or fixed and may vary depending on a number of factors including:

- a combination of stresses acting together or in opposition,
- indirect effects, such as for example the destruction of highly productive vegetative substrate by temperature alterations, sludge deposits, turbidity, chemical weed control,
- the physical characteristics of the stressed environment, particularly in relation to substrate and current velocity.

Data on standing crop and numbers of taxa may be presented in simple tabular form or pictorially with bar and line graphs, pie diagrams, and histograms. Whatever the method of presentation, the number of replicates and the sampling variability must be shown in the tables or graphs. Sampling variability may be shown as a range of values or as a calculated standard deviation, as discussed in the Biometrics Section of this manual.

Data on standing crop and number of taxa are amenable to simple but powerful statistical techniques of evaluation. Under grossly stressed situations, such analyses may be unnecessary; however, in some cases, the effects of environmental perturbations may be so subtle in comparison with sampling variation that statistical comparisons are a helpful and necessary tool for the evaluative process. For this purpose, biologists engaged in studies of macroinvertebrates should familiarize themselves with the simple statistical tools discussed in the Biometrics Section of this manual.

5.1.3 Diversity

Diversity indices are an additional tool for measuring the quality of the environment and the effect of induced stress on the structure of a community of macroinvertebrates. Their use is based on the generally observed phenomenon that relatively undisturbed environments support communities having large numbers of species with no individual species present in overwhelming abundance. If the species in such a community are ranked on the basis of their numerical abundance, there will be relatively few species with large numbers of individuals and large numbers of species represented by only a few individuals. Many forms of stress tend to reduce diversity by making the environment unsuitable for some species or by giving other species a competitive advantage.

The investigator must be aware that there are naturally occurring extreme environments in which the diversity of macroinvertebrate communities may be low, as for example the profundal fauna of a deep lake or the black fly-dominated communities of the high gradient, bed rock section of a torrential stream. Furthermore, because colonization is by chance, diversity may be highly variable in a successional community; for this reason, diversity indices calculated from the fauna of artificial substrate samplers must be evaluated with caution. These confounding factors can be reduced by comparing diversity in similar habitats and by exposing artificial substrate samplers long enough for a relatively stable, climax community to develop.

Indices, such as $\frac{S}{N}$, $\frac{S}{\text{Log N}}$, and $\frac{S\text{-}1}{\text{Log N}}$ where S = number of taxa and N = total number of individuals, are merely additional means of summarizing data on total numbers and total taxa in a single numerical form for evaluation and summarization. They add no new dimension to the methods of data presentation and analyses discussed above and, in addition, are highly influenced by sample size. Sample size in this context relates to the total number of organisms collected (an uncontrollable variable in most macroinvertebrate sampling), not to the area or volume of habitat sampled. Do not use such indices for summarizing and evaluating data on aquatic macroinvertebrate communities.

There are two components of species diversity:

- richness of species
- distribution of individuals among the species.

It is immediately obvious that the second component adds a new dimension that was not considered in the methods for evaluating data discussed above. The distribution of individuals among the species may be readily presented in frequency distribution tables or graphs; but for any appreciable number of samples, such methods of presentation are so voluminous that they are virtually impossible to compare and interpret.

Indices of diversity based on information theory, as originally proposed by Margalef (39) and subsequently utilized by numerous workers, include both components of species diversity as enumerated above. Additionally, a measure of the component of diversity due to the distribution of individuals among the species can readily be extracted from the overall index. For purposes of uniformity, the Shannon-Weaver function is provisionally recommended for calculating mean diversity $\bar{d}$.

The machine formula presented by Lloyd, Zar, and Karr (34) is:

$$\bar{d} = \frac{C}{N}(N \log_{10} N - \Sigma n_i \log_{10} n_i)$$

where C = 3.321928 (converts base 10 log to base 2 [bits]); N = total number of individuals; and n_i = total number of individuals in the i^{th} species. When their tables (reproduced in Table 5) are used, the calculations are simple and straightforward, as shown by the following example:

	Number of individuals in each taxa (n_i's)	$n_i \log_{10} n_i$ (from Table 5)
	41	66.1241
	5	3.4949
	18	22.5949
	3	1.4314
	1	.0000
	22	29.5333
	1	.0000
	2	.6021
	12	12.9502
	4	2.4082
Total	109	139.1391

Total number of taxa, s = 10
Total number of individuals, N = 109

$N \log_{10} N$ = 222.0795 (from Table 5)
$\Sigma n_i \log_{10} n_i$ = 139.1391

$$= \frac{3.321928}{109}(222.0795 - 139.1391)$$
$$= 0.030476 \times 82.9404$$
$$= 2.5$$

Mean diversity, $\bar{d}$, as calculated above is affected both by richness of species and by the distribution of individuals among the species and may range from zero to 3.321928 log N.

To evaluate the component of diversity due to the distribution of individuals among the species, compare the calculated $\bar{d}$ with a hypothetical maximum $\bar{d}$ based on an arbitrarily selected distribution. The measure of redundancy proposed by Margalef (39) is based on the ratio between $\bar{d}$ and a hypothetical maximum computed as though all species were equally abundant. In nature, equality of species is quite unlikely, so Lloyd and Ghelardi (33) proposed the term "equitability" and compared $\bar{d}$ with a maximum based on the distribution obtained from MacArthur's (36) broken stick model. The MacArthur model results in a distribution quite frequently observed in nature – one with a few relatively abundant species and increasing numbers of species represented by only a few individuals. Sample data are not expected to conform to the MacArthur model, since it is only being used as a yardstick against which the distribution of abundances is being compared. Lloyd and Ghelardi (33) devised a table for determining equitability by comparing the number of species (s) in the sample with the number of species expected (s') from a community that conforms to the MacArthur model. In the table (reproduced as Table 6 of this Section), the proposed measure of equitability is:

$$e = \frac{s'}{s}$$

where s = number of taxa in the sample, and s' = the tabulated value. For the example given above (without interpolation in the table):

$$e = \frac{s'}{s} = \frac{8}{10} = 0.8.$$

Equitability "e," as calculated, may range from 0 to 1 except in the unusual situation where the distribution in the sample is more equitable than the distribution resulting from the MacArthur model. Such an eventuality will result in values of e greater than 1, and this occasionally occurs in samples containing only a few specimens with several taxa represented. The estimate of $\bar{d}$ and e improves with increased sample size, and samples containing less than 100 specimens should be evaluated with caution, if at all.

When Wilhm (59) evaluated values calculated from data that numerous authors had collected from a variety of polluted and unpolluted waters, he found that in unpolluted waters $\bar{d}$ was generally between 3 and 4, whereas in polluted water, $\bar{d}$ was generally less than 1. However, collected data from southeastern U. S. waters by EPA biologists has shown that where degradation is at slight to moderate levels, $\bar{d}$ lacks the sensitivity to demonstrate differences. Equitability e, on the contrary, has been found to be very sensitive to even slight levels of degradation. Equitability levels below 0.5 have not been encountered in southeastern streams known to be unaffected by oxygen-demanding wastes, and in such streams, e generally ranges between 0.6 and 0.8. Even slight levels of degradation have been found to reduce equitability below 0.5 and generally to a range of 0.0 to 0.3.

Agency biologists are encouraged to calculate both mean diversity $\bar{d}$ and equitability e for samples collected in the course of macroinvertebrate studies. (If the mean and range of values found by different sampling methods and under varying levels and types of pollution are reported to the Biological Methods Branch, these data will be included in tabular form in future revisions of this Section.)

5.2 Qualitative Data

As previously defined, qualitative data result from samples collected in such a manner that no estimate of numerical abundance or biomass can be calculated. The output consists of a list of taxa collected in the various habitats of the environment being studied. The numerous schemes advanced for the analysis of qualitative data may be grouped in two categories:

5.2.1 Indicator-organism scheme

For this technique, individual taxa are classified on the basis of their tolerance or intolerance to various levels of putrescible wastes (4, 5, 30, 42, 48). Taxa are classified according to their presence or absence in different environments as determined by field studies. Beck (6) reduced data based on the presence or absence of indicator organisms to a simple numerical form for ease in presentation.

5.2.2 Reference station methods

Comparative or *control station* methods compare the qualitative characteristics of the fauna in *clean water* habitats with those of fauna in habitats subject to stress. Patrick (46) compared stations on the basis of richness of species and Wurtz (61) used indicator organisms in comparing stations.

If adequate background data are available to an experienced investigator, both of these techniques can prove quite useful—particularly for the purpose of demonstrating the effects of gross to moderate organic contamination on the macroinvertebrate community. To detect more subtle changes in the macroinvertebrate community, collect quantitative data on numbers or biomass of organisms. Data on the presence of tolerant and intolerant taxa and richness of species may be effectively summarized for evaluation and presentation by means of line graphs, bar graphs, pie diagrams, histograms, or pictoral diagrams (27).

The calssification by various authors of representative macroinvertebrates according to their tolerance of organic wastes is presented in Table 7. In most cases, the taxonomic nomenclature used in the table is that of the original authors. The pollutional classifications of the authors were arbitrarily placed in three categories – tolerant, facultative, and intolerant – defined as follows:

- Tolerant: Organisms frequently associated with gross organic contamination and are generally capable of thriving under anaerobic conditions.

- Facultative: Organisms having a wide range of tolerance and frequently are associated with moderate levels of organic contamination.
- Intolerant: Organisms that are not found associated with even moderate levels of organic contaminants and are generally intolerant of even moderate reductions in dissolved oxygen.

When evaluating qualitative data in terms of material such as that contained in Table 7, the investigator should keep in mind the pitfalls mentioned earlier, as well as the following:

- Since tolerant species may be found in both clean and degraded habitats, a simple record of their presence or absence is of no significance. Therefore, the indicator-organism technique can provide positive evidence of only one condition—clean water—and this only if taxa classified as intolerant are collected. An exception to this rule would occur where sensitive species may be totally absent because of the discharge of toxic substances or waste heat.
- Because evaluations are based on the mere presence or absence or organisms, a single specimen has as much weight as a large population. Therefore, data for the original classification and from field studies may be biassed by the drift of organisms into the study area.
- The presence or absence of a particular taxa may depend more on characteristics of the environment, such as velocity and substrate, than on the level of degradation by organic wastes. This affects both the original placement of the taxa in the classificatory scheme and its presence in study samples.
- Technique is totally subjective and quite dependent upon the skill and experience of the individual who makes the field collections. Therefore, results of one investigator are difficult to compare with those of another, particularly where data are summarized in an index such as that proposed by Beck (6).

TABLE 5. FUNCTIONS FOR CALCULATING SPECIES DIVERSITY AND (FOR PERFECTLY RANDOM SAMPLING) ITS STANDARD ERROR LOGARITHMS ARE TO BASE 10. TABLE VALUES ARE ACCURATE TO WITHIN ±1 IN THE EIGHTH SIGNIFICANT FIGURE. (REPRODUCED WITH PERMISSION FROM LLOYD, ZAR, AND KARR, 1968.)

n	log n!	n log n	n log² n
1	.0000	.0000	.0000
2	.3010	.6021	.1812
3	.7782	1.4314	.6829
4	1.3802	2.4082	1.4499
5	2.0792	3.4949	2.4428
6	2.8573	4.6689	3.6331
7	3.7024	5 9157	4.9993
8	4.6055	7.2247	6.5246
9	5.5598	8.5882	8.1952
10	6.5598	10.0000	10.0000
11	7.6012	11.4553	11.9295
12	8.6803	12.9502	13.9756
13	9.7943	14.4813	16.1313
14	10.9404	16.0458	18.3905
15	12.1165	17.6414	20.7479
16	13 3206	19.2659	23.1985
17	14.5511	20.9176	25.7381
18	15 8063	22.5949	28.3628
19	17.0851	24.2963	31.0690
20	18 3861	26.0206	33.8536
21	19.7083	27.7666	36.7135
22	21.0508	29.5333	39.6462
23	22.4125	31.3197	42.6490
24	23.7927	33.1251	45.7196
25	25.1906	34.9485	48.8559
26	26.6056	36.7893	52.0559
27	28.0370	38.6468	55 3177
28	29.4841	40 5204	58.6395
29	30.9465	42.4095	62.0196
30	32.4237	44.3136	65.4566
31	33.9150	46.2322	68.9490
32	35.4202	48.1648	72.4952
33	36.9387	50.1110	76.0942
34	38.4702	52.0703	79 7445
35	40.0142	54.0424	83.4451
36	41.5705	56.0269	87 1948
37	43.1387	58.0235	90.9925
38	44.7185	60.0318	94.8372
39	46.3096	62 0515	98 7280
40	47.9116	64.0824	102.6638
41	49.5244	66.1241	106.6439
42	51.1477	68.1765	110 6674
43	52.7811	70.2391	114.7334
44	54.4246	72.3119	118.8412
45	56.0778	74.3946	122.9900
46	57.7406	76.4869	127.1791
47	59.4127	78.5886	131.4078
48	61.0939	80.6996	135 6755
49	62.7841	82.8196	139.9814
50	64.4831	84.9485	144.3250
51	66.1906	87.0861	148.7056
52	67.9066	89.2322	153.1227
53	69.6309	91.3866	157.5757
54	71.3633	93.5493	162.0642
55	73.1037	95 7199	166 5874
56	74.8519	97.8985	171.1450
57	76.6077	100.0849	175.7365
58	78.3712	102.2788	180.3613
59	80.1420	104.4803	185.0191
60	81.9202	106.6891	189.7093
61	83.7055	108.9051	194.4316
62	85.4979	111.1283	199.1854
63	87.2972	113.3585	203.9705
64	89.1034	115.5955	208.7863
65	90.9163	117.8394	213 6326
66	92.7359	120.0899	218 5088
67	94.5619	122.3470	223.4148
68	96.3945	124.6106	228.3500
69	98.2333	126.8806	233.3143
70	100.0784	129.1569	238.3071
71	101.9297	131.4393	243.3282
72	103.7870	133.7279	248.3772
73	105.6503	136.0226	253.4540
74	107.5196	138.3231	258 5580
75	109.3946	140.6296	263.6891
76	111.2754	142.9418	268.8469
77	113.1619	145.2598	274.0312
78	115.0540	147.5834	279.2417
79	116.9516	149.9125	284.4781
80	118.8547	152.2472	289.7401
81	120.7632	154.5873	295.0275
82	122.6770	156.9327	300.3400
83	124.5961	159.2835	305.6774
84	126.5204	161 6395	311.0395
85	128.4498	164 0006	316.4259
86	130.3843	166.3669	321.8364
87	132.3238	168.7382	327 2709
88	134.2683	171.1145	332.7291
89	136.2177	173.4957	338.2108
90	138.1719	175.8818	343.7157
91	140.1310	178.2728	349.2437
92	142.0948	180.6685	354.7946
93	144.0632	183.0689	360 3680
94	146.0364	185.4740	365.9640
95	148.0141	187.8837	371.5821
96	149.9964	190.2980	377.2223
97	151.9831	192.7169	382.8844
98	153.9744	195.1402	388.5682
99	155.9700	197.5679	394.2734
100	157.9700	200.0000	400.0000
101	159.9743	202.4365	405.7477
102	161.9829	204.8772	411.5164
103	163.9958	207 3222	417.3059
104	166.0128	209 7715	423.1160
105	168.0340	212.2249	428.9466
106	170.0593	214.6824	434.7976
107	172.0887	217.1441	440.6686
108	174.1221	219.6098	446.5597
109	176.1595	222.0795	452.4706
110	178.2009	224.5532	458.4013
111	180.2462	227.0309	464.3514
112	182.2955	229.5124	470.3210
113	184.3485	231.9979	476.3098
114	186.4054	234 4872	482.3178
115	188.4661	236.9803	488.3447
116	190.5306	239.4771	494.3905
117	192.5988	241.9777	500.4550
118	194.6707	244.4821	506.5380
119	196.7462	246.9901	512.6395
120	198.8254	249.5017	518.7594
121	200.9082	252.0170	524 8974
122	202.9945	254.5359	531.0535
123	205 0844	257.0583	537.2275
124	207.1779	259.5843	543.4194
125	209.2748	262.1138	549.6290
126	211.3751	264.6467	555.8561
127	213.4790	267.1831	562.1007
128	215.5862	269.7229	568.3627
129	217.6967	272.2661	574.6420
130	219.8107	274 8126	580.9383
131	221 9280	277.3625	587.2517
132	224.0485	279.9158	593.5821
133	226.1724	282.4723	599.9292
134	228 2995	285.0320	606 2930
135	230.4298	287.5951	612.6735
136	232.5634	290.1613	619.0704
137	234.7001	292.7307	625.4837
138	236.8400	295.3033	631.9134
139	238.9830	297 8791	638.3592
140	241.1291	300.4579	644.8212

TABLE 5. (Continued)

n	log n!	n log n	n $\log^2$ n
141	243.2783	303.0399	651.2991
142	245.4306	305.6249	657.7930
143	247.5860	308.2131	664.3027
144	249.7443	310.8042	670.8281
145	251.9057	313.3984	677.3692
146	254.0700	315.9955	683.9258
147	256.2374	318.5956	690.4979
148	258.4076	321.1987	697.0853
149	260.5808	323.8048	703.6880
150	262.7569	326.4137	710.3060
151	264.9359	329.0255	716.9390
152	267.1177	331.6402	723.5871
153	269.3024	334.2578	730.2501
154	271.4899	336.8782	736.9280
155	273.6803	339.5014	743.6207
156	275.8734	342.1274	750.3281
157	278.0693	344.7562	757.0501
158	280.2679	347.3878	763.7867
159	282.4693	350.0221	770.5377
160	284.6735	352.6592	777.3032
161	286.8803	355.2990	784.0830
162	289.0898	357.9414	790.8770
163	291.3020	360.5866	797.6852
164	293.5168	363.2344	804.5075
165	295.7343	365.8849	811.3438
166	297.9544	368.5379	818.1941
167	300.1771	371.1936	825.0582
168	302.4024	373.8520	831.9362
169	304.6303	376.5129	838.8280
170	306.8608	379.1763	845.7334
171	309.0938	381.8423	852.6524
172	311.3293	384.5109	859.5850
173	313.5674	387.1820	866.5311
174	315.8079	389.8556	873.4906
175	318.0509	392.5317	880.4634
176	320.2965	395.2102	887.4496
177	322.5444	397.8913	894.4489
178	324.7948	400.5748	901.4615
179	327.0477	403.2607	908.4871
180	329.3030	405.9491	915.5257
181	331.5606	408.6398	922.5774
182	333.8207	411.3330	929.6419
183	336.0832	414.0285	936.7193
184	338.3480	416.7265	943.8096
185	340.6152	419.4268	950.9125
186	342.8847	422.1294	958.0282
187	345.1565	424.8344	965.1564
188	347.4307	427.5417	972.2973
189	349.7071	430.2513	979.4506
190	351.9859	432.9632	986.6164
191	354.2669	435.6774	993.7946
192	356.5502	438.3938	1000.9852
193	358.8358	441.1126	1008.1880
194	361.1236	443.8335	1015.4031
195	363.4136	446.5567	1022.6304
196	365.7059	449.2822	1029.8698
197	368.0003	452.0098	1037.1213

n	log n!	n log n	n $\log^2$ n
198	370.2970	454.7397	1044.3849
199	372.5959	457.4718	1051.6604
200	374.8969	460.2060	1058.9478
201	377.2001	462.9424	1066.2471
202	379.5054	465.6810	1073.5583
203	381.8129	468.4217	1080.8812
204	384.1226	471.1646	1088.2159
205	386.4343	473.9095	1095.5622
206	388.7482	476.6566	1102.9202
207	391.0642	479.4059	1110.2897
208	393.3822	482.1572	1117.6709
209	395.7024	484.9106	1125.0635
210	398.0246	487.6661	1132.4675
211	400.3489	490.4236	1139.8829
212	402.6752	493.1832	1147.3098
213	405.0036	495.9449	1154.7479
214	407.3340	498.7085	1162.1973
215	409.6664	501.4743	1169.6579
216	412.0009	504.2420	1177.1296
217	414.3373	507.0118	1184.6126
218	416.6758	509.7835	1192.1066
219	419.0162	512.5573	1199.6116
220	421.3587	515.3330	1207.1277
221	423.7031	518.1107	1214.6547
222	426.0494	520.8904	1222.1926
223	428.3977	523.6720	1229.7415
224	430.7480	526.4556	1237.3011
225	433.1002	529.2411	1244.8716
226	435.4543	532.0285	1252.4528
227	437.8103	534.8179	1260.0447
228	440.1682	537.6091	1267.6473
229	442.5281	540.4023	1275.2606
230	444.8898	543.1974	1282.8844
231	447.2534	545.9944	1290.5188
232	449.6189	548.7932	1298.1637
233	451.9862	551.5939	1305.8192
234	454.3555	554.3965	1313.4850
235	456.7265	557.2009	1321.1613
236	459.0994	560.0072	1328.8479
237	461.4742	562.8154	1336.5448
238	463.8508	565.6253	1344.2521
239	466.2292	568.4371	1351.9696
240	468.6094	571.2507	1359.6973
241	470.9914	574.0661	1367.4352
242	473.3752	576.8833	1375.1833
243	475.7608	579.7023	1382.9415
244	478.1482	582.5231	1390.7098
245	480.5374	585.3457	1398.4881
246	482.9283	588.1700	1406.2764
247	485.3210	590.9961	1414.0747
248	487.7154	593.8240	1421.8829
249	490.1116	596.6536	1429.7010
250	492.5096	599.4850	1437.5291
251	494.9093	602.3181	1445.3669
252	497.3107	605.1529	1453.2146
253	499.7138	607.9895	1461.0720
254	502.1186	610.8278	1468.9392

n	log n!	n log n	n $\log^2$ n
255	504.5252	613.6677	1476.8161
256	506.9334	616.5094	1484.7026
257	509.3433	619.3528	1492.5988
258	511.7549	622.1979	1500.5047
259	514.1682	625.0446	1508.4201
260	516.5832	627.8931	1516.3450
261	518.9999	630.7432	1524.2795
262	521.4182	633.5949	1532.2234
263	523.8381	636.4484	1540.1769
264	526.2597	639.3034	1548.1397
265	528.6830	642.1602	1556.1119
266	531.1078	645.0185	1564.0936
267	533.5344	647.8785	1572.0845
268	535.9625	650.7401	1580.0847
269	538.3922	653.6034	1588.0943
270	540.8236	656.4682	1596.1130
271	543.2566	659.3347	1604.1410
272	545.6912	662.2027	1612.1782
273	548.1273	665.0724	1620.2245
274	550.5651	667.9437	1628.2800
275	553.0044	670.8165	1636.3446
276	555.4453	673.6909	1644.4182
277	557.8878	676.5669	1652.5009
278	560.3318	679.4445	1660.5927
279	562.7774	682.3236	1668.6934
280	565.2246	685.2042	1676.8031
281	567.6733	688.0865	1684.9217
282	570.1235	690.9702	1693.0492
283	572.5753	693.8556	1701.1856
284	575.0287	696.7424	1709.3309
285	577.4835	699.6308	1717.4850
286	579.9399	702.5207	1725.6479
287	582.3977	705.4121	1733.8196
288	584.8571	708.3050	1742.0001
289	587.3180	711.1995	1750.1893
290	589.7804	714.0954	1758.3871
291	592.2443	716.9929	1766.5937
292	594.7097	719.8918	1774.8089
293	597.1766	722.7922	1783.0327
294	599.6449	725.6941	1791.2651
295	602.1147	728.5975	1799.5061
296	604.5860	731.5023	1807.7557
297	607.0588	734.4087	1816.0138
298	609.5330	737.3164	1824.2803
299	612.0087	740.2257	1832.5554
300	614.4858	743.1364	1840.8389
301	616.9644	746.0485	1849.1308
302	619.4444	748.9621	1857.4312
303	621.9258	751.8771	1865.7399
304	624.4087	754.7936	1874.0570
305	626.8930	757.7115	1882.3824
306	629.3787	760.6308	1890.7162
307	631.8659	763.5515	1899.0582
308	634.3544	766.4736	1907.4085
309	636.8444	769.3972	1915.7670
310	639.3357	772.3221	1924.1337
311	641.8285	775.2485	1932.5087

n	log n!	n log n	n $\log^2$ n
312	644.3226	778.1762	1940.8918
313	646.8182	781.1054	1949.2831
314	649.3151	784.0359	1957.6825
315	651.8134	786.9678	1966.0900
316	654.3131	789.9011	1974.5056
317	656.8142	792.8358	1982.9293
318	659.3166	795.7718	1991.3610
319	661.8204	798.7092	1999.8007
320	664.3255	801.6480	2008.2484
321	666.8320	804.5881	2016.7041
322	669.3399	807.5296	2025.1678
323	671.8491	810.4724	2033.6394
324	674.3596	813.4166	2042.1189
325	676.8715	816.3621	2050.6064
326	679.3847	819.3089	2059.1016
327	681.8993	822.2571	2067.6048
328	684.4152	825.2066	2076.1157
329	686.9324	828.1575	2084.6345
330	689.4509	831.1096	2093.1611
331	691.9707	834.0631	2101.6954
332	694.4918	837.0178	2110.2375
333	697.0143	839.9739	2118.7874
334	699.5380	842.9313	2127.3449
335	702.0631	845.8900	2135.9102
336	704.5894	848.8500	2144.4831
337	707.1170	851.8113	2153.0636
338	709.6460	854.7738	2161.6518
339	712.1762	857.7377	2170.2477
340	714.7076	860.7028	2178.8510
341	717.2404	863.6692	2187.4620
342	719.7744	866.6369	2196.0806
343	722.3097	869.6059	2204.7067
344	724.8463	872.5761	2213.3403
345	727.3841	875.5476	2221.9814
346	729.9232	878.5203	2230.6299
347	732.4635	881.4943	2239.2860
348	735.0051	884.4696	2247.9495
349	737.5479	887.4461	2256.6204
350	740.0920	890.4238	2265.2988
351	742.6373	893.4028	2273.9845
352	745.1838	896.3830	2282.6776
353	747.7316	899.3645	2291.3780
354	750.2806	902.3472	2300.0858
355	752.8308	905.3311	2308.8009
356	755.3823	908.3162	2317.5233
357	757.9349	911.3026	2326.2531
358	760.4888	914.2901	2334.9900
359	763.0439	917.2789	2343.7342
360	765.6002	920.2689	2352.4857
361	768.1577	923.2601	2361.2444
362	770.7164	926.2525	2370.0102
363	773.2764	929.2461	2378.7832
364	775.8375	932.2409	2387.5634
365	778.3997	935.2369	2396.3508
366	780.9632	938.2341	2405.1453
367	783.5279	941.2324	2413.9469
368	786.0937	944.2320	2422.7556

TABLE 5. (Continued)

n	log n!	n log n	n $\log^2$ n
369	788.6608	947.2327	2431.5714
370	791.2290	950.2346	2440.3942
371	793.7983	953.2377	2449.2241
372	796.3689	956.2420	2458.0610
373	798.9406	959.2474	2466.9050
374	801.5135	962.2540	2475.7559
375	804.0875	965.2617	2484.6139
376	806.6627	968.2706	2493.4787
377	809.2390	971.2807	2502.3506
378	811.8165	974.2919	2511.2294
379	814.3952	977.3043	2520.1151
380	816.9749	980.3178	2529.0077
381	819.5559	983.3324	2537.9072
382	822.1379	986.3482	2546.8135
383	824.7211	989.3651	2555.7268
384	827.3055	992.3832	2564.6469
385	829.8909	995.4024	2573.5738
386	832.4775	998 4227	2582.5075
387	835.0652	1001.4441	2591.4480
388	837.6540	1004.4667	2600.3953
389	840.2440	1007.4904	2609.3493
390	842.8351	1010.5152	2618.3102
391	845.4272	1013.5411	2627.2777
392	848.0205	1016.5681	2636.2520
393	850.6149	1019.5963	2645.2330
394	853.2104	1022.6255	2654.2206
395	855.8070	1025.6558	2663.2150
396	858.4047	1028.6873	2672.2160
397	861.0035	1031.7198	2681.2237
398	863.6034	1034.7534	2690.2380
399	866.2044	1037.7882	2699.2589
400	868.8064	1040.8240	2708.2865
401	871.4096	1043.8609	2717.3206
402	874.0138	1046.8989	2726.3613
403	876.6191	1049.9379	2735.4086
404	879.2255	1052.9781	2744.4624
405	881.8329	1056.0193	2753.5228
406	884.4415	1059.0616	2762.5897
407	887.0510	1062.1049	2771.6631
408	889.6617	1065.1493	2780.7430
409	892.2734	1068.1948	2789.8293
410	894.8862	1071.2414	2798.9222
411	897.5001	1074.2890	2808.0215
412	900.1150	1077.3376	2817.1272
413	902.7309	1080.3874	2826.2394
414	905.3479	1083.4381	2835.3580
415	907.9660	1086.4900	2844.4830
416	910.5850	1089.5428	2853.6143
417	913.2052	1092.5967	2862.7521
418	915.8264	1095.6517	2871.8962
419	918.4486	1098.7077	2881.0467
420	921.0718	1101.7647	2890.2035
421	923.6961	1104.8228	2899.3666
422	926.3214	1107.8819	2908.5360
423	928.9478	1110.9420	2917.7117
424	931.5751	1114.0031	2926.8938
425	934.2035	1117.0653	2936.0820

n	log n!	n log n	n $\log^2$ n
426	936.8329	1120.1285	2945.2766
427	939.4633	1123.1927	2954.4774
428	942.0948	1126.2579	2963.6844
429	944 7272	1129.3242	2972.8976
430	947.3607	1132.3914	2982.1171
431	949.9952	1135.4597	2991.3428
432	952.6307	1138.5290	3000.5746
433	955.2672	1141.5993	3009.8126
434	957.9047	1144.6705	3019.0568
435	960.5431	1147.7428	3028.3071
436	963.1826	1150.8161	3037.5636
437	965.8231	1153.8904	3046.8261
438	968.4646	1156.9657	3056.0948
439	971.1071	1160.0419	3065.3696
440	973 7505	1163.1192	3074.6505
441	976.3949	1166.1974	3083.9374
442	979.0404	1169.2766	3093.2305
443	981.6868	1172.3568	3102.5295
444	984.3342	1175.4380	3111.8346
445	986.9825	1178.5202	3121.1458
446	989.6318	1181.6033	3130.4629
447	992 2822	1184.6875	3139.7861
448	994.9334	1187.7725	3149.1152
449	997.5857	1190.8586	3158.4504
450	1000.2389	1193.9456	3167.7915
451	1002.8931	1197.0336	3177.1385
452	1005.5482	1200.1226	3186.4915
453	1008.2043	1203.2125	3195.8505
454	1010.8614	1206 3033	3205.2154
455	1013.5194	1209.3952	3214.5862
456	1016.1783	1212.4880	3223.9629
457	1018 8382	1215.5817	3233.3455
458	1021.4991	1218.6764	3242.7339
459	1024.1609	1221.7720	3252.1283
460	1026.8237	1224.8686	3261.5284
461	1029.4874	1227.9661	3270.9345
462	1032.1520	1231.0646	3280.3464
463	1034.8176	1234.1640	3289.7641
464	1037.4841	1237.2643	3299.1876
465	1040.1516	1240.3656	3308.6169
466	1042.8200	1243.4678	3318.0521
467	1045.4893	1246.5710	3327.4930
468	1048.1595	1249.6750	3336.9396
469	1050.8307	1252.7801	3346.3921
470	1053 5028	1255.8860	3355.8503
471	1056.1758	1258.9928	3365.3142
472	1058.8498	1262.1006	3374.7838
473	1061.5246	1265.2093	3384.2592
474	1064.2004	1268.3189	3393.7403
475	1066.8771	1271 4295	3403.2271
476	1069.5547	1274.5409	3412.7196
477	1072.2332	1277.6533	3422.2177
478	1074.9126	1280 7665	3431.7216
479	1077.5930	1283.8807	3441.2310
480	1080.2742	1286.9958	3450.7462
481	1082.9564	1290.1118	3460.2669
482	1085.6394	1293.2287	3469.7933

n	log n!	n log n	n $\log^2$ n
483	1088.3234	1296.3465	3479 3253
484	1091.0082	1299.4651	3488.8630
485	1093.6940	1302 5847	3498 4062
486	1096.3806	1305.7052	3507 9550
487	1099.0681	1308.8266	3517 5094
488	1101.7565	1311.9489	3527 0693
489	1104.4458	1315 0720	3536.6349
490	1107.1360	1318.1961	3546.2059
491	1109.8271	1321.3210	3555.7825
492	1112.5191	1324 4468	3565.3646
493	1115.2119	1327.5735	3574.9523
494	1117.9057	1330.7011	3584.5454
495	1120.6003	1333.8296	3594.1441
496	1123.2957	1336.9589	3603.7482
497	1125.9921	1340.0891	3613.3578
498	1128.6893	1343 2202	3622.9730
499	1131.3874	1346.3522	3632.5935
500	1134.0864	1349.4850	3642.2195
501	1136.7862	1352.6187	3651.8510
502	1139.4869	1355.7533	3661.4879
503	1142.1885	1358.8887	3671.1302
504	1144.8909	1362.0250	3680.7779
505	1147.5942	1365 1621	3690.4310
506	1150.2984	1368.3002	3700.0896
507	1153.0034	1371.4390	3709.7535
508	1155.7093	1374.5788	3719 4228
509	1158.4160	1377.7193	3729.0974
510	1161.1235	1380.8608	3738.7775
511	1163.8320	1384.0031	3748.4629
512	1166.5412	1387.1462	3758.1536
513	1169.2514	1390.2902	3767.8496
514	1171.9623	1393.4350	3777.5510
515	1174.6741	1396.5807	3787.2577
516	1177.3868	1399.7272	3796.9697
517	1180.1003	1402.8746	3806 6870
518	1182.8146	1406.0228	3816.4095
519	1185.5298	1409.1718	3826.1374
520	1188.2458	1412.3217	3835.8705
521	1190.9626	1415.4724	3845 6089
522	1193.6803	1418.6240	3855.3526
523	1196.3988	1421.7764	3865.1015
524	1199.1181	1424.9296	3874.8556
525	1201.8383	1428.0836	3884.6150
526	1204.5592	1431.2385	3894.3795
527	1207.2811	1434.3942	3904.1493
528	1210.0037	1437.5507	3913.9243
529	1212.7271	1440.7080	3923.7045
530	1215.4514	1443.8662	3933.4899
531	1218.1765	1447.0252	3943.2804
532	1220.9024	1450.1850	3953.0761
533	1223.6292	1453.3456	3962.8770
534	1226.3567	1456.5070	3972.6830
535	1229.0851	1459.6693	3982.4942
536	1231.8142	1462.8323	3992.3105
537	1234.5442	1465.9962	4002.1319
538	1237.2750	1469.1609	4011.9584
539	1240.0066	1472.3263	4021.7901

n	log n!	n log n	n $\log^2$ n
540	1242.7390	1475.4926	4031.6268
541	1245 4722	1478.6597	4041.4687
542	1248.2061	1481.8276	4051.3156
543	1250.9409	1484.9963	4061.1676
544	1253.6765	1488.1658	4071.0247
545	1256.4129	1491.3361	4080.8868
546	1259.1501	1494.5072	4090.7541
547	1261.8881	1497.6791	4100.6263
548	1264.6269	1500.8517	4110.5035
549	1267.3665	1504.0252	4120 3859
550	1270.1068	1507.1995	4130.2732
551	1272.8480	1510.3745	4140.1655
552	1275.5899	1513 5504	4150 0629
553	1278.3327	1516.7270	4159.9652
554	1281.0762	1519.9044	4169.8726
555	1283.8205	1523.0826	4179.7849
556	1286.5655	1526.2616	4189.7021
557	1289.3114	1529.4413	4199.6245
558	1292.0580	1532.6219	4209.5516
559	1294.8054	1535.8032	4219 4838
560	1297.5536	1538.9853	4229.4210
561	1300.3026	1542.1682	4239.3630
562	1303.0523	1545 3518	4249 3099
563	1305.8028	1548.5362	4259.2618
564	1308.5541	1551.7214	4269.2187
565	1311.3062	1554.9074	4279.1804
566	1314.0590	1558.0941	4289.1470
567	1316.8126	1561.2816	4299.1185
568	1319.5669	1564.4698	4309 0949
569	1322.3220	1567.6589	4319.0762
570	1325.0779	1570.8487	4329.0623
571	1327.8345	1574.0392	4339.0533
572	1330.5919	1577.2305	4349.0492
573	1333.3501	1580 4226	4359 0499
574	1336.1090	1583.6154	4369.0554
575	1338.8687	1586.8090	4379.0658
576	1341.6291	1590 0033	4389.0810
577	1344.3903	1593.1984	4399.1010
578	1347.1522	1596.3943	4409.1258
579	1349.9149	1599.5909	4419.1555
580	1352.6783	1602.7882	4429 1898
581	1355.4425	1605.9863	4439.2291
582	1358.2074	1609.1852	4449.2731
583	1360.9731	1612.3848	4459.3218
584	1363.7395	1615.5851	4469.3754
585	1366.5066	1618.7862	4479 4337
586	1369.2745	1621.9880	4489.4967
587	1372.0432	1625.1906	4499.5645
588	1374.8125	1628.3939	4509.6370
589	1377.5827	1631.5979	4519.7143
590	1380.3535	1634.8027	4529.7963
591	1383.1251	1638.0082	4539.8830
592	1385.8974	1641.2144	4549.9744
593	1388.6705	1644.4214	4560.0706
594	1391.4443	1647.6291	4570.1714
595	1394.2188	1650.8376	4580.2769
596	1396.9940	1654.0468	4590.3871

TABLE 5. (Continued)

n	log n!	n log n	n log² n	n	log n!	n log n	n log² n	n	log n!	n log n	n log² n	n	log n!	n log n	n log² n
597	1399.7700	1657.2567	4600.5020	654	1559.1662	1841.3878	5184.5706	711	1720.7210	2027.6793	5782.6769	768	1884.2611	2215 9574	6393.8376
598	1402.5467	1660.4673	4610.6215	655	1561.9824	1844.6380	5194.9459	712	1723.5735	2030.9657	5793.2892	769	1887.1470	2219.2773	6404.6710
599	1405.3241	1663.6787	4620.7457	656	1564.7993	1847.8889	5205.3254	713	1726.4265	2034.2528	5803.9055	770	1890.0335	2222.5978	6415.5081
600	1408.1023	1666.8907	4630.8746	657	1567.6169	1851.1404	5215.7092	714	1729.2802	2037.5405	5814.5257	771	1892.9205	2225.9189	6426.3489
601	1410.8811	1670.1035	4641.0081	658	1570.4351	1854.3926	5226.0973	715	1732.1346	2040 8288	5825.1500	772	1895.8082	2229.2405	6437 1935
602	1413.6608	1673.3171	4651.1463	659	1573.2540	1857.6455	5236.4897	716	1734.9895	2044.1177	5835.7783	773	1898.6963	2232.5627	6448.0419
603	1416.4411	1676.5313	4661.2891	660	1576.0735	1860.8990	5246.8865	717	1737.8450	2047.4072	5846 4105	774	1901.5851	2235.8855	6458.8940
604	1419.2221	1679.7463	4671.4365	661	1578.8938	1864.1532	5257.2875	718	1740.7011	2050.6973	5857.0468	775	1904.4744	2239.2088	6469 7498
605	1422.0039	1682.9620	4681.5886	662	1581.7146	1867.4080	5267.6927	719	1743.5578	2053.9881	5867 6870	776	1907.3642	2242.5327	6480.6094
606	1424.7863	1686.1784	4691.7452	663	1584.5361	1870.6635	5278.1022	720	1746.4152	2057.2794	5878.3312	777	1910.2547	2245.8571	6491.4727
607	1427.5695	1689.3955	4701.9065	664	1587.3583	1873.9196	5288.5161	721	1749.2731	2060.5713	5888.9794	778	1913.1456	2249.1821	6502.3396
608	1430.3534	1692.6134	4712.0724	665	1590.1811	1877.1764	5298.9341	722	1752.1316	2063.8638	5899.6316	779	1916.0372	2252.5077	6513.2103
609	1433.1380	1695.8319	4722.2429	666	1593 0046	1880.4338	5309.3564	723	1754.9908	2067.1570	5910.2877	780	1918 9293	2255.8338	6524.0847
610	1435.9234	1699.0512	4732.4180	667	1595.8287	1883.6919	5319.7830	724	1757.8505	2070 4507	5920.9478	781	1921.8219	2259.1604	6534.9628
611	1438.7094	1702.2712	4742.5977	668	1598.6535	1886.9507	5330.2138	725	1760.7109	2073.7450	5931 6118	782	1924.7151	2262 4877	6545.8446
612	1441.4962	1705.4919	4752.7819	669	1601.4789	1890.2101	5340.6489	726	1763.5718	2077.0400	5942.2797	783	1927.6089	2265.8154	6556 7301
613	1444.2836	1708.7133	4762.9707	670	1604.3050	1893.4701	5351.0881	727	1766.4333	2080.3355	5952.9517	784	1930.5032	2269.1438	6567.6193
614	1447.0718	1711.9354	4773.1641	671	1607.1317	1896.7308	5361.5317	728	1769.2955	2083.6316	5963 6275	785	1933.3981	2272.4727	6578.5122
615	1449.8607	1715.1582	4783.3620	672	1609.9591	1899.9921	5371.9794	729	1772.1582	2086.9283	5974 3073	786	1936.2935	2275.8021	6589.4088
616	1452.6503	1718.3817	4793.5645	673	1612 7871	1903.2541	5382.4313	730	1775.0215	2090.2257	5984.9910	787	1939.1895	2279.1321	6600 3090
617	1455.4405	1721.6059	4803.7715	674	1615.6158	1906.5168	5392.8875	731	1777.8854	2093 5236	5995 6786	788	1942.0860	2282.4626	6611.2129
618	1458.2315	1724.8309	4813.9831	675	1618.4451	1909.7800	5403.3478	732	1780 7499	2096.8221	6006.3701	789	1944.9831	2285.7937	6622.1205
619	1461.0232	1728.0565	4824.1992	676	1621.2750	1913.0440	5413.8124	733	1783.6150	2100.1212	6017 0656	790	1947 8807	2289.1254	6633.0317
620	1463.8156	1731.2828	4834.4198	677	1624 1056	1916.3085	5424.2812	734	1786.4807	2103.4209	6027.7650	791	1950.7789	2292.4576	6643.9467
621	1466.6087	1734.5099	4844.6450	678	1626 9368	1919.5737	5434.7542	735	1789.3470	2106.7212	6038.4683	792	1953.6776	2295.7903	6654.8652
622	1469.4025	1737.7376	4854.8746	679	1629.7687	1922.8396	5445.2313	736	1792.2139	2110 0221	6049 1754	793	1956.5769	2299.1236	6665.7875
623	1472.1970	1740.9660	4865.1088	680	1632.6012	1926.1060	5455.7125	737	1795.0814	2113.3235	6059 8865	794	1959.4767	2302.4575	6676.7134
624	1474.9922	1744.1952	4875.3475	681	1635 4344	1929.3732	5466.1981	738	1797.9494	2116.6256	6070.6015	795	1962.3771	2305.7918	6687 6429
625	1477.7880	1747.4250	4885.5906	682	1638.2681	1932.6409	5476.6877	739	1800.8181	2119.9282	6081 3203	796	1965.2780	2309.1268	6698 5761
626	1480.5846	1750.6555	4895.8383	683	1641.1026	1935.9093	5487.1815	740	1803.6873	2123.2314	6092.0430	797	1968.1794	2312.4623	6709.5129
627	1483.3819	1753.8867	4906.0905	684	1643 9376	1939.1784	5497.6794	741	1806.5571	2126.5353	6102.7697	798	1971.0814	2315.7983	6720.4534
628	1486.1798	1757.1187	4916.3470	685	1646.7733	1942.4480	5508.1816	742	1809.4275	2129 8397	6113.5002	799	1973.9840	2319.1349	6731.3975
629	1488.9785	1760.3512	4926.6082	686	1649 6096	1945.7183	5518.6878	743	1812.2985	2133 1447	6124.2345	800	1976.8871	2322.4720	6742.3452
630	1491.7778	1763.5845	4936.8737	687	1652.4466	1948.9893	5529.1982	744	1815.1701	2136 4503	6134.9727	801	1979.7907	2325.8096	6753.2965
631	1494.5779	1766.8185	4947.1437	688	1655 2842	1952.2608	5539.7128	745	1818.0422	2139.7564	6145 7140	802	1982.6949	2329.1478	6764.2515
632	1497.3786	1770.0532	4957.4182	689	1658.1224	1955.5330	5550.2314	746	1820.9150	2143.0631	6156 4608	803	1985.5996	2332.4866	6775.2100
633	1500.1800	1773.2885	4967.6971	690	1660.9612	1958.8059	5560.7542	747	1823.7883	2146 3705	6167.2105	804	1988.5049	2335.8258	6786.1722
634	1502.9821	1776.5246	4977.9804	691	1663.8007	1962.0793	5571.2811	748	1826 6622	2149 6784	6177.9642	805	1991.4106	2339.1657	6797.1380
635	1505.7849	1779.7613	4988.2682	692	1666.6408	1965.3534	5581.8122	749	1829.5367	2152.9869	6188 7216	806	1994.3170	2342.5060	6808.1074
636	1508.5883	1782.9987	4998.5604	693	1669.4816	1968.6281	5592.3474	750	1832 4117	2156.2959	6199.4829	807	1997.2239	2345.8469	6819.0804
637	1511.3924	1786.2368	5008.8571	694	1672.3229	1971.9035	5602.8866	751	1835.2874	2159.6056	6210.2480	808	2000.1313	2349.1884	6830.0569
638	1514.1973	1789.4756	5019.1581	695	1675.1649	1975.1794	5613.4299	752	1838.1636	2162 9158	6221 0170	809	2003.0392	2352.5303	6841.0371
639	1517.0028	1792.7150	5029.4636	696	1678.0075	1978.4560	5623.9774	753	1841.0404	2166 2266	6231.7898	810	2005.9477	2355.8729	6852.0209
640	1519.8089	1795.9552	5039.7734	697	1680.8507	1981.7332	5634.5289	754	1843.9178	2169 5380	6242 5664	811	2008.8567	2359.2159	6863.0082
641	1522.6158	1799.1960	5050 0877	698	1683.6946	1985.0111	5645.0845	755	1846.7957	2172 8499	6253 3469	812	2011.7663	2362 5595	6873.9992
642	1525.4233	1802.4375	5060.4064	699	1686 5391	1988.2895	5655.6442	756	1849.6742	2176 1625	6264 1311	813	2014.6764	2365 9036	6884.9937
643	1528.2316	1805.6796	5070.7294	700	1689.3842	1991.5686	5666.2079	757	1852.5533	2179.4756	6274 9191	814	2017.5870	2369 2483	6895.9918
644	1531.0404	1808.9225	5081.0568	701	1692 2299	1994.8483	5676.7758	758	1855.4330	2182 7892	6285.7110	815	2020.4982	2372 5934	6906 9935
645	1533.8500	1812.1660	5091.3886	702	1695.0762	1998.1286	5687.3477	759	1858.3132	2186.1035	6296 5066	816	2023.4099	2375 9391	6917.9987
646	1536.6602	1815.4102	5101.7248	703	1697.9232	2001.4096	5697.9236	760	1861.1941	2189.4183	6307.3060	817	2026.3221	2379.2854	6929.0074
647	1539.4711	1818.6551	5112.0653	704	1700.7708	2004.6911	5708.5037	761	1864.0754	2192.7337	6318.1093	818	2029.2348	2382.6322	6940 0198
648	1542 2827	1821.9006	5122.4102	705	1703 6189	2007.9733	5719.0878	762	1866.9574	2196.0497	6328.9163	819	2032.1481	2385 9795	6951 0357
649	1545.0950	1825.1468	5132.7594	706	1706.4678	2011.2561	5729.6758	763	1869.8399	2199.3662	6339.7271	820	2035.0619	2389.3273	6962.0551
650	1547.9079	1828.3937	5143.1130	707	1709.3172	2014.5395	5740.2680	764	1872.7230	2202.6833	6350 5416	821	2037 9763	2392.6757	6973.0781
651	1550.7215	1831.6412	5153.4709	708	1712.1672	2017.8235	5750.8642	765	1875.6067	2206 0010	6361 3600	822	2040.8911	2396.0246	6984 1047
652	1553 5357	1834.8894	5163.8331	709	1715.0179	2021.1082	5761.4644	766	1878.4909	2209.3192	6372.1821	823	2043.8065	2399.3741	6995 1347
653	1556.3506	1838.1383	5174.1997	710	1717.8691	2024.3934	5772.0686	767	1881.3757	2212.6380	6383 0079	824	2046.7225	2402.7240	7006 1683

TABLE 5. (Continued)

n	log n!	n log n	n log² n
825	2049 6389	2406 0745	7017.2054
826	2052.5559	2409.4255	7028.2462
827	2055.4734	2412.7770	7039.2903
828	2058.3914	2416.1291	7050.3380
829	2061.3100	2419.4817	7061.3893
830	2064.2291	2422.8348	7072.4440
831	2067 1487	2426 1884	7083 5023
832	2070 0688	2429.5426	7094.5640
833	2072 9894	2432.8973	7105 6293
834	2075.9106	2436.2525	7116 6980
835	2078 8323	2439 6082	7127.7703
836	2081 7545	2442.9644	7138.8460
837	2084 6772	2446.3212	7149 9252
838	2087 6005	2449.6785	7161 0079
839	2090.5242	2453.0363	7172 0942
840	2093 4485	2456.3946	7183.1838
841	2096 3733	2459.7534	7194.2770
842	2099 2986	2463.1128	7205 3736
843	2102 2244	2466.4726	7216.4736
844	2105 1508	2469.8330	7227.5772
845	2108.0776	2473.1939	7238 6842
846	2111 0050	2476.5553	7249 7946
847	2113 9329	2479 9172	7260.9086
848	2116 8613	2483.2797	7272 0259
849	2119 7902	2486 6426	7283 1467
850	2122 7196	2490 0061	7294.2709
851	2125.6495	2493.3700	7305.3986
852	2128 5800	2496 7345	7316.5297
853	2131.5109	2500.0995	7327.6642
854	2134.4424	2503.4650	7338.8022
855	2137 3744	2506 8310	7349 9436
856	2140 3068	2510.1975	7361.0884
857	2143.2398	2513.5645	7372.2366
858	2146 1733	2516.9321	7383 3882
859	2149.1073	2520.3001	7394.5433
860	2152.0418	2523.6686	7405 7018
861	2154 9768	2527.0377	7416.8636
862	2157 9123	2530 4073	7428 0289
863	2160 8483	2533.7773	7439.1975
864	2163.7848	2537.1479	7450.3696
865	2166 7218	2540.5189	7461.5450
866	2169 6594	2543.8905	7472 7238
867	2172 5974	2547.2625	7483 9060
868	2175 5359	2550.6351	7495 0916
869	2178.4749	2554 0082	7506.2805
870	2181.4144	2557.3817	7517.4728
871	2184 3544	2560.7558	7528.6686
872	2187.2950	2564.1304	7539.8676
873	2190 2360	2567 5054	7551.0700
874	2193 1775	2570 8810	7562.2758
875	2196 1195	2574 2570	7573.4849
876	2199.0620	2577.6336	7584.6974
877	2202.0050	2581 0106	7595.9133
878	2204.9485	2584 3882	7607.1324
879	2207 8925	2587 7662	7618.3549
880	2210 8370	2591 1447	7629.5808
881	2213.7820	2594 5238	7640 8100

n	log n!	n log n	n log² n
882	2216.7274	2597.9033	7652.0425
883	2219.6734	2601 2833	7663.2784
884	2222 6198	2604 6638	7674.5175
885	2225 5668	2608.0448	7685.7600
886	2228 5142	2611 4263	7697.0059
887	2231 4621	2614 8082	7708.2549
888	2234.4105	2618 1907	7719.5074
889	2237 3594	2621 5736	7730 7632
890	2240 3088	2624 9571	7742.0222
891	2243 2587	2628 3410	7753.2846
892	2246 2091	2631.7254	7764.5502
893	2249 1599	2635 1104	7775.8192
894	2252 1113	2638 4957	7787.0914
895	2255 0631	2641 8816	7798.3670
896	2258 0154	2645 2680	7809.6458
897	2260 9682	2648 6548	7820 9279
898	2263 9214	2652 0421	7832.2133
399	2266 8752	2655.4300	7843.5020
900	2269 8295	2658 8182	7854 7939
901	2272 7842	2662 2070	7866 0891
902	2275 7394	2665 5963	7877.3876
903	2278 6951	2668 9860	7888 6893
904	2281 6512	2672 3763	7899.9943
905	2284 6079	2675 7669	7911.3026
906	2287 5650	2679 1581	7922 6141
907	2290 5226	2682 5498	7933 9288
908	2293 4807	2685 9419	7945 2468
909	2296 4393	2689 3346	7956 5681
910	2299 3983	2692 7277	7967.8926
911	2302 3578	2696 1212	7979 2203
912	2305 3178	2699 5153	7990.5513
913	2308 2783	2702 9098	8001 8855
914	2311 2393	2706 3048	8013 2230
915	2314 2007	2709 7003	8024 5636
916	2317 1626	2713 0963	8035 9075
917	2320 1249	2716 4927	8047.2546
918	2323 0878	2719 8896	8058 6049
919	2326 0511	2723 2869	8069 9584
920	2329 0149	2726 6848	8081.3152
921	2331 9792	2730 0831	8092 6752
922	2334 9439	2733 4819	8104 0383
923	2337 9091	2736 8812	8115 4047
924	2340 8748	2740.2809	8126.7742
925	2343 8409	2743 6811	8138.1470
926	2346 8075	2747 0818	8149.5229
927	2349 7746	2750 4829	8160 9021
928	2352.7421	2753.8845	8172 2844
929	2355 7101	2757 2866	8183.6699
930	2358.6786	2760 6891	8195 0586
931	2361 6476	2764 0921	8206 4504
932	2364 6170	2767.4956	8217.8455
933	2367 5869	2770 8996	8229.2438
934	2370 5572	2774 3040	8240.6451
935	2373 5280	2777 7088	8252 0497
936	2376 4993	2781 1142	8263 4574
937	2379.4710	2784 5200	8274.8683
938	2382 4433	2787.9262	8286 2823

n	log n!	n log n	n log² n
939	2385.4159	2791.3330	8297 6995
940	2388.3890	2794 7402	8309 1199
941	2391.3626	2798 1478	8320 5433
942	2394.3367	2801.5559	8331 9700
943	2397 3112	2804 9645	8343 3998
944	2400.2862	2808 3735	8354 8326
945	2403.2616	2811 7831	8366 2687
946	2406 2375	2815 1930	8377.7079
947	2409.2138	2818.6034	8389.1503
948	2412.1906	2822 0143	8400 5957
949	2415.1679	2825.4256	8412 0442
950	2418 1456	2828.8374	8423 4960
951	2421.1238	2832.2497	8434.9507
952	2424.1024	2835 6624	8446 4087
953	2427.0815	2839.0755	8457 8698
954	2430 0611	2842 4891	8469 3339
955	2433 0411	2845.9032	8480 8011
956	2436 0216	2849 3177	8492.2715
957	2439 0025	2852 7327	8503.7450
958	2441 9838	2856 1481	8515.2216
959	2444 9657	2859 5640	8526 7012
960	2447.9479	2862 9804	8538 1840
961	2450.9307	2866 3972	8549.6698
962	2453 9138	2869 8144	8561.1588
963	2456 8975	2873 2321	8572.6508
964	2459 8815	2876 6502	8584 1459
965	2462 8661	2880 0688	8595 6442
966	2465 8510	2883.4879	8607.1454
967	2468 8365	2886 9074	8618 6498
968	2471.8223	2890.3273	8630 1571
969	2474 8087	2893 7477	8641.6676
970	2477.7954	2897.1686	8653 1812
971	2480 7827	2900 5898	8664 6973
972	2483.7703	2904 0116	8676 2174
973	2486 7584	2907 4338	8687 7402
974	2489.7470	2910 8564	8699 2660
975	2492.7360	2914 2795	8710 7948
976	2495 7254	2917 7030	8722.3267
977	2498 7153	2921 1270	8733 8617
978	2501 7057	2924.5514	8745 3997
979	2504.6965	2927 9762	8756 9407
980	2507 6877	2931.4016	8768 4847
981	2510.6794	2934.8273	8780 0319
982	2513.6715	2938 2535	8791 5819
983	2516 6640	2941 6801	8803 1351
984	2519.6570	2945 1071	8814 6913
985	2522 6505	2948 5347	8826 2505
986	2525.6443	2951.9626	8837 8127
987	2528 6386	2955.3910	8849 3781
988	2531.6334	2958.8199	8860 9463
989	2534.6286	2962 2491	8872.5176
990	2537.6242	2965.6788	8884.0918
991	2540.6203	2969.1090	8895.6692
992	2543.6168	2972 5396	8907 2495
993	2546.6138	2975.9706	8918 8328
994	2549.6111	2979.4020	8930 4191

n	log n!	n log n	n log² n
995	2552 6090	2982.8340	8942.0084
996	2555.6072	2986.2663	8953 6007
997	2558 6059	2989.6991	8965 1960
998	2561 6051	2993.1323	8976 7944
999	2564 6046	2996.5659	8988.3956
1000	2567 6046	3000 0000	9000.0000
1001	2570 6051	3003 4345	9011.6072
1002	2573 6059	3006.8694	9023 2174
1003	2576.6072	3010 3048	9034.8307
1004	2579.6090	3013 7406	9046.4469
1005	2582 6111	3017.1769	9058 0660
1006	2585 6137	3020.6136	9069 6881
1007	2588.6168	3024 0507	9081.3132
1008	2591.6202	3027.4882	9092.9413
1009	2594 6241	3030.9262	9104.5724
1010	2597.6284	3034 3646	9116 2063
1011	2600 6332	3037.8034	9127.8433
1012	2603 6384	3041 2427	9139.4832
1013	2606 6440	3044 6823	9151.1260
1014	2609 6500	3048 1225	9162.7719
1015	2612 6565	3051.5630	9174 4205
1016	2615 6634	3055 0040	9186 0723
1017	2618.6707	3058 4454	9197.7269
1018	2621.6784	3061 8872	9209 3845
1019	2624.6866	3065 3295	9221.0450
1020	2627.6952	3068.7722	9232 7084
1021	2630.7043	3072.2153	9244.3749
1022	2633.7137	3075.6588	9256.0441
1023	2636 7236	3079.1028	9267.7163
1024	2639 7339	3082.5471	9279 3915
1025	2642.7446	3085 9919	9291 0696
1026	2645 7557	3089 4372	9302.7506
1027	2648 7673	3092.8828	9314 4346
1028	2651.7793	3096 3289	9326 1213
1029	2654.7917	3099 7754	9337 8110
1030	2657.8046	3103 2223	9349.5038
1031	2660 8178	3106 6697	9361 1993
1032	2663.8315	3110 1174	9372.8977
1033	2666.8456	3113.5656	9384 5991
1034	2669.8601	3117.0142	9396 3033
1035	2672 8751	3120.4633	9408 0105
1036	2675 8904	3123 9127	9419 7206
1037	2678 9062	3127 3625	9431.4336
1038	2681 9224	3130.8128	9443 1494
1039	2684 9390	3134 2635	9454.8682
1040	2687 9560	3137.7147	9466 5897
1041	2690.9735	3141.1662	9478.3142
1042	2693 9914	3144 6181	9490 0416
1043	2697 0096	3148.0705	9501.7719
1044	2700.0284	3151 5233	9513 5050
1045	2703.0475	3154 9765	9525.2410
1046	2706 0670	3158 4301	9536.9799
1047	2709.0869	3161.8842	9548 7216
1048	2712.1073	3165.3386	9560 4662
1049	2715.1281	3168.7935	9572 2136
1050	2718.1493	3172.2487	9583 9640

TABLE 6. THE DIVERSITY OF SPECIES, $\bar{d}$, CHARACTERISTIC OF MacARTHUR'S MODEL FOR VARIOUS NUMBERS OF HYPOTHETICAL SPECIES, s'*

s'	$\bar{d}$	s'	$\bar{d}$	s'	$\bar{d}$	s'	$\bar{d}$
1	0.0000	51	5.0941	102	6.0792	205	7.0783
2	0.8113	52	5.1215	104	6.1069	210	7.1128
3	1.2997	53	5.1485	106	6.1341	215	7.1466
4	1.6556	54	5.1749	108	6.1608	220	7.1796
5	1.9374	55	5.2009	110	6.1870	225	7.2118
6	2.1712	56	5.2264	112	6.2128	230	7.2434
7	2.3714	57	5.2515	114	6.2380	235	7.2743
8	2.5465	58	5.2761	116	6.2629	240	7.3045
9	2.7022	59	5.3004	118	6.2873	245	7.3341
10	2.8425	60	5.3242	120	6.3113	250	7.3631
11	2.9701	61	5.3476	122	6.3350	255	7.3915
12	3.0872	62	5.3707	124	6.3582	260	7.4194
13	3.1954	63	5.3934	126	6.3811	265	7.4468
14	3.2960	64	5.4157	128	6.4036	270	7.4736
15	3.3899	65	5.4378	130	6.4258	275	7.5000
16	3.4780	66	5.4594	132	6.4476	280	7.5259
17	3.5611	67	5.4808	134	6.4691	285	7.5513
18	3.6395	68	5.5018	136	6.4903	290	7.5763
19	3.7139	69	5.5226	138	6.5112	295	7.6008
20	3.7846	70	5.5430	140	6.5318	300	7.6250
21	3.8520	71	5.5632	142	6.5521	310	7.6721
22	3.9163	72	5.5830	144	6.5721	320	7.7177
23	3.9779	73	5.6027	146	6.5919	330	7.7620
24	4.0369	74	5.6220	148	6.6114	340	7.8049
25	4.0937	75	5.6411	150	6.6306	350	7.8465
26	4.1482	76	5.6599	152	6.6495	360	7.8870
27	4.2008	77	5.6785	154	6.6683	370	7.9264
28	4.2515	78	5.6969	156	6.6867	380	7.9648
29	4.3004	79	5.7150	158	6.7050	390	8.0022
30	4.3478	80	5.7329	160	6.7230	400	8.0386
31	4.3936	81	5.7506	162	6.7408	410	8.0741
32	4.4381	82	5.7681	164	6.7584	420	8.1087
33	4.4812	83	5.7853	166	6.7757	430	8.1426
34	4.5230	84	5.8024	168	6.7929	440	8.1757
35	4.5637	85	5.8192	170	6.8099	450	8.2080
36	4.6032	86	5.8359	172	6.8266	460	8.2396
37	4.6417	87	5.8524	174	6.8432	470	8.2706
38	4.6792	88	5.8687	176	6.8596	480	8.3009
39	4.7157	89	5.8848	178	6.8758	490	8.3305
40	4.7513	90	5.9007	180	6.8918	500	8.3596
41	4.7861	91	5.9164	182	6.9076	550	8.4968
42	4.8200	92	5.9320	184	6.9233	600	8.6220
43	4.8532	93	5.9474	186	6.9388	650	8.7373
44	4.8856	94	5.9627	188	6.9541	700	8.8440
45	4.9173	95	5.9778	190	6.9693	750	8.9434
46	4.9483	96	5.9927	192	6.9843	800	9.0363
47	4.9787	97	6.0075	194	6.9992	850	9.1236
48	5.0084	98	6.0221	196	7.0139	900	9.2060
49	5.0375	99	6.0366	198	7.0284	950	9.2839
50	5.0661	100	6.0510	200	7.0429	1000	9.3578

*The data in this table are reproduced, with permission, from Lloyd and Ghelardi, Reference 33.

TABLE 7. CLASSIFICATION, BY VARIOUS AUTHORS, OF THE TOLERANCE OF VARIOUS MACROINVERTEBRATE TAXA TO DECOMPOSABLE ORGANIC WASTES; TOLERANT (T), FACULTATIVE (F), AND INTOLERANT (I)

Macroinvertebrate	T	F	I
Porifera			
Demospongiae			
Monaxonida			
Spongillidae			42*
Spongilla fragilis		48	
Bryozoa			
Ectoprocta			
Phylactolaemata			
Plumatellidae			
Plumatella repens		51	
P. princeps var. *mucosa*	48		
P. p. var. *mucosa spongiosa*		48	
P. p. var. *fruticosa*	48		
P. polymorpha var. *repens*			48
Cristatellidae			
Cristatella mucedo		51	
Lophopodidae			
Lophopodella carteri			42
Pectinatella magnifica			48,42
Endoprocta			
Urnatellidae			
Urnatella gracilis		48,42	
Gymnolaemata			
Ctenostomata			
Paludicellidae			
Paludicella ehrenbergi		48	
Coelenterata			
Hydrozoa			
Hydroida			
Hydridae			
Hydra		42	
Clavidae			
Cordylophora lacustris		42	
Platyhelminthes			
Turbellaria		42	
Tricladida			
Planariidae			
Planaria		48	
Nematoda		42	
Nematomorpha			
Gordioida			
Gordiidae		48	
Annelida			
Oligochaeta	5,4	48	
Plesiopora			
Naididae		48	
Nais		42	
Dero		48	
Ophidonais	60		
Stylaria		42	
Tubificidae			
Tubifex tubifex	48,42		
Tubifex	48,18,60		
Limnodrilus hoffmeisteri	48,3,42		
L. claparedianus	48		
Limnodrilus	48,18,60		
Branchiura sowerbyi	42		
Prosopora			
Lumbriculidae	60		
Hirudinea			
Rhynchobdellida			
Glossiphoniidae			
Glossiphonia complanata	48		
Helobdella stagnalis	48,42		
H. nepheloidea	48		
Placobdella montifera	60		
P. rugosa		48	
Placobdella		42	
Piscicolidae			
Piscicola punctata		60	
Gnathobdellida			
Hirudidae			
Macrobdella	28		
Pharyngobdellida			
Erpobdellidae			
Erpobdella punctata	48		
Dina parva	48		
D. microstoma	48		
Dina		42	
Mooreobdella microstoma	42		
Hydracarina			5
Arthropoda			
Crustacea			
Isopoda			
Asellidae			
Asellus intermedius		48	
Asellus	60	42	5,4
Lirceus		42	
Amphipoda		4	
Talitridae			
Hyallela azteca		5,3,4,42	
H. knickerbockeri	48		
Gammaridae			
Gammarus		42	
Crangonyx pseudogracilis		42	
Decapoda			
Palaemonidae			
Palaemonetes paludosus		5,3,4	
P. exilipes	48		
Astacidae			
Cambarus striatus	25		
C. fodiens	1		
C. bartoni bartoni		1	1
C. b. cavatus		1	
C. conasaugaensis			1
C. asperimanus			1
C. latimanus		1	
C. acuminatus			1
C. hiwassensis			1
C. extraneus			1
C. diogenes diogenes	1		
C. cryptodytes†			1

*Numbers refer to references enumerated in the "Literature" section immediately following this table.

†Albinistic

TABLE 7. (Continued)

Macroinvertebrate	T	F	I
C. floridanus		1	
C. carolinus‡	1		
C. longulus longirostris			1
Procambarus raneyi			1
P. acutus acutus	1		
P. paeninsulanus		1	
P. spiculifer			1
P. versutus			1
P. pubescens		1	
P. litosternum		1	
P. enoplosternum		1	
P. angustatus		1	
P. seminolae		1	
P. truculentus‡	1		
P. advena‡	1		
P. pygmaeus‡	1		
P. pubischelae		1	
P. barbatus		1	
P. howellae		1	
P. troglodytes	1		
P. epicyrtus		1	
P. fallax	1		
P. chacei		1	
P. lunzi		1	
Orconectes propinquus		42	
O. rusticus		42	
O. juvenilis			1
O. erichsonianus		1	
Faxonella clypeata		1	
Insecta			
Diptera			
Chironomidae			
Pentaneura inculta		60	3,4
P. carneosa		60,44	60,12
P. flavifrons	5		
P. melanops	44,12		
P. americana			44,12
Pentaneura			42,44
Ablabesmyia janta		3,4, 42	
A. americana		48,60	5
A. illinoense	12	44	
A. mallochi		42	4
A. ornata			4
A. aspera			4
A. peleensis		4	
A. auriensis			4
A. rhamphe		42	
Ablabesmyia			42
Procladius culiciformis	60	44,12	
P. denticulatus	42		
Procladius	12	4,44, 12	
Labrundinia floridana			4
L. pilosella			42
L. virescens			4
Guttipelopia		42	
Conchapelopia		42	
Coelotanypus scapularis		42	
C. concinnus	42	48,60, 44,12	44
Psilotanypus bellus	42		
Tanypus stellatus	44,12	18,60	5
T. carinatus		42	
T. punctipennis		44,12	
Tanypus		44,12	
Psectrotanypus dyari	44,12	48	
Psectrotanypus		44	
Larsia lurida		4	
Clinotanypus caliginosus			44,12
Clinotanypus		4	
Orthocladius obumbratus			60
Orthocladius		5,48	60,42, 44,12
Nanocladius			4,42
Psectrocladius niger		42	
P. julia		42	
Psectrocladius			4,44
Metriocnemus lundbecki			4
Cricotopus bicinctus			3,4, 44,12
C. bicinctus group	42		
C. exilis		44	12
C. exilis group		42	
C. trifasciatus		44	12
C. trifasciatus group		42	
C. politus			44,12
C. tricinctus		44	12
C. absurdus			18,44, 12
Cricotopus			44
Corynoneura taris			4
C. scutellata			44,12
Corynoneura			5,42, 12
Thienemanniella xena			4,42
Thienemanniella			4,44
Trichocladius robacki			3,4
Brillia par			4
Diamesa nivoriunda			18,42, 44
Diamesa			60
Prodiamesa olivacea			12
Chironomus attenuatus group	5,4, 42,12		44
C. riparius	18,44, 12		
C. riparius group	42		
C. tentans			12
C. tentans-plumosus	60		
C. plumosus	48,18, 60		48,12
C. plumosus group	42		
C. carus	4		
C. crassicaudatus	4		
C. stigmaterus	4		
C. flavus		60	
C. equisitus		60	
C. fulvipilus	4		
C. anthracinus			12
C. paganus			12
C. staegeri		12	

‡Not usually inhabitant of open water; are burrowers.

TABLE 7. (Continued)

Macroinvertebrate	T	F	I
Chironomus	5	60	
Kiefferullus dux	4		44,12
Cryptochironomus fulvus	3,4		44,12
C. fulvus group		42	
C. digitatus		48	12
C. sp. B (Joh.)			5
C. blarina		42	12
C. psittacinus			60
C. nais		42	
Cryptochironomus	5		
Chaetolabis atroviridis			12
C. ochreatus			12
Endochironomus nigricans		4,42	44,12
Stenochironomus macateei			42,44
S. hilaris			3,4
Stictochironomus devinctus			4,12
S. varius			44
Xenochironomus xenolabis			42
X. rogersi		42	
X. scopula			44,12
Pseudochironomus richardson			44,12
Pseudochironomus			12
Parachironomus abortivus group		42	
P. pectinatellae		42	
Cryptotendipes emorsus		42	
Microtendipes pedellus			44,12
Microtendipes			12
Paratendipes albimanus			44,12
Tribelos jucundus			12
T. fuscicornis			42
Harnischia collator		42	
H. tenuicaudata			44
Phaenopsectra			42
Dicrotendipes modestus		42	
D. neomodestus		44	42,12
D. nervosus		42	12
D. incurvus	42		
D. fumidus			42,12
Glyptotendipes senilis			42
G. paripes	4		12
G. meridionalis		42	
G. lobiferus	48,4, 42		44,12
G. barbipes	42		
G. amplus		42	
Glyptotendipes	12		
Polypedilum halterale		42	4,12
P. fallax		5,44, 12	4
P. scalaenum	4	42	
P. illinoense		3,4, 42,44	44,12
P. tritum		42	
P. simulans		42	12
P nubeculosum			12
P. vibex			44
Polypedilum		48,44	12
Tanytarsus neoflavellus		44,12	18
T. gracilentus			12
T. dissimilis			42
Rheotanytarsus exiguus	5		3,4
Rheotanytarsus		42	

Macroinvertebrate	T	F	I
Cladotanytarsus		42	
Micropsectra dives		60	12
M deflecta			42
M. nigripula			44,12
Calopsectra gregarius	5		
Calopsectra			44,12
Stempellina johannseni		44	12
Culicidae	4		
Culex pipiens	18,44		
Anopheles punctipennis			44
Chaoboridae			
Chaoborus punctipennis		60,42	44
Ceratopogonidae	5,4	42	
Palpomyia tibialis		60	
Palpomyia		48,60	
Bezzia glabra	44		
Stilobezzia antenalis	44		
Tipulidae	4	42	
Tipula caloptera			44
T. abdominalis			44
Pseudolimnophila luteipennis			44
Hexatoma			44
Eriocera		60	
Psychodidae	4		
Psychoda alternata	44		
P. schizura	44		
Psychoda	42		
Telmatoscopus albipunctatus	60		
Telmatoscopus			44
Simuliidae	42	44	5,4
Simulium vittatum		18,44	
S. venustrum			44
Simulium			3
Prosimulium johannseni			44
Cnephia pecuarum			44
Stratiomyidae	4		
Stratiomys discalis	44		
S. meigeni	44		
Odontomyia cincta		44	
Tabanidae	4		
Tabanus atratus	18	44	
T. stygius		44	
T. benedictus	44		
T. giganteus			44
T. lineola	44		
T. variegatus			44
Tabanus			44
Syrphidae	4		
Syrphus americanus	44		
Eristalis bastardi	18,44		
E. aenaus	44		
E. brousi	44		
Eristalis	44		
Empididae		42	
Ephydridae			
Brachydeutera argentata	44		
Anthomyiidae		42	
Lepidoptera			
Pyralididae		5,4	
Trichoptera			
Hydropsychidae			
Hydropsyche orris		42	

TABLE 7. (Continued)

Macroinvertebrate	T	F	I
H. bifida group		42	
H. simulans			42
H. frisoni			42
H. incommoda		48	5,3,4
Hydropsyche			5,4
Cheumatopsyche		5,18, 3,4, 42	
Macronemum carolina			5,3,4
Macronemum			42
Potamyia flava		42	
Psychomyiidae			
Psychomyia			42
Neureclipsis crepuseularis			42
Polycentropus		42	5,48, 4
Cyrnellus fraternus		42	
Oxyethira			5,4
Rhyacophilidae			
Rhyacophila			48
Hydroptilidae			
Hydroptila waubesiana			42
Hydroptila			5,3,4
Ochrotrichia			42
Agraylea			42
Leptoceridae			48
Leptocella		5,4	42
Athripsodes			42
Oecetis		5,4	
Philopotamidae			
Chimarra perigua			3,4
Chimarra			5,4
Brachycentridae			
Brachycentrus			4
Molannidae			48
Ephemeroptera			
Heptageniidae			
Stenonema integrum		32,42	
S. rubromaculatum			32
S. fuscum			32
S. pulchellum		32	
S. ares		32	
S. scitulum		42	
S. femoratum		18,42	32
S. terminatum			42
S. interpunctatum			32,42
S. i. ohioense			32
S. i. canadense			32
S. i. heterotarsale		32	
S. exiguum			5,3,4
S. smithae			5,3,4
S. proximum			3
S. tripunctatum			32
Stenonema			32
Hexageniidae			
Hexagenia limbata			42
H. bilineata		60	48
Pentagenia vittgera			42
Baetidae			
Baetis vagans			42
Callibaetis floridanus	4		
Callibaetis		18	

Macroinvertebrate	T	F	I
Caenidae			
Caenis diminuta	4		
Caenis		42	48
Tricorythidae		42	
Siphlonuridae			
Isonychia			42
Plecoptera			5,4
Perlidae			
Perlesta placida		18	3
Acroneuria abnormis		42	
A. arida			42
Nemouridae			
Taeniopteryx nivalis			42
Allocapnia viviparia		18	
Perlodidae			
Isoperla bilineata			42
Neuroptera			
Sisyridae			
Climacia areolaris			42
Megaloptera			
Corydalidae			
Corydalis cornutus		42	5,3,4
Sialidae			
Sialis infumata			48
Sialis		42	
Odonata			
Calopterygidae			
Hetaerina titia			4
Agrionidae			
Argia apicalis		42	
A. translata		42	
Argia			5,4
Ischnura verticalis	48	42	
Enallagma antennatum		42	
E. signatum		42	48
Aeshnidae			
Anax junius			48
Gomphidae			
Gomphus pallidus		5,3,4	
G. plagiatus			48
G. externus			48
G. spiniceps		42	
G. vastus		42	
Gomphus		5,4	
Progomphus			5,4
Dromogomphus		42	
Erpetogomphus		42	
Libellulidae			
Libellula lydia		18	
Neurocordulia moesta		42	
Plathemis		42	
Macromia		5,42	4
Hemiptera	4		
Corixidae		42	
Corixa	18		
Hesperocorixa	18		
Gerridae			
Gerris	18		
Belostomatidae			
Belostoma	18,3		
Hydrometridae			
Hydrometra martini	3		

TABLE 7. (Continued)

Macroinvertebrate	T	F	I
Coleoptera	4§		
Elmidae			
Stenelmis crenata			18,50
S. sexlineata		42,50	18
S. decorata	50		
Dubiraphia		42,50	
Promoresia			50
Optioservus		50	
Macronychus glabratus			50
Anacyronyx variegatus			50
Microcylloepus pusillus			50
Gonielmis dietrichi		50	
Hydrophilidae			
Berosus	42		
Tropisternus natator	18		
T. lateralis	3		
T. dorsalis			48
Dytiscidae			
Laccophilus maculosus	18		
Gyrinidae			
Gyrinus floridanus	3		
Dineutus americanus	18		
Dineutus		42	
Mollusca			
Gastropoda			
Mesogastropoda			
Valvatidae			
Valvata tricarinata		28	48,28
V. piscinalis		28	
V. bicarinata			48
V. b. var. *normalis*			48
Viviparidae			
Vivaparus contectoides			48
V. subpurpurea			48
Campeloma integrum		28	
C. rufum		28	
C. contectus		28	
C. fasciatus		28	
C. decisum			28
C. subsolidum		48,28	
Campeloma		60	
Lioplax subcarinatus			48
Pleuroceridae			
Pleurocera acuta		48,28	
P. elevatum		28	
P. e. lewisi		28	
Pleurocera		28	
Goniobasis livescens		48,28	
G. virginica	28		
Goniobasis		28	5,4
Anculosa		28	
Bulimidae			
Bulimus tentaculatus		28	
Amnicola emarginata			48
A. limosa			48
Somatogyrus subglobosus			48
Basommatophora			
Physidae			
Physa integra	18,28	28	
P. heterostropha	28	28	
P. gyrina		28	
P. acuta		28	28
P. fontinalis		28	28
P. anatina	28		
P. halei	28		
P. cubensis	28		
P. pumilia	3		
Physa	5,4		
Aplexa hypnorum		28	28
Lymnaeidae			
Lymnaea ovata	28		
L. peregra		28	
L. caperata		28	
L. humilis		28	
L. obrussa		28	
L. polustris		28	28
L. auricularia		28	
L. stagnalis		28	28
L. s. appressa			28
Lymnaea	4	42	
Pseudosuccinea columella		28	
Galba catascopium	28		
Fossaria modicella	28		
Planorbidae			
Planorbis carinatus			28
P. trivolvis	28		
P. panus	28		
P. corneus		28	28
P. marginatus			28
Planorbis		28	
Segmentina armigera	28		
Helisoma anceps		28	
H. trivolvis		28	
Helisoma	3,4		
Gyraulus arcticus		28	
Gyraulus		28	
Ancylidae			
Ancylus lacustris		28	28
A. fluviatilis		28	28
Ferrissia fusca		28	
F. tarda		28	
F. rivularis			28
Ferrissia	5,3,4	42	
Bivalvia			
Eulamellibranchia			
Margaritiferidae			
Margaritifera margaritifera			28
Unionidae			
Unio complanata	28		
U. gibbosus	28	28	
U. batavus			28
U. pictorum			28
U. tumidus		28	
Lampsilis luteola		28	
L. alata		28	
L. anadontoides		28	
L. gracilis		48	
L. parvus			48
Lampsilis		48,42	
Quadrula pustulosa		28,42	

§Except riffle bettles

TABLE 7. (Continued)

Macroinvertebrate	T	F	I
Q. undulata		28	
Q. rubiginosa		28	
Q. lachrymosa		28	
Q. plicata		28	
Truncilla donaciformis			48
T. elegans			48
Tritigonia tuberculata		28	
Symphynota costata		28	
Strophitus edentulus		28	
Anodonta grandis		28,42	
A. imbecillis		48,28	
A. mutabilis			28
Alasmodonta costata		28	
Proptera alata			42
Leptodea fragilis			42
Amblema undulata		28	
Lasmigona complanata		28	
Obliquaria reflexa			60
Heterodonta			
Corbiculidae			
Corbicula manilensis			42
Sphaeriidae	5,4		
Sphaerium notatum	28		
S. corneum		28	
S. rhomboideum		28	
S. striatinum		28	
S. s. var. *corpulentum*		48	
S. s. var. *lilycashense*		48	
S. sulcatum		28	
S. stamineum		48,28	
S. moenanum		28	28
S. vivicolum		28	28
S. solidulum			28
Sphaerium		42	
Musculium securis		28	
M. transversum	48,28	28	
M. truncatum	48	28	
Musculium	60		
Pisidium abditum	28		
P. fossarinum			28
P. pauperculum crystalense		48,28	
P. amnicum		28	28
P. casertanum			
P. compressum	48	28	
P. fallax		28	
P. henslorvanum		28	
P. idahoensis	28		
P. complanatum	48,28	48,28	
P. subtruncatum		28	
Pisidium		48	
Dresisseniidae			
Mytilopsis leucophaeatus		28	
Mactridae			
Rangia cuneata		28	

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FISH

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1.0 INTRODUCTION

To the public, the condition of the fishery is the most meaningful index of water quality. Fish occupy the upper levels of the aquatic food web and are directly and indirectly affected by chemical and physical changes in the environment. Water quality conditions that significantly affect the lower levels of the food web will affect the abundance, species composition, and condition of the fish population. In some cases, however, the fish are more sensitive to the pollutant(s) than are the lower animals and plants; they may be adversely affected even when the lower levels of the food web are relatively unharmed.

Many species of fish have stringent dissolved oxygen and temperature requirements and are intolerant of chemical and physical contaminants resulting from agricultural, industrial, and mining operations. The discharge of moderate amounts of degradable organic wastes may increase the nutrient levels in the habitat and result in an increase in the standing crop of fish. This increase, however, usually occurs in only one or a few species and results in an imbalance in the population. The effects of toxic wastes may range from the elimination of all fish to a slight reduction in reproductive capacity, growth, or resistance to disease and parasitism.

Massive and complete fish kills are dramatic signs of abrupt, adverse changes in environmental conditions. Fish, however, can repopulate an area rapidly if the niche is not destroyed, and the cause of the kill may be difficult to detect by examination of the fish community after it has recovered from the effects of the pollutant. Chronic pollution, on the other hand, is more selective in its effects and exerts its influence over a long period of time and causes recognizable changes in the species composition and relative abundance of the fish.

The principal characteristics of interest in field studies of fish populations include: (1) species present, (2) relative and absolute abundance of each species, (3) size distribution, (4) growth rate, (5) condition, (6) success of reproduction, (7) incidence of disease and parasitism, and (8) palatability. Observations of fish behavior can also be valuable in detecting environmental problems; e.g. ventilation rates, position in the current, and erratic movement. Fish may also be collected for use in laboratory bioassays, for tissue analyses to measure the concentrations of metals and pesticides, and for histopathologic examination.

Fisheries data have some serious limitations. Even if the species composition of the fish in a specific area were known before and after the discharge of pollutants, the real significance of changes in the catch could not be properly interpreted unless the life histories of the affected species were understood, especially the spawning, seasonal migration, temperature gradient and stream-flow responses, diurnal movements, habitat preferences, and activity patterns. Without this knowledge, fish presence or absence cannot be correlated with water quality. Of course, any existing data on the water quality requirements of fish would be of great value in interpreting field data.

Fisheries data have been found useful in enforcement cases and in long-term water quality monitoring (Tebo, 1965). Fishery surveys are costly, however, and a careful and exhaustive search should be conducted for existing information on the fisheries of the area in question before initiating a field study. State and Federal fishery agencies and universities are potential sources of information which, if available, may save time and expense. Most states require a collecting permit, and the state fishery agency must usually be contacted before fish can be taken in a field study. If data are not available and a field study must be conducted, other Federal and State agencies will often join the survey and pool their resources because they have an interest in the data and have found that a joint effort is more economical and efficient.

2.0 SAMPLE COLLECTION

2.1 General Considerations

Fish can be collected actively or passively. Active sampling methods include the use of seines, trawls, electrofishing, chemicals, and hook and line. Passive methods involve entanglement (gill nets and trammel nets) and entrapment (hoop nets, traps, etc.) devices. The chief limitations in obtaining qualitative and quantitative data on a fish population are gear selectivity and the mobility and rapid recruitment of the fish. Gear selectivity refers to the greater success of a particular type of gear in collecting certain species, or sizes of fish, or both. All sampling gear is selective to some extent. Two factors that affect gear selectivity are: (1) the habitat or portion of habitat (niches) sampled and (2) the actual efficiency of the gear. A further problem is that the efficiency of gear for a particular species in one area does not necessarily apply to the same species in another area. Even if nonselective gear could be developed, the problem of adequately sampling an area is difficult because of the nonrandom distribution of fish populations.

Temporal changes in the relative abundance of a single species can be assessed under a given set of conditions if that species is readily taken with a particular kind of gear, but the data are not likely to reflect the true abundance of the species occurring in nature.

Passive collection methods are very selective and do not obtain representative samples of the total population. Active methods are less selective and more efficient, but usually require more equipment and manpower. Although the choice of method depends on the objectives of the particular fishery investigation, active methods are generally preferred.

2.2 Active Sampling Techniques

2.2.1 Seines

A haul seine is essentially a strip of strong netting hung between a stout cork or float line at the top and a strong, heavily-weighted lead line at the bottom (Figure 1). The wings of the net are often of larger mesh than the middle portion, and the wings may taper so that they are shallower on the ends. The center portion of the net may be formed into a bag to aid in confining the fish. At the ends of the wings, the cork and lead lines are often fastened to a short stout pole or brail. The hauling lines are then attached to the top and bottom of the brail by a short bridle.

Figure 1. The common haul seine. (From Dumont and Sundstrom, 1961.)

Deepwater seining usually requires a boat. One end of one of the hauling lines is anchored on shore and the boat pays out the line until it reaches the end. The boat then changes direction and lays out the net parallel to the beach. When all of the net is in the water, the boat brings the end of the second hauling line ashore. The net is then beached rapidly.

The straight seines (without bags), such as the common-sense minnow seines, can usually be handled quite easily by two people. The method of paying out the seine and bringing it in is similar to the haul seine, except the straight seine is generally used in shallow water where one member of the party can wade offshore with lines.

Bag and straight seines vary considerably in dimensions and mesh size. The length varies from 3 to 70 meters, and mesh size and net width vary with the size of the fish and the depth of the water to be sampled.

Nylon seines are recommended because of the ease of maintenance. Cotton seines should be treated with a fungicide to prevent decay.

Seining is not effective in deep water because the fish can escape over the floats and under the lead line. Nor is it effective in areas that have snags and sunken debris. Although the results are expressed as number of fish captured per unit area seined, quantitative seining is very difficult. The method is more useful in determining the variety rather than the number of fish inhabiting the water.

2.2.2 Trawls

Trawls are specialized submarine seines used in large, open-water areas of reservoirs, lakes, large rivers, estuaries, and in the oceans. They may be of considerable size and are towed by boats at speeds sufficient to overtake and enclose the fish. Three basic types are: (1) the beam trawl used to capture bottom fish (Figure 2), (2) the otter trawl used to capture near-bottom and bottom fish (Figure 3), and (3) the mid-water trawl used to collect schooling fish at various depths.

The beam trawls have a rigid opening and are difficult to operate from a small boat. Otter trawls have vanes or "otter boards," which are attached to the forward end of each wing and are used to keep the mouth of the net open while it is being towed. The otter boards are approximately rectangular and usually made of wood, with steel strapping. The lower edge is shod with a steel runner to protect the wood when the otter slides along the bottom. The leading edge of the otter is rounded near the bottom to aid in riding over obstructions.

The towing bridle or warp is attached to the board by four heavy chains or short heavy metal rods. The two forward rods are shorter so that, when towed, the board sheers to the outside and down. Thus, the two otters sheer in opposite directions and keep the mouth of the trawl open and on the bottom. Floats or corks along the headrope keep the net from sagging, and the weights on the lead-line keep the net on the bottom. The entrapped fish are funneled back into the bag of the trawl (cod end).

A popular small trawl consists of a 16- to 20-foot (5- to 6-m) headrope, semiballoon modified shrimp (otter) trawl with 3/4-inch (1.9 cm) bar mesh in the wings and cod end. A 1/4-inch (0.6 cm) bar mesh liner may be installed in the cod end if smaller fish are desired. This small trawl uses otter boards, the dimensions of which, in inches, are approximately 24 to 30 (61 to 76 cm) X 12 to 18 (30 to 46 cm) X 3/4 to 1-1/4 inches (0.9 to 3.2 cm), and the trawl can be operated out of a medium-sized boat.

The midwater trawl resembles an otter trawl with modified boards and vanes for controlling the trawling depth. Such trawls are cumbersome for freshwater and inshore areas.

Trawling data are usually expressed in weight of catch per unit of time.

The use of trawls requires experienced personnel. Boats deploying large trawls must be equipped with power winches and large motors. Also, trawls can not be used effectively if the bottom is irregular or harbors snags or other debris. Trawls are best used to gain information on a particular species of fish rather than to estimate the overall fish population. See Rounsefell and Everhart (1953), Massman, Ladd and McCutcheon (1952) and Trent (1967) for further information on trawls.

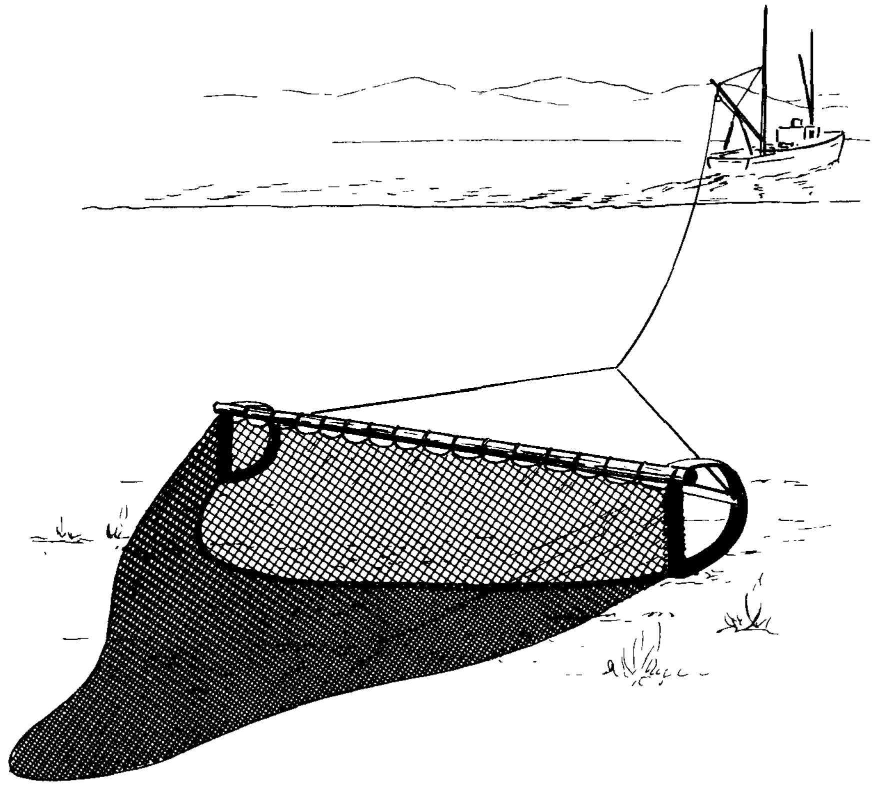

Figure 2. The beam trawl. (From Dumont and Sundstrom, 1961.)

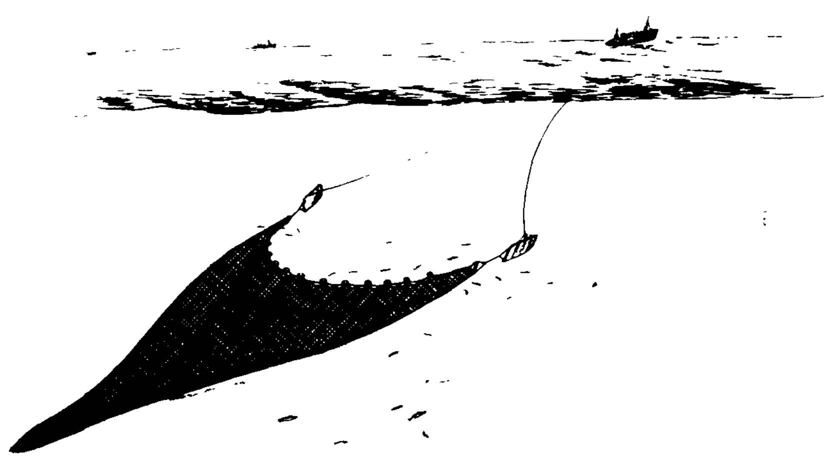

Figure 3. The otter trawl. (From Dumont and Sundstrom, 1961.)

2.2.3 Electrofishing

Electrofishing is a sampling method in which alternating (AC) or direct (DC) electrical current is applied to water that has a resistance different from that of fish. The difference in the resistance of the water and the fish to pulsating DC stimulates the swimming muscles for short periods of time, causing the fish to orient towards and be attracted to the positive electrode. An electrical field of sufficient potential to immobilize the fish is present near the positive electrode.

The electrofishing unit may consist of a 110-volt, 60-cycle, heavy-duty generator, an electrical control section consisting of a modified, commercially-sold, variable-voltage pulsator, and electrodes. The electrical control section permits the selection of any AC voltage between 50 to 700 and any DC voltage between 25 to 350 and permits control of the size of the electrical field required by various types of water. The alternating current serves as a standby for the direct current and is used in cases of extremely low water resistance.

Decisions on the use of AC, DC, pulsed DC, or alternate polarity forms of electricity and the selection of the electrode shape, electrode spacing, amount of voltage, and proper equipment depend on the resistance, temperature, and total dissolved solids of the water. Light-weight conductivity meters are recommended for field use. Lennon (1959) provides a comprehensive table and describes the system or combination of systems that worked best for him.

Rollefson (1958, 1961) thoroughly tested and evaluated AC, DC, and pulsating DC, and discussed basic electrofishing principles, wave forms, voltage -- current relationships, electrode types and designs, and differences between AC and DC and their effects in hard and soft waters. He concluded that pulsating DC was best in terms of power economy and fishing ability when correctly used. Haskell and Adelman (1955) found that slowly pulsating DC worked best in leading fish to the anode. Pratt (1951) also found the DC shocker to be more effective than the AC shocker.

Fisher (1950) found that brackish water requires much more power (amps) than freshwater, even though the voltage drops may be identical. Seehorn (1968) recommended the use of an electrolyte (salt blocks) when sampling in some soft waters to produce a large enough field with the electric shocker. Frankenberger (1960), Larimore, Durham and Bennett (1950) and Latta and Meyers (1961) have excellent papers on boat shockers. Frankenberger and Latta and Meyers used a DC shocker and Larimore et al. an AC shocker. Stubbs (1966), used DC or pulsed DC, and has his (aluminum) boat wired as the negative pole. In his paper, he also shows the design and gives safety precautions that emphasize the use of the treadle switch or "deadman switch" in case a worker falls overboard.

Backpack shockers that are quite useful for small, wadeable streams have been described by Blair (1958) and McCrimmon and Berst (1963), as has a backpack shocker for use by one man (Seehorn, 1968). Most of these papers give diagrams for wiring and parts needed.

There are descriptions of electric trawls (AC) (Haskell, Geduliz, and Snolk, 1955, and Loeg, 1955); electric seines (Funk, 1947; Holton, 1954; and Larimore, 1961); and a fly-rod electrofishing device employing alternating polarity current (Lennon, 1961).

The user must decide which design is most adaptable to his particular needs. Before deciding which design to use, the biologist should carefully review the literature. The crew should wear rubber boots and electrician's gloves and adhere strictly to safety precautions.

Night sampling was found to be much more effective than day sampling. Break sampling efforts into time units so that unit effort data are available for comparison purposes.

2.2.4 Chemical fishing

Chemicals used in fish sampling include rotenone, toxaphene, cresol, copper sulfate, and sodium cyanide. Rotenone has generally been the most acceptable because of its high degradability; freedom from such problems as precipitation (as with copper sulfate) and persistant toxicity (as with toxaphene); and relative safety for the user.

Rotenone, obtained from the derris root (*Deguelia elliptica*, East Indies) and cube root

(*Lonchocarpus nicour*, South America), has been used extensively in fisheries work throughout the United States and Canada since 1934 (Krumholz, 1948). Although toxic to man and warm-blooded animals (132 mg/kg), rotenone has not been considered hazardous in the concentrations used for fish eradication (0.025 to 0.050 ppm active ingredient) (Hooper, 1960), and has been employed in waters used for bathing and in some instances in drinking water supplies (Cohen et al., 1960, 1961). Adding activated carbon not only effectively removes rotenone, but it also removes the solvents, odors, and emulsifiers present in all commercial rotenone formulations.

Rotenone obtained as an emulsion containing approximately 5 percent active ingredient, is recommended because of the ease of handling. It is a relatively fast-acting toxicant. In most cases, the fish will die within 1 to 2 hours after exposure. Rotenone decomposes rapidly in most lakes and ponds and is quickly dispersed in streams. At summer water temperatures, toxicity lasts 24 hours or less. Detoxification is brought about by five principal factors: dissolved oxygen, light, alkalinity, heat, and turbidity. Of these, light and oxygen are the most important factors.

Although the toxicity threshold for rotenone differs slighly among fish species, it has not been widely used as a selective toxicant. It has, however, been used at a concentration of 0.1 ppm of the 5 percent emulsion to control the gizzard shad (Bowers, 1955).

Chemical sampling is usually employed on a spot basis, e.g. a short reach of river or an embayment of a lake. A concentration of 0.5 ppm active ingredient will provide good recovery of most species of fish in acidic or slightly alkaline waters. If bullheads and carp are suspected of being present, however, a concentration of 0.7 ppm active ingredient is recommended. If the water is turbid and strongly alkaline, and resistant species (i.e., carp and bullheads) are present, use 1-2 ppm. To obtain a rapid kill, local concentrations of 2 ppm can be used; however, caution is advised because rotenone dispersed into peripheral water areas may kill fish as long as the concentration is above 0.1 ppm.

A very efficient method of applying emulsion products is to pump the emulsion from a drum mounted in the bottom of a boat. The emulsion is suctioned by a venturi pump (Amundson boat bailer) clamped on the outboard motor. The flow can be metered by a valve at the drum hose connection. This method gives good dispersion of the chemical and greater boat handling safety, since the heavy drum can be mounted in the bottom of a boat rather than above the gunwales, as required for gravity flow.

Spraying equipment needed to apply a rotenone emulsion efficiently varies according to the size of the job. For small areas of not more than a few acres, a portable hand pump ordinarily used for garden spraying or fire fighting is sufficient. The same size pump is also ideal for sampling the population of a small area.

A power-driven pump is recommended for a large-scale or long-term sampling program. A detailed description of spraying equipment can be found in Mackenthun and Ingram (1967). The capacity of the pump need not be greater than 200 liters per minute. Generally speaking, a 1-1/2 H.P. engine is adequate.

The power application of rotenone emulsives requires a pressure nozzle, or a spray boom, or both, and sufficient plumbing and hose to connect with the pump. The suction line of the pump should be split by a "Y" to attach two intake lines. One line is used to supply the toxicant from the drum, and the other line, to supply water from the lake or embayment. The valves are adjusted so the water and toxicant are drawn into the pumping system in the desired proportion and mixed.

In sampling a stream, select a 30- to 100-meter reach depending on the depth and width of the stream; measure the depth of the section selected, calculate the area, and determine the amount of chemical required. Block off the area upstream and downstream with seines. To detoxify the area downstream from the rotenone, use potassium permanganate. Care must be exercised, however, because potassium permanganate is toxic to fish at about 3 ppm.

2.2.5 Hook and line

Fish collection by hook and line can be as simple as using a hand-held rod or trolling baited hooks or other lures, or it may take the form of long trot lines or set lines with many baited hooks. Generally speaking, the hook and line method is not acceptable for conducting a fishery survey, because it is too highly selective in the size and species captured and the catch per unit of effort is too low. Although it can only be used as a supporting technique, it may be the best method to obtain a few adult specimens for heavy metal analysis, etc., where sampling with other gear is impossible.

2.3 Passive Sampling Techniques

2.3.1 Entanglement nets

Gill and trammel nets are used extensively to sample fish populations in estuaries, lakes, reservoirs, and larger rivers.

A gill net is usually set as an upright fence of netting and has a uniform mesh size. Fish attempt to swim through the net and are caught in the mesh (Figure 4). Because the size of the mesh determines the species and size of the fish to be caught, gill nets are considered selective. The most versatile type is an experimental gill net consisting of five different mesh size sections. Gill nets can be set at the surface, in midwater, or at the bottom, and they can be operated as stationary or movable gear. Gill nets made of multifilament or monofilament nylon are recommended. Multifilament nets cost less and are easier to use, but monofilament nets generally capture more fish. The floats and leads usually supplied with the nets can cause net entanglement. To reduce this problem, replace the individual floats with a float line made with a core of expanded foam and use a lead-core leadline instead of individual lead weights.

The trammel net (Figure 5) has a layer of large mesh netting on each side of loosely-hung, smaller gill netting. Small fish are captured in the gill netting and large fish are captured in a "bag" of the gill netting that is formed as the smaller-mesh gill netting is pushed through an opening in the larger-mesh netting. Trammel nets are not used as extensively as are gill nets in sampling fish.

Results for both nets are expressed as the number or weight of fish taken per length of net per day.

Stationary gill and trammel nets are fished at right angles to suspected fish movements and at any depth from the surface to the bottom. They may be held in place by poles or anchors. The anchoring method must hold the net in position against any unexpected water movements such as, runoff, tides, or seiches.

Drifting gill or trammel nets are also set and fished the same as stationary gear, except that they are not held in place but are allowed to drift with the currents. This method requires constant surveillance when fishing. They are generally set for a short period of time, and if currents are too great, stationary gear is used.

The use of gill nets in the estuaries may present special problems, and consideration should be given to tidal currents, predation, and optimum fishing time, and to anchors, floats, and line.

The gunnels of any boat used in a net fishing operation should be free of rivets, cleats, etc., on which the net can catch.

2.3.2 Entrapment devices

With entrapment devices, the fish enter an enclosed area (which may be baited) through a series of one or more funnels and cannot escape.

The hoop net and trap net are the most common types of entrapment devices used in fishery surveys. These traps are small enough to be deployed from a small open boat and are relatively simple to set. They are held in place with anchors or poles and are used in water deep enough to cover the nets, or to a depth up to 4 meters.

The hoop net (Figure 6) is constructed by covering hoops or frames with netting. It has one or more internal funnels and does not have wings or a lead. The first two sections can be made square to prevent the net from rolling in the currents.

The fyke net (Figure 7) is a hoop net with wings, or a lead, or both attached to the first frame. The second and third frames can each hold funnel throats, which prevent fish from escaping as they enter each section. The opposite (closed) end of the net may be tied with a slip cord to facilitate fish removal.

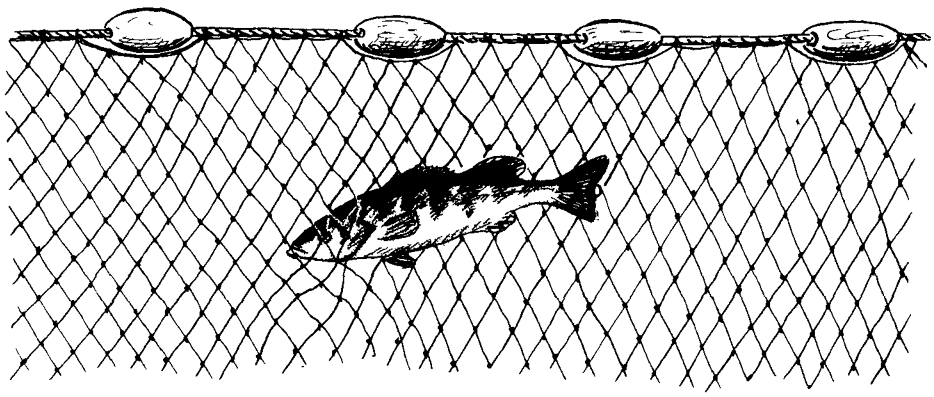

Figure 4. Gill net. (From Dumont and Sundstrom, 1961.)

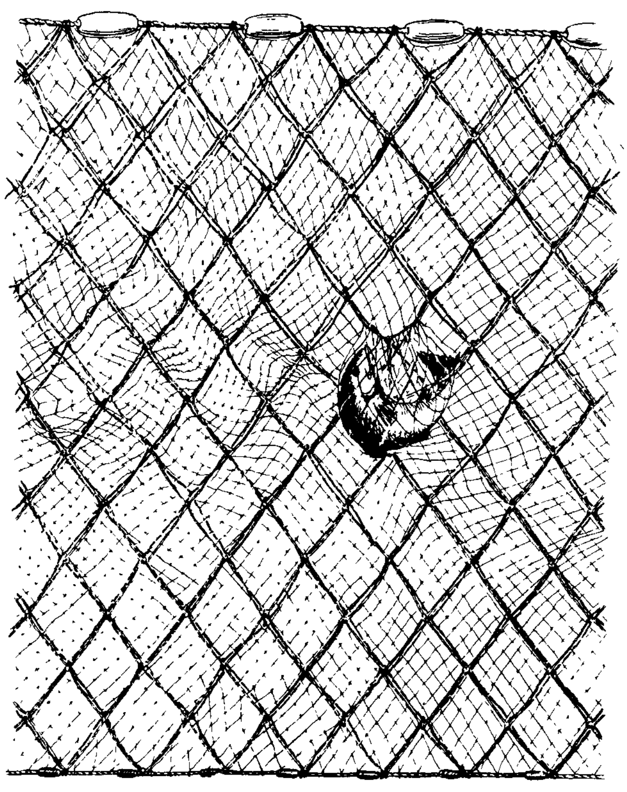

Figure 5. Trammel net. (From Dumont and Sundstrom, 1961.)

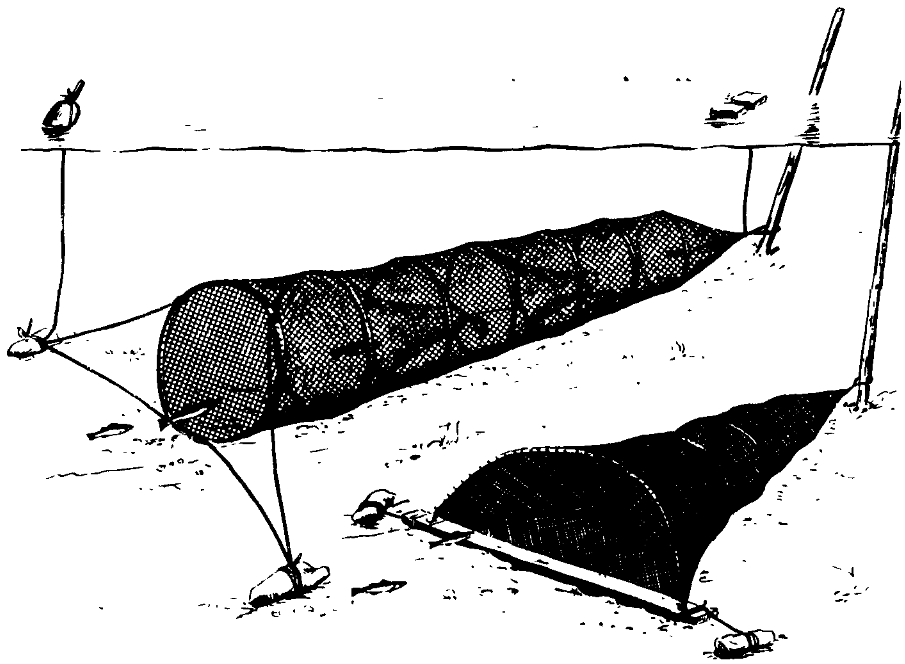

Figure 6. Hoop net. (From Dumont and Sundstrom, 1961.)

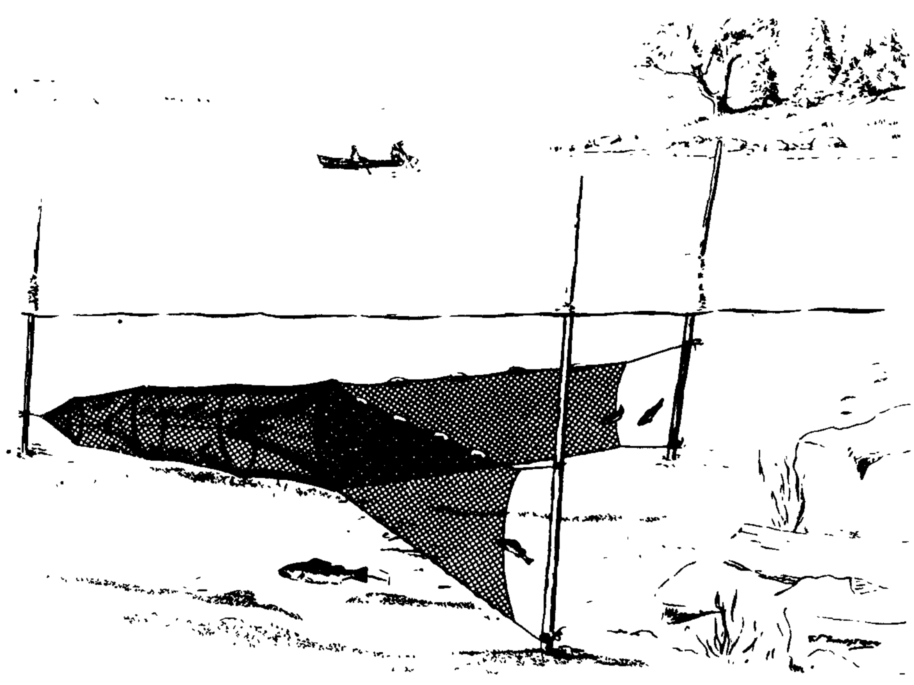

Figure 7. Fyke net. (From Dumont and Sundstrom, 1961.)

Hoop nets are fished in rivers and other waters where fish move in predictable directions, whereas the fyke net is used when fish movement is more random such as in lakes, impoundments, and estuaries. Hoop and fyke nets can be obtained with hoops from 2 to 6 feet (0.6 to 1.8 meters) in diameter, but any net over 4 feet (1.2 meters) in diameter is too large to be used in a fishery survey.

Trap nets use the same principle as hoop nets for capturing fish, but their construction is more complex. Floats and weights instead of hoops give the net its shape. The devices are expensive, require considerable experience, and are fished in waters deep enough to cover them.

One of the most simple types is the minnow trap, usually made of wire mesh or glass, with a single inverted funnel. The bait is suspended in a porous bag. A modification of this type is the slat trap; this employs long wooden slats in a cylindrical trap, and when baited with cheese bait, cottonseed cake, etc., it is used very successfully in sampling catfish in large rivers (Figure 8).

Most fish can be sampled by setting trap and hoop nets of varying mesh sizes in a variety of habitats. Hoop and trap nets are made of cotton or nylon, but nets made of nylon have a longer life and are lighter when wet. Protect cotton nets from decay by treatment. Catch is recorded as numbers or weight per unit of effort, usually fish per net day.

3.0 SAMPLE PRESERVATION

Preserve fish in the field in 10 percent formalin. Add 3 grams borax and 50 ml glycerin per liter of formalin. Specimens larger than 7.5 cm should be slit on the side at least one-third of the length of the body cavity to permit the preservative to bathe the internal organs. Slit the fish on the right side, because the left side is generally used for measurements, scale sampling, and photographic records.

Fixation may take from a few hours with small specimens to a week or more with large forms. After fixation, the fish may be washed in running water or by several changes of water for at least 24 hours and placed in 40 percent isopropyl alcohol. One change of alcohol is necessary to remove the last traces of formalin. Thereafter, they may be permanently preserved in the 40 percent isopropyl alcohol.

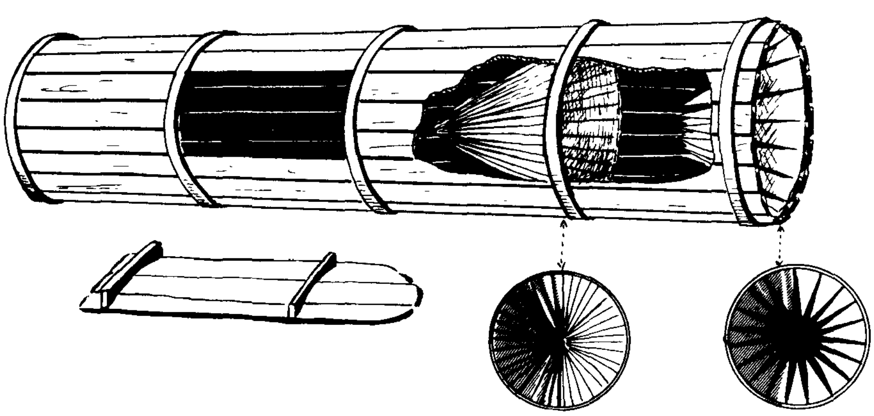

Figure 8. Slat trap. (From Dumont and Sundstrom, 1961.)

4.0 SAMPLE ANALYSIS

4.1 Data Recording

The sample records should include collection number, name of water body, date, locality, and other pertinent information associated with the sample. Make adequate field notes for each collection. Write with water-proof ink and paper to ensure a permanent record. Place the label inside the container with the specimens and have the label bear the same number or designation as the field notes, including the locality, date, and collector's name. Place a numbered tag on the outside of the container to make it easier to find a particular collection. Place any detailed observations about a collection on the field data sheet. Record fishery catch data in standard units such as number or weight per area or unit of effort. Use the metric system for length and weight measurements.

4.2 Identification

Proper identification of fishes to species is important in analysis of the data for water quality interpretation. A list of regional and national references for fish identification is located at the end of this chapter. Assistance in confirming questionable identification is available from State, Federal, and university fishery scientists.

4.3 Age, Growth, and Condition

Changes in water quality can be detected by studying the growth rate of fishes. Basic methods used to determine the age and growth of fish include:

- Study of fish length-frequencies, and
- Study of seasonal ring formations in hard bony parts such as scales and bones.

The length-frequency method of age determination depends on the fact that fish size varies with age. When the number of fish per length interval is plotted on graph paper, peaks generally appear for each age group. This method works best for young fish.

The seasonal ring-formation method depends on the fact that fish are cold-blooded animals and the rates of their body processes are affected by the temperature of the water in which they live. Growth is rapid during the warm season and slows greatly or stops in winter. This seasonal change in growth rate of fishes is often reflected in zones or bands (annual rings) in hard bony structures, such as scales, otoliths (ear stone), and vertebrae. The scales of fish may indicate exposure to adverse conditions such as injury, poor food supply, disease, and possibly water quality.

Note the general well being of the fish – do they appear emaciated? diseased from fungus? have open sores, ulcers, or fin rot? parasitized? Check the gill condition, also. Healthy fish will be active when handled, reasonably plump, and not diseased. Dissect a few specimens and check the internal organs for disease or parasites. The stomachs can be checked at this time to determine if the fish are actively feeding.

5.0 SPECIAL TECHNIQUES

5.1 Flesh Tainting

Sublethal concentrations of chemicals, such as phenols, benzene, oil, 2, 4-D, are often responsible for imparting an unpleasant taste to fish flesh, even when present in very low concentrations. Flesh tainting is nearly as detrimental to the fisheries as a complete kill.

A method has been developed (Thomas, 1969) in which untainted fish are placed in cages upstream and downstream from suspected waste sources. This procedure will successfully relate the unacceptable flavor produced in native fish if exposed to a particular waste source.

To ensure uniform taste quality before exposure, all fish are held in pollution-free water for a 10-day period. After this period, a minimum of three fish are cleaned and frozen with dry ice as control fish. Test fish are then transferred to the test sites, and a minimum of three fish are placed in each portable cage. The cages are suspended at a depth of 0.6 meter for 48 to 96 hours.

After exposure, the fish are dressed, frozen on dry ice, and stored to 0°F until tested. The control and exposed samples are shipped to a fish-tasting panel, such as is available at the food science and technology departments in many of the major universities, and treated as follows: (a) The fish are washed, wrapped in aluminum foil, placed on slotted, broiler-type pans, and cooked in a gas oven at 400°F for 23 to 45 minutes depending on the size of the fish. (b) Each sample is boned and the flesh is flaked and mixed to ensure a uniform sample. (c) The samples are served in coded cups to judges. Known and coded references or control samples are included in each test. The judges score the flavor and desirability of each sample on a point scale. The tasting agency will establish a point on the scale designated as the acceptable and desirable level.

5.2 Fish Kill Investigations

Fish mortalities result from a variety of causes, some natural and some man-induced. Natural fish kills are caused by phenomena such as acute temperature change, storms, ice and snow cover, decomposition of natural organic materials, salinity changes, spawning mortalities, and bacterial, parasitic, and viral epidemics. Man-induced fish kills may be attributed to municipal or industrial wastes, agricultural activities, and water manipulations. Winter kills occur in northern areas where ice on shallow lakes and ponds becomes covered with snow, and the resulting opaqueness stops photosynthesis. The algae and vascular plants die because of insufficient light, and their decomposition results in oxygen depletion. Oxygen depletion and extreme pH variation can be caused also by the respiration or decay of algae and higher plants during summer months in very warm weather. Kills resulting from such causes are often associated with a series of cloudy days that follow a period of hot, dry, sunny days.

Occasionally fish may be killed by toxins released from certain species of living or decaying algae that reached high population densities because of the increased fertility resulting from organic pollution.

Temperature changes, either natural or the result of a heated water discharge, will often result in fish kills. Long periods of very warm, dry weather may raise water temperatures above lethal levels for particular species. A wind-induced seiche may be hazardous to certain temperature-sensitive, deep-lake, cold-water fish, or fish of shallow coastal waters.

Disease, a dense infestation of parasites, or natural death of weakened fish at spawning time must always be suspected as contributory factors in fish mortalities.

Explosions, abrupt water level fluctuations, hurricanes, extreme turbidity or siltation, discharges of toxic chemicals, certain insecticides, algicides, and herbicides may each cause fish kills.

Recent investigations in Tennessee have shown that the leaking of small amounts of very toxic chemicals from spent pesticide-containing barrels used as floats for piers and diving rafts in lakes and reservoirs can produce extensive fish kills.

Fish die of old age, but the number so afflicted at any one time is usually small.

All possible speed must be exercised in conducting the initial phases of any fish kill investigation because fish disintegrate rapidly in hot weather and the cause of death may disappear or become unidentifiable within minutes. Success in solving a fish kill problem is usually related to the speed with which investigators can arrive at the scene after a fish kill begins. The speed of response in the initial investigation is enhanced through the training of qualified personnel who will report immediately the location of observed kills, the time that the kill was first observed, the general kinds of organisms affected, an estimate of the number of dead fish involved, and any unusual phenomena associated with the kill.

Because there is always the possibility of legal liability associated with a fish kill, lawyers, judges, and juries may scrutinize the investigation report. The investigation, therefore, must be made with great care.

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BIOASSAY

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1.0 GENERAL CONSIDERATIONS

The term BIOASSAY includes any test in which organisms are used to detect or measure the presence or effect of one or more substances or conditions. The organism responses measured in these tests include: mortality, growth rate, standing crop (biomass), reproduction, stimulation or inhibition of metabolic or enzyme systems, changes in behavior, histopathology, and flesh tainting (in shellfish and fish). The ultimate purpose of bioassays is to predict the response of native populations of aquatic organisms to specific changes within the natural environment. Whenever possible, therefore, tests should be carried out with species that are native (indigenous) to the receiving water used as the diluent for the bioassay. Bioassays are important because in most cases the success of a water pollution control program must be judged in terms of the effects of water quality on the condition of the indigenous communities of aquatic organisms. Also, in many cases, bioassays are more sensitive than chemical analyses.

Two general kinds of bioassays are recognized:

- laboratory tests conducted to determine the effects of a substance on a species; more or less arbitrary conditions are employed;
- in situ tests conducted to determine the effects of a specific natural environment; the test organisms are held in "containers" through which the water circulates freely.

The general principles and methods of conducting laboratory bioassays presented in Standard Methods for the Examination of Water and Waste Water, 13th edition (APHA, 1971) apply to most bioassays, and the described methods can be used with many types of aquatic organisms with only slight modification.

The following are suggested improvements to the methods given in Standard Methods, 13th edition (APHA, 1971).

- The 48- and 96-hour LC50 values are presently important for determining compliance with water quality standards as established by various pollution control authorities. Short-term threshold information can be derived by reporting LC50 values at 24-hour intervals to demonstrate the shape of the toxicity curve.
- Reports of LC50's should state the method of calculation used and the statistical confidence limits when possible.
- Rubber or plastic materials should be used in bioassay equipment only after consideration has been given to the possibility of the leaching of substances such as plasticizers or sorption of toxicants.
- Test materials should be administered in such a way that their physical and chemical behavior approximates that in natural systems.

Biological tests can be conducted in any kind of water with proper precautions, and although most tests have been conducted in freshwater, the same general principles apply to brackish and salt waters. The literature contains a great many formulations for artificial seawater. Of these, a modification of the Kester et al. (1967) formulation (LaRoche et al., 1970; Zaroogian et al., 1969) seems to support the greatest variety of marine organisms. When metal-containing wastes are to be bioassayed, omitting EDTA and controlling trace metals, as described by Davey et al. (1970), is recommended.

Using a standard toxicant and a parallel series in a standard medium is recommended to help assess variations due to experimental technique and the condition of the organisms. Such tests are also useful in distinguishing effects due to an altered character of the effluent from changes in the sensitivity of the organism, or from changes in the quality of the receiving water.

When making waste management decisions, it is important to consider and tentatively define the persistence of a pollutant. Materials that have half lives less than 48 hours can be termed as rapidly decaying compounds; those with half lives greater than 48 hours but less than 6 months, as slowly decaying; and those compounds in natural waters with half lives longer than 6 months, as long-lived persistent materials.

Bioassays can be conducted over almost any interval of time, but the test duration must be appropriate to the life stage or life cycle of the test organisms and the objectives of the investigation. The purpose of short-term tests, such as acute mortality tests, is to determine toxicant concentrations lethal to a given fraction (usually 50 percent) of the organisms during a short period of their life cycle. Acute mortality tests with fish generally last about 4 to 7 days. Most toxicants, however, cause adverse effects at levels below those that cause mortality. To meet this need, long-term (chronic) tests are designed to expose test organisms to the toxicant over their entire life cycle and measure the effects of the toxicant on survival, growth, and reproduction. Sometimes only a portion of the life cycle is tested, such as studies involving growth or emergence of aquatic insects. With fish, such tests usually last for 30, 60, or 90 days and are often termed subacute.

Laboratory bioassays may be conducted on a "static" or "continuous flow" basis. The specific needs of the investigator and available test facilities determine which technique should be used. The advantages and applications of each have been described in Standard Methods, (APHA, 1971) and by the National Technical Advisory Committee (1968). Generally, the continuous-flow technique should be used where possible. Apparatus advantageous for conducting flow-through tests includes diluters (Mount and Warner, 1965; Mount and Brungs, 1967), valve controlling systems (Jackson and Brungs, 1966) and chemical metering pumps (Symons, 1963).

The biological effects of many industrial wastes are best evaluated in the field; transporting large volumes of industrial wastes to a laboratory for bioassay purposes can be impractical. Testing facilities are best located at the site of the waste discharge. A bioassay trailer (Zillich, 1969) has proven useful for this purpose. *In situ* bioassay procedures are also a good method for defining the impact to aquatic life below the source of industrial waste discharges (Basch, 1971).

Biomonitoring, a special application of biological tests, is the use of organisms to provide information about a surface water, effluent, or mixtures thereof on a periodic or continuing basis. For the best results, biomonitoring should maintain continuous surveillance with the use of indigenous species in a flow-through system under conditions that approximate the natural environment.

2.0 PHYTOPLANKTON – ALGAL ASSAY

The Algal Assay Procedure: Bottle Test was published by the National Eutrophication Research Program (USEPA, 1971) after 2 years of intensive evaluation, during which excellent agreement of the data was obtained among the 8 participating laboratories. This test is the only algal bioassay that has undergone sufficient evaluation and refinement to be considered reliable. The following material represents only a brief outline of the test. For more explicit details, see the references.

2.1 Principle

An algal assay is based on the principle that growth is limited by the nutrient that is present in shortest supply with respect to the needs of the organism. The test can be used to identify algal growth-limiting nutrients, to determine biologically the availability of algal growth-limiting nutrients, to quantify the biological response (algal growth response) to changes in concentrations of algal growth-limiting nutrients, and to determine whether or not various compounds or water samples are toxic or inhibitory to algae.

2.2 Planning Algal Assays

The specific experimental design of each algal assay is dictated by the particular problem to be solved. All pertinent ecological factors must be considered in planning a given assay to ensure that valid results and conclusions are obtained.

Water quality may vary greatly with time and location in lakes, impoundments and streams. If meaningful data are to be obtained, therefore, *the sampling program must take these variations* into account.

2.3 Apparatus and Test Conditions

2.3.1 Glassware

Use good-quality borosilicate glassware. When studing trace nutrients, use special glassware such as Vycor or polycarbonate containers. Although container size is not critical, the surface to volume ratios are critical because of possible carbon limitation. The recommended sample volumes for use in Erlenmeyer flasks are: 40 ml in a 125 ml flask; 60 ml in a 250 ml flask; and 100 ml in a 500 ml flask. Use culture closures such as loose-fitting aluminum foil or inverted beakers to permit good gas exchange and prevent contamination.

2.3.2 Illumination

After inoculation, incubate the flasks at 24 ± 2°C under cool-white fluorescent lighting: 200 ft-c (2152 lux) ± 10 percent for blue-green algae and diatom test species, and 400 ft-c (4304 lux) ± 10 percent for green algae test species. Measure the light intensity adjacent to the flask at the liquid level.

2.3.3 pH

To ensure the availability of carbon dioxide, maintain the pH of the incubating cultures below 8.5 by using the sample volumes mentioned above and shaking the cultures at 100 oscillations per minute. In samples containing high concentrations of nutrients, such as highly-productive surface waters or domestic waste effluents, it may be necessary to bubble air or an air/carbon dioxide mixture through the culture to maintain the pH below 8.5.

2.4 Sample Preparation

Two alternate methods of sample preparation are recommended, depending upon the type of information to be obtained from the sample:

- membrane filtration (0.45 pore diameter) – remove the indigenous algae by filtration if you wish to determine the growth response to growth-limiting nutrients which have not been taken up by filterable organisms, or if you wish to predict the effect of adding nutrients to a test water at a specific time.
- autoclaving -- autoclave samples if you wish to determine the amount of algal biomass that can be grown from *all* nutrients in the water, including those in the plankton. Autoclaving solubilizes the nutrients in the indigenous filterable organisms and releases them for use by the test organisms.

2.5 Inoculum

The algal test species may be one of those recommended in the Bottle Test or another that has been obtained in unialgal culture. Grow the test species in a culture medium that minimizes the intracellular carryover of nutrients in the test species when transferred from the stock culture to the test water (Table I.) When taken from the stock culture, centrifuge the test cells and discard the supernatant. Resuspend the sedimented cells in an appropriate volume of glass-distilled water containing 15 mg sodium bicarbonate per liter and recentrifuge. Decant the supernatant, resuspend the algae in fresh bicarbonate solution, and use as the inoculum. The amount of inoculum depends upon the algal test species used. The following initial cell concentrations are recommended:

Test organism	Initial cell count/ml
Selenastrum capricornutum	1000/ml
Anabaena flos-aquae	50000/ml
Microcystis aeruginosa	50000/ml

Prepare test flasks in triplicate.

2.6 Growth Response Measurements

The method used to determine growth response during incubation depends on the equipment available. Cells may be counted with a microscope, using a hemacytometer or a Palmer-Maloney or Sedgwick-Rafter plankton counting chamber. The amount of algal biomass may be determined by measuring the optical density of the culture at 600 -750 nm with a colorimeter or spectrophotometer. The amount of chlorophyll contained in the algae may be

TABLE 1. STOCK CULTURE AND CONTROL NUTRIENT MEDIUM

MACROELEMENTS:			
Compound	Final concentration (mg/l)	Element furnished	Element concentration (mg/l)
$NaNO_3$	25.500	N	4.200
K_2HPO_4	1.044	P	0.186
		K	0.468
$MgCl_2$	5.700	Mg	1.456
$MgSO_4{\cdot}7H_2O$	14.700	Mg	1.450
		S	1.911
$CaCl_2{\cdot}2H_2O$	4.410	Ca	1.203
$NaHCO_3$	15.000	Na	11.004

(If the medium is to be filtered, add the following trace-element-iron-EDTA solution from a single combination stock solution after filtration. With no filtration, K_2HPO_4 should be added last to avoid iron precipitation. Stock solutions of individual salts may be made up in 1000 x's final concentration or less.)

MICROELEMENTS:			
	(μg/l)		(μg/l)
H_3BO_3	185.64	B	33
$MnCl_2$	264.27	Mn	114
$ZnCl_2$	32.70	Zn	15
$CoCl_2$	0.78	Co	0.35
$CuCl_2$	0.009	Cu	0.003
$Na_2MoO_4{\cdot}2H_2O$	7.26	Mo	2.88
$FeCl_3$	96	Fe	33
$Na_2EDTA{\cdot}2H_2O$	333		

measured either directly *(in vivo)* by fluorometry or after extraction by fluorometry or spectrophotometry. If available, an electronic particle counter will provide an accurate and rapid count of the cells. All methods used for determining the algal biomass should be related to a dry weight measurement (mg/l) determined gravimetrically. (See the Plankton Section of the manual for analytical details.)

2.7 Data Evaluation

Two parameters are used to describe the growth of a test alga: maximum specific growth rate and maximum standing crop. The maximum specific growth rate (μ_{max}) for an individual flask is the largest specific growth rate (μ) occurring at any time during incubation. The μ_{max} for a set of replicates is determined by averaging the μ_{max} of the individual flasks. The specific growth rate, μ, is defined by:

$$\mu = \frac{\ln(X_2/X_1)}{t_2 - t_1}$$

where:
ln = log to the base "e"
X_2 = biomass concentration at the end of the selected time interval
X_1 = biomass concentration at the beginning of the selected time interval
$t_2 - t_1$ = elapsed time (days) between selected determinations of biomass

Because the maximum specific growth rate (μ_{max}) occurs during the logarithmic phase of growth (usually between day 3 and day 5), the biomass must be measured at least daily during the first 5 days of incubation.

The maximum standing crop in any flask is defined as the maximum algal biomass achieved during incubation. For practical purposes, the maximum standing crop is assumed to have been achieved when the rate of increase in biomass has declined to less than 5 percent per day.

2.8 Additions (Spikes)

The quantity of cells produced in a given medium is limited by the nutrient present in the lowest relative quantity with respect to the needs of the organism. If a quantity of the limiting substance were added to the test flasks, cell production would increase until this additional supply was depleted or until some other substance became limiting to the organism. Adding substances other than the limiting substance would not increase algal growth. Nutrient additions may be made singly or in combination, and the growth response can be compared with that of unspiked controls to identify those substances that limit growth rate or cell production.

In all instances, the volume of a spike should be as small as possible. The concentration of spikes will vary and must be matched to the waters being tested. When selecting the spike concentration, keep in mind that (1) the concentration should be kept small to minimize alterations of the sample, but at the same time, be sufficiently large to yield a potentially measureable response; and (2) the concentration should be related to the fertility of the sample.

2.9 Data Analysis and Interpretation

Present the results of spiking assays together with the results from two types of reference samples: the assay reference medium and unspiked samples of the water under consideration. Preferably, the entire growth curves should be presented for each of the two types of reference samples. Present the results of individual assays in the form of the maximum specific growth rate (with time of occurrence) and maximum standing crop (with time at which it was reached), both with the confidence interval indicated.

Growth rate limiting nutrients can be determined by spiking a number of replicate flasks with single nutrients, determining the maximum specific growth rate for each flask, and comparing the averages by a Students' t-test or other appropriate statistical tests.

Data analysis for multiple nutrient spiking can be performed by analysis of variance calculations. In multiple nutrient spiking, accounting for the possible interaction between different nutrients is important and can readily be done by factorial analysis. The same methods described above can be used to determine the nutrient limiting growth of the maximum standing crop.

2.10 Assays to Determine Toxicity

As previously pointed out, the assay may be used to determine whether or not various compounds or water samples are either toxic or inhibitory to algal growth. In this case the substance to be tested for toxicity is added to the standard algal culture medium in varying concentrations, the algal test species is added, and either the maximum standing crop or maximum specific growth rate (or both) determined. These are then compared to those obtained in the standard culture medium without the additions (controls). The LC50, or that concentration at which either 50% of the maximum standing crop or maximum specific growth rate is obtained, as compared with the controls, is then calculated.

3.0 PERIPHYTON

Uniform methods for conducting bioassays with periphyton have not been developed, and their environmental requirements and toxicology are still relatively unknown. Many of the common species have not been successfully cultured, and the bioassays that have been carried out with the algae and other microorganisms occurring in this community were conducted principally to screen potential algicides, fungicides, and other control agents. Two kinds of tests can be conducted with periphyton: static and continuous flow.

3.1 Static

Because the techniques currently employed in the Algal Assay Procedure: Bottle Test (USEPA, 1971) have been more rigorously tested than any procedure previously used for periphyton, this method is recommended for static bioassays with the periphyton.

3.2 Continuous Flow

Many periphyton grow well only in flowing water and can be studied only *in situ* or in artificial streams (Whitford, 1960; Whitford *et al.*, 1964). The following procedure, which is similar to the method described by McIntire *et al.* (1964), is tentatively recommended at this time.

- Test Chamber – Twin, inter-connected channels, each approximately 4" X 4" X 36", with two inches of water circulated by a paddle wheel. Duplicate chambers should be provided for each condition tested (Figures 1 and 2).
- Current velocity – 30 cm/sec.
- Temperature – 20°C
- Light – 400 fc, cool-white (daylight) fluorescent lamps
- Culture medium – Optional
  a. Algal Assay Medium (Table 1).
  b. Natural surface water supply
  Where direct flow-through is not provided, the water exchange rate should ensure a complete change at least six times daily.

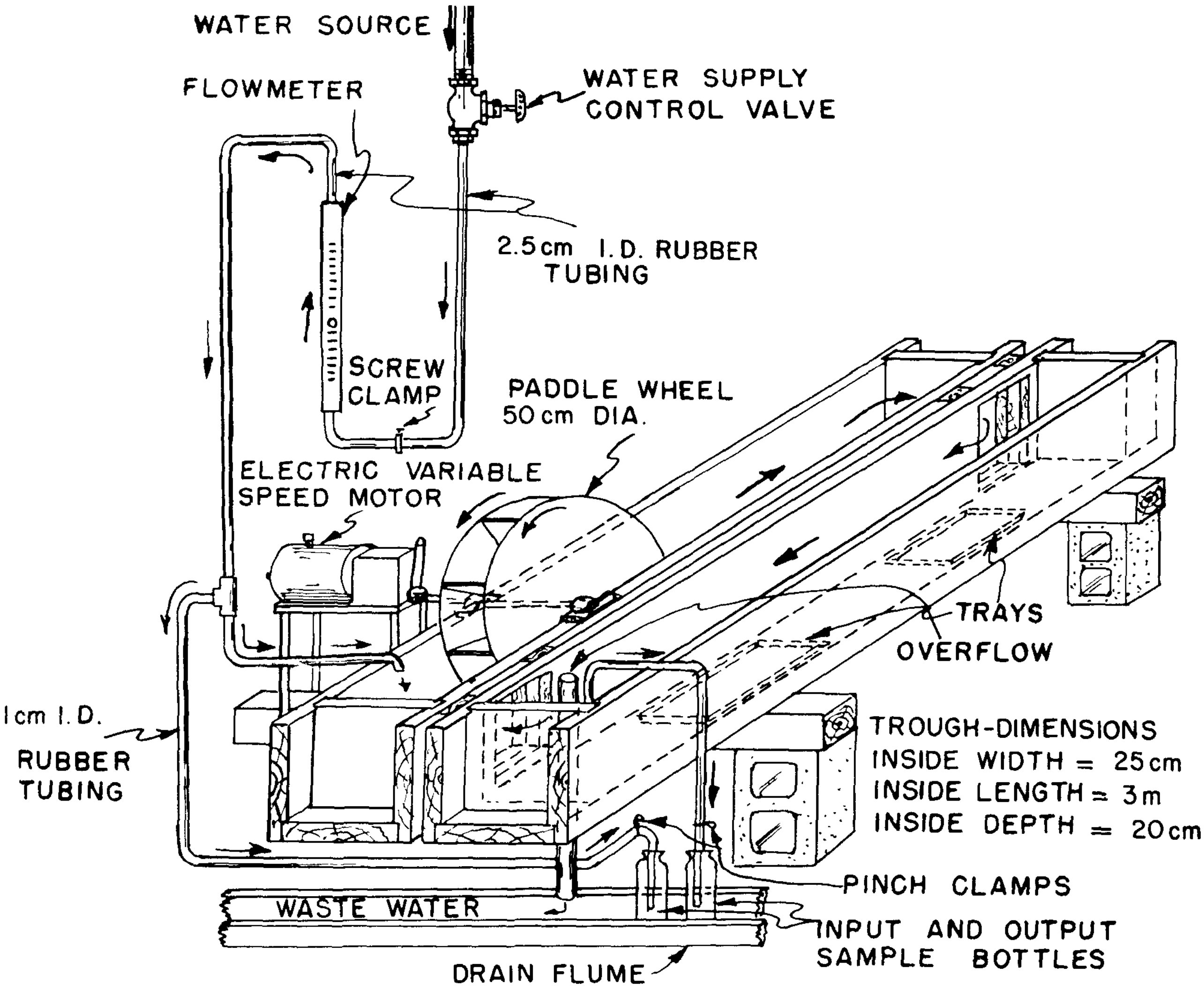

Figure 1. Diagram of laboratory stream, showing the paddle wheel for circulating the water between the two interconnected troughs and the exchange water system. (From McIntire *et al.*, 1964).

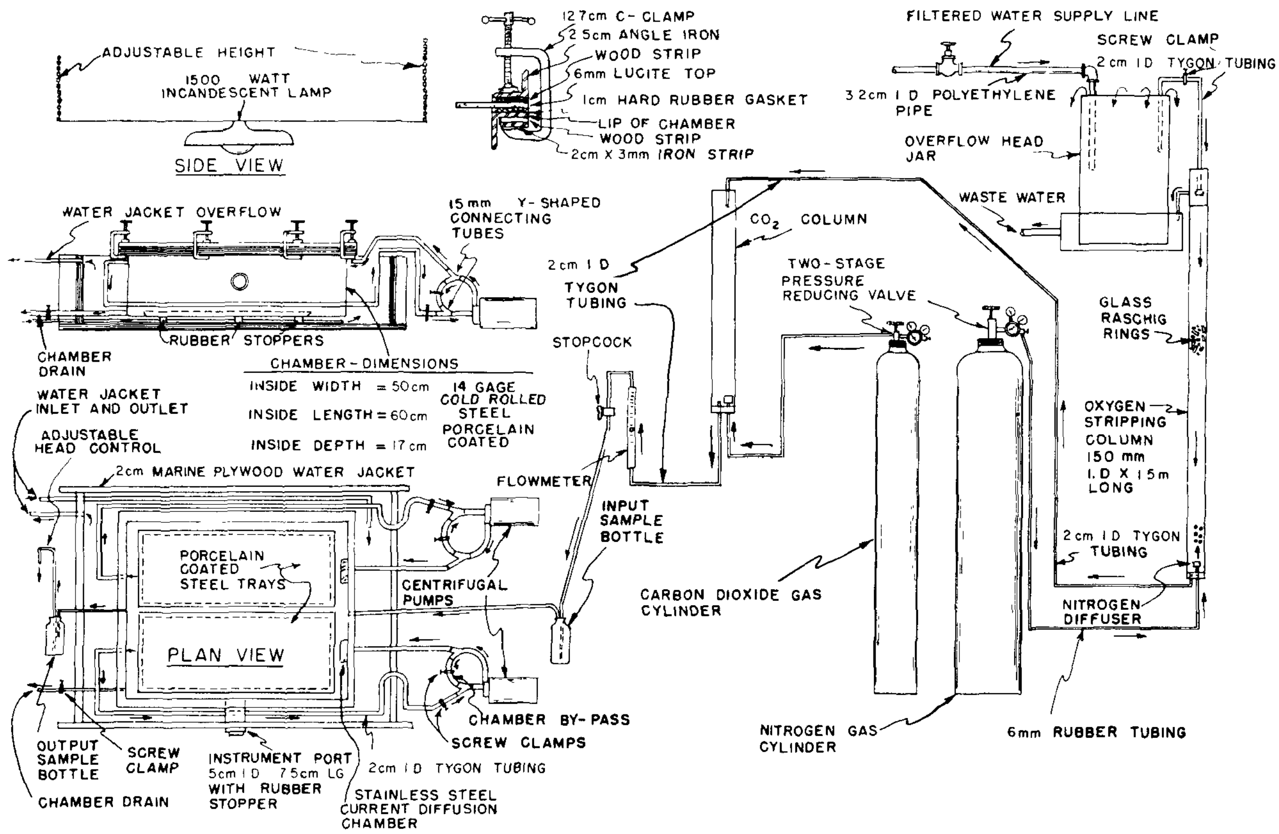

Figure 2. Diagram of photosynthesis-respiration chamber, showing the chamber with its circulating and exchange water systems, the water jacket for temperature control, the nutrient and gas concentration control system, and the light source.

- Test organism(s) – Optional; filamentous blue-green or green algae or diatoms.
  - a. Unialgal culture – No standard test organisms are available
  - b. Periphyton community – Use "seed" of periphyton from the water resource for which the data are being developed.
- Acclimatization period – The culture (or community) should be allowed to develop in the test chambers for a minimum of two weeks before introducing the test condition.
- *Maintaining test conditions* – Chemicals are added to the water supply prior to flow into the test chamber. Temperature control may be maintained by placing thermostatically controlled heating (or cooling) elements in the channel.
- Substrate – A minimum of eight 1" X 3" plain glass slides should be placed on the bottom of each channel.
- Test duration – Two weeks
- Evaluation – The effects of the test condition are evaluated at the end of the test period by comparing the biomass and community structure in the test chambers with that of the control chambers. (See Periphyton Section for methodology.)

  - a. Biomass – Use four of the eight slides; analyze individually.
    - (1) Chlorophyll *a* (mg/m^2)
    - (2) Organic matter (Ash-free weight, g/m^2)
  - b. Cell count and identification – Use four pooled slides.
    - (1) Cell density ($cells/mm^2$)
    - (2) Species proportional count
    - (3) Community diversity (Diversity Index)
- Toxicity – The toxicity of a chemical or effluent is expressed as the LC50, which is

the concentration of toxicant resulting in a 50% reduction in the biomass or cell count. Community diversity is not affected in the same manner as biomass and cell counts, and would yield a much different value.

4.0 MACROINVERTEBRATES

In general, most of the considerations covered by Standard Methods (APHA, 1971) apply equally well to macroinvertebrate tests in fresh and marine waters. Recent refinements in acute and chronic methodology for aquatic insects, amphipods, mussels, and *Daphnia* have been described by Gaufin (1971), Bell and Nebeker (1969), Arthur and Leonard (1970), Dimick and Breese (1965), Woelke (1967), and Biesinger and Christensen (1971), respectively.

5.0 FISH

The general principles and methods for acute and chronic laboratory fish toxicity tests are presented in Standard Methods (APHA, 1971) and in the report of the National Technical Advisory Committee (1968). Sprague (1969, 1970) has recently reviewed many of the problems and the terminology associated with fish toxicity tests.

Chronic tests are becoming increasingly important as sublethal adverse effects of more and more toxic agents are found to be significant. At present, a chronic fish bioassay test is a relatively sophisticated research procedure and entails large allocations of manpower, time, and expense. Important contributions in this area include those by Mount and Stephan (1969), Brungs (1969), Eaton (1970), and McKim *et al.* (1971).

Two procedures for chronic toxicity tests using the fathead minnow, *Pimephales promelas* Rafinesque, and the brook trout, *Salvelinus fontinales* (Mitchell), developed by the staff of the National Water Quality Laboratory, U.S. Environmental Protection Agency, Duluth, Minn., are presented following the references in this section.

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RECOMMENDED BIOASSAY PROCEDURES
NATIONAL WATER QUALITY LABORATORY
DULUTH, MINNESOTA

Recommended Bioassay Procedures are established by the approval of both the Committee on Aquatic Bioassays and the Director of the National Water Quality Laboratory. The main reasons for establishing them are: (1) to permit direct comparison of test results, (2) to encourage the use of the best procedures available, and (3) to encourage uniformity. These procedures should be used by National Water Quality Laboratory personnel whenever possible, unless there is a good reason for using some other procedure.

Recommended Bioassay Procedures consider the basic elements that are believed to be important in obtaining reliable and reproducible results in laboratory bioassays. An attempt has been made to adopt the best acceptable procedures based on current evidence and opinion, although it is recognized that alternative procedures may be adequate. Improvements in the procedures are being considered and tested, and revisions will be made when necessary. Comments and suggestions are encouraged.

Director, National Water Quality Lab (NWQL)

Committee on Aquatic Bioassays, NWQL

Fathead Minnow *Pimephales promelas* Rafinesque Chronic Tests
April, 1971
(Revised January, 1972)

1.0 PHYSICAL SYSTEM

1.1 Diluter

Proportional diluters (Mount and Brungs, 1967) should be employed for all long-term exposures. Check the operation of the diluter daily, either directly or through measurement of toxicant concentrations. A minimum of five toxicant concentrations and one control should be used for each test with a dilution factor of not less than 0.30. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter, temperature control or water supply failure.

1.2 Toxicant Mixing

A container to promote mixing of toxicant-bearing and w-cell water should be used between diluter and tanks for each concentration. Separate delivery tubes should run from this container to each duplicate tank. Check at least once every month to see that the intended amounts of water are going to each duplicate tank or chamber.

1.3 Tank

Two arrangements of test tanks (glass, or stainless steel with glass ends) can be utilized:

a. Duplicate spawning tanks measuring 1 X 1 X 3 ft. long with a one sq. ft. portion at one end screened off and divided in half for the progeny. Test water is to be delivered separately to the larval and spawning chambers of each tank, with about one-third the water volume going to the former chamber as to the latter.
b. Duplicate spawning tanks measuring 1 X 1 X 2 ft. long with a separate duplicate progeny tank for each spawning tank. The larval tank for each spawning tank should be a minimum of 1 cu. ft. dimensionally and divided to form two separate larval chambers with separate standpipes, or separate 1/2 sq. ft. tanks may be used. Test water is to be supplied by delivery tubes from the mixing cells described in Step 2 above.

Test water depth in tanks and chambers for both a and b above should be 6 inches.

1.4 Flow Rate

The flow rate to each chamber (larval or adult) should be equal to 6 to 10 tank volumes/24 hr.

1.5 Aeration

Total dissolved oxygen levels should never be allowed to drop below 60% of saturation, and flow rates must be increased if oxygen levels do drop below 60%. As a first alternative, flow rates can be increased above those specified in 1.4. Only aerate (with oil free air) if testing a non-volatile toxic agent, and then as a last resort to maintain dissolved oxygen at 60% of saturation.

1.6 Cleaning

All adult tanks, and larvae tanks and chambers after larvae swim-up, must be siphoned a minimum of 2 times weekly and brushed or scraped when algal or fungus growth becomes excessive.

1.7 Spawning Substrate

Use spawning substrates made from inverted cement and asbestos halved, 3-inch ID drain tile, or the equivalent, each of these being 3 inches long.

1.8 Egg Cup

Egg incubation cups are made from either 3-inch sections of 2-inch OD (1 1/2-inch ID) polyethylene water hose or 4-oz., 2-inch OD round glass jars with the bottoms cut off. One end of the jar or hose sections is covered with stainless steel or nylon screen (with a minimum of 40 meshes per inch). Cups are oscillated in the test water by means of a rocker arm apparatus driven by a 2 r.p.m. electric motor (Mount, 1968). The vertical-travel distance of the cups should be 1 to 1 1/2 inches.

1.9 Light

The lights used should simulate sunlight as nearly as possible. A combination of Duro-Test (Optima FS)[1,2] and wide spectrum Grow-lux[3] fluorescent tubes has proved satisfactory at the NWQL.

1.10 Photoperiod

The photoperiods to be used (Appendix A) simulate the dawn to dusk times of Evansville, Indiana. Adjustments in day-length are to be made on the first and fifteenth day of every Evansville test month. The table is arranged so that adjustments need be made only in the dusk times. Regardless of the *actual* date that the experiment is started, the Evansville *test* photoperiod should be adjusted so that the mean or estimated hatching date of the fish used to start the experiment corresponds to the Evansville test day-length for December first. Also, the dawn and dusk times listed in the table need *not* correspond to the actual times where the experiment is being conducted. To illustrate these points, an experiment started with 5-day-old larvae in Duluth, Minnesota, on August 28 (actual date), would require use of a December 5 Evansville test photoperiod, and the lights could go on anytime on that day just so long as they remained on for 10 hours and 45 minutes. Ten days later (Sept. 7 actual date, Dec. 15 Evansville test date) the day-length would be changed to 10 hours and 30 minutes. Gradual changes in light intensity at dawn and dusk (Drummond and Dawson, 1970), if desired, should be included within the day-lengths shown, and should not last for more than 1/2 hour from full on to full off and vice versa.

[1]Mention of trade names does not constitute endorsement.
[2]Duro-Test, Inc., Hammond, Ind.
[3]Sylvania, Inc., New York, N. Y.

1.11 Temperature

Temperature should not deviate instantaneously from 25°C by more than 2°C and should not remain outside the range of 24 to 26°C for more than 48 hours at a time. Temperature should be recorded continuously.

1.12 Disturbance

Adults and larvae should be shielded from disturbances such as people continually walking past the chambers, or from extraneous lights that might alter the intended photoperiod.

1.13 Construction Materials

Construction materials which contact the diluent water should not contain leachable substances and should not sorb significant amounts of substances from the water. Stainless steel is probably the preferred construction material. Glass absorbs some trace organics significantly. Rubber should not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents should not contain leachable materials and should not sorb significant amounts of most substances. Unplasticized polyethylene and polypropylene should not contain leachable substances, but may sorb very significant amounts of trace organic compounds.

1.14 Water

The water used should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used. If it is thought that the water supply could be conceivably contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system.

2.0 BIOLOGICAL SYSTEM

2.1 Test Animals

If possible, use stocks of fathead minnows from the National Water Quality Laboratory in Duluth, Minnesota or the Fish Toxicology

Laboratory in Newtown, Ohio. Groups of starting fish should contain a mixture of approximately equal number of eggs or larvae from at least three different females. Set aside enough eggs or larvae at the start of the test to supply an adequate number of fish for the acute mortality bioassays used in determining application factors.

2.2 Beginning Test

In beginning the test, distribute 40 to 50 eggs or 1- to 5-day-old larvae per duplicate tank using a stratified random assignment (see 4.3). All acute mortality tests should be conducted when the fish are 2 to 3 months old. If eggs or 1-to 5-day-old larvae are not available, fish up to 30 days of age may be used to start the test. If fish between 20 and 60 days old are used, the exposure should be designated a partial chronic test. Extra test animals may be added at the beginning so that fish can be removed periodically for special examinations (see 2.12.) or for residue analysis (see 3.4).

2.3 Food

Feed the fish a frozen trout food (e.g., Oregon Moist). A minimum of once daily, fish should be fed *ad libitum* the largest pellet they will take. Diets should be supplemented weekly with live or frozen-live food (e.g., *Daphnia,* chopped earthworms, fresh or frozen brine shrimp, etc.). Larvae should be fed a fine trout starter a minimum of 2 times daily, ad libitum; one feeding each day of live young zooplankton from mixed cultures of small copepods, rotifers, and protozoans is highly recommended. Live food is especially important when larvae are just beginning to feed, or about 8 to 10 days after egg deposition. Each batch of food should be checked for pesticides (including DDT, TDE, dieldrin, lindane, methoxychlor, endrin, aldrin, BHC, chlordane, toxaphene, 2,4-D, and PCBs), and the kinds and amounts should be reported to the project officer or recorded.

2.4 Disease

Handle disease outbreaks according to their nature, with all tanks receiving the same treatment whether there seems to be sick fish in all of them or not. The frequency of treatment should be held to a minimum.

2.5 Measuring Fish

Measure total lengths of all starting fish at 30 and 60 days by the photographic method used by McKim and Benoit (1971). Larvae or juveniles are transferred to a glass box containing 1 inch of test water. Fish should be moved to and from this box in a water-filled container, rather than by netting them. The glass box is placed on a translucent millimeter grid over a fluorescent light platform to provide background illumination. Photos are then taken of the fish over the millimeter grid and are enlarged into 8 by 10 inch prints. The length of each fish is subsequently determined by comparing it to the grid. Keep lengths of discarded fish separate from those of fish that are to be kept.

2.6 Thinning

When the starting fish are sixty (± 1 or 2) days old, impartially reduce the number of surviving fish in each tank to 15. Obviously injured or crippled individuals may be discarded before the selection so long as the number is not reduced below 15; be sure to record the number of deformed fish discarded from each tank. As a last resort in obtaining 15 fish per tank, 1 or 2 fish may be selected for transfer from one duplicate to the other. Place five spawning tiles in each duplicate tank, separated fairly widely to reduce interactions between male fish guarding them. One should also be able to look under tiles from the end of the tanks. During the spawning period, sexually maturing males must be removed at weekly intervals so there are no more than four per tank. An effort should be made *not* to remove those males having well established territories under tiles where recent spawnings have occurred.

2.7 Removing Eggs

Remove eggs from spawning tiles starting at 12:00 noon Evansville test time (Appendix A) each day. As indicated in Step 1.10, the test time need not correspond to the actual time where the test is being conducted. Eggs are loosened from the spawning tiles and at the same time separated from one another by lightly placing a finger on the egg mass and moving it in a circular pattern with increasing pressure until the eggs begin to roll. The groups of eggs should then be washed into separate, appropriately marked containers and subsequently handled (counted, selected for incubation, or discarded) as soon as possible after all eggs have been removed and the spawning tiles put back into the test tanks. All egg batches must be checked initially for different stages of development. If it is determined that there is more than one distinct stage of development present, then each stage must be considered as one spawning and handled separately as described in Step 2.8.

2.8 Egg Incubation and Larval Selection

Impartially select 50 unbroken eggs from spawnings of 50 eggs or more and place them in an egg incubator cup for determining viability and hatchability. Count the remaining eggs and discard them. Viability and hatchability determinations must be made on each spawning (>49 eggs) until the number of spawnings (>49 eggs) in each duplicate tank equals the number of females in that tank. Subsequently, only eggs from every third spawning (>49 eggs) and none of those obtained on weekends need be set up to determine hatchability; however, weekend spawns must still be removed from tiles and the eggs counted. If unforeseen problems are encountered in determining egg viability and hatchability, additional spawnings should be sampled before switching to the setting up of eggs from every third spawning. Every day, record the live and dead eggs in the incubator cups, remove the dead ones, and clean the cup screens. Total numbers of eggs accounted for should always add up to within two of 50 or the entire batch is to be discarded. When larvae begin to hatch, generally after 4 to 6 days, they should not be handled again or removed from the egg-cups until all have hatched. Then, if enough are still alive, 40 of these are eligible to be transferred immediately to a larval test chamber. Those individuals selected out to bring the number kept to 40 should be chosen impartially. Entire egg-cup-groups not used for survival and growth studies should be counted and discarded.

2.9 Progeny Transfer

Additional important information on hatchability and larval survival is to be gained by transferring control eggs immediately after spawning to concentrations where spawning is reduced or absent, or to where an affect is seen on survival of eggs or larvae, and by transferring eggs from these concentrations to the control tanks. One larval chamber in, or corresponding to, each adult tank should always be reserved for eggs produced in that tank.

2.10 Larval Exposure

From early spawnings in each duplicate tank, use the larvae hatched in the egg incubator cups (Step 2.8. above) for 30 or 60 day growth and survival exposures in the larval chambers. Plan ahead in setting up eggs for hatchability so that a new group of larvae is ready to be tested for 30 or 60 days as soon as possible after the previously tested group comes out of the larval chambers. Record mortalities, and measure total lengths of larvae at 30 and, if they are kept, 60 days posthatch. At the time the larval test is terminated they should also be weighed. No fish (larvae, juveniles, or adults) should be fed within 24 hr's. of when they are to be weighed.

2.11 Parental Termination

Parental fish testing should be terminated when, during the receding day-length photoperiod, a one week period passes in which no spawning occurs in any of the tanks. Measure total lengths and weights of parental fish; check sex and condition of gonads. The gonads of most parental fish will have begun to regress from the spawning condition, and thus the differences between the sexes will be less distinct now than previously. Males and females that are readily distinguishable from one another because of their external characteristics should be selected initially for determining how to differentiate between testes and ovaries. One of the more obvious external characteristics of females that have spawned is an extended, transparent anal canal (urogenital papilla). The gonads of both sexes will be located just ventral to the kidneys. The ovaries of the females at this time will appear transparent, but perhaps containing some yellow pigment, coarsely granular, and larger than testes. The testes of males will appear as slender, slightly milky, and very finely granular strands. Fish must not be frozen before making these examinations.

2.12 Special Examinations

Fish and eggs obtained from the test should be considered for physiological, biochemical, histological and other examinations which may indicate certain toxicant-related effects.

2.13 Necessary Data

Data that must be reported for each tank of a chronic test are:

a. Number and individual total length of normal and deformed fish at 30 and 60 days; total length, weight and number of either sex, both normal and deformed, at end of test.
b. Mortality during the test.
c. Number of spawns and eggs.
d. Hatchability.
e. Fry survival, growth, and deformities.

3.0 CHEMICAL SYSTEM

3.1 Preparing a Stock Solution

If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or an organic solvent. Acetone has been the most widely used solvent, but dimethylformanide (DMF) and triethylene glycol may be preferred in many cases. If none of these solvents are acceptable, other water-miscible solvents such as methanol, ethanol, isopropanol, acetonitrile, dimethylacetamide (DMAC), 2-ethoxyethanol, glyme (dimethylether of ethylene glycol, diglyme (dimethyl ether of diethylene glycol) and propylene glycol should be considered. However, dimethyl sulfoxide (DMSO) should not be used if at all possible because of its biological properties.

Problems of rate of solubilization or solubility limit should be solved by mechanical means if at all possible. Solvents, or as a last resort, surfactants, can be used for this purpose, only after they have been proven to be necessary in the actual test system. The suggested surfactant is p-tert-octylphenoxynonaethoxy-ethanol (p-1, 1, 3, 3-tetramethylbutylphenoxynonaethoxy-ethanol, OPE_{10}) (Triton X-100, a product of the Rohm and Haas Company, or equivalent).

The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent grade or better should be used. The amount of an additive used should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one one-thousandth of the 96-hr. LC50 for test species under the test conditions and must never exceed one gram per liter of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed one-tenth gram per liter of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed.

3.2 Measurement of Toxicant Concentration

As a minimum, the concentration of toxicant must be measured in one tank at each toxicant concentration every week for each set of duplicate tanks, alternating tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank. Equivolume daily grab samples can be composited for a week if it has been shown that the results of the analysis are not affected by storage of the sample.

Enough grouped grab samples should be analyzed periodically throughout the test to determine whether or not the concentration of toxicant is reasonably constant from day to day in one tank and from one tank to its duplicate. If not, enough samples must be analyzed weekly throughout the test to show the variability of the toxicant concentration.

3.3 Measurement of Other Variables

Temperature must be recorded continuously (see 1.11.).

Dissolved oxygen must be measured in the tanks daily, at least five days a week on an alternating basis, so that each tank is analyzed once each week. However, if the toxicant or an additive causes a depression in dissolved oxygen, the toxicant concentration with the lowest dissolved oxygen concentration must be analyzed daily in addition to the above requirement.

A control and one test concentration must be analyzed weekly for pH, alkalinity, hardness, acidity, and conductance, or more often, if necessary, to show the variability in the test water. However, if any of these characteristics are affected by the toxicant, the tanks must be analyzed for that characteristic daily, at least five days a week, on an alternating basis so that each tank is analyzed once every other week.

At a minimum, the test water must be analyzed at the beginning and near the middle of the test for calcium, magnesium, sodium, potassium, chloride, sulfate, total solids, and total dissolved solids.

3.4 Residue Analysis

When possible and deemed necessary, mature fish, and possibly eggs, larvae, and juveniles, obtained from the test, should be analyzed for toxicant residues. For fish, muscle should be analyzed, and gill, blood, brain, liver, bone, kidney, GI tract, gonad, and skin should be considered for analysis. Analyses of whole organisms may be done in addition to, but should not be done in place of, analyses of individual tissues, especially muscle.

3.5 Methods

When they will provide the desired information with acceptable precision and accuracy, methods described in Methods for Chemical Analysis of Water and Wastes (EPA, 1971) should be used unless there is another method which requires much less time and can provide the desired information with the same or better precision and accuracy. At a minimum, accuracy should be measured using the method of known additions for all analytical methods for toxicants. If available, reference samples should be analyzed periodically for each analytical method.

4.0 STATISTICS

4.1 Duplicates

Use true duplicates for each level of toxic agent, i.e., no water connections between duplicate tanks.

4.2 Distribution of Tanks

The tanks should be assigned to locations by stratified random assignment (random assignment of one tank for each level of toxic agent in a row followed by random assignment of the second tank for each level of toxic agent in another or an extension of the same row).

4.3 Distribution of Test Organisms

The test organisms should be assigned to tanks by stratified random assignment (random assignment of one test organism to each tank, random assignment of a second test organism to each tank, etc.).

5.0 MISCELLANEOUS

5.1 Additional Information

All routine bioassay flow-through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater, (American Public Health Association, 1971), or information requested from appropriate persons at Duluth or Newtown.

5.2 Acknowledgments

These procedures for the fathead minnow were compiled by John Eaton for the Committee on Aquatic Bioassays. The participating members of this committee are: Robert Andrew, John Arthur, Duane Benoit, Gerald Bouck, William Brungs, Gary Chapman, John Eaton, John Hale, Kenneth Hokanson, James McKim, Quentin Pickering, Wesley Smith, Charles Stephan, and James Tucker.

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Appendix A
Test (Evansville, Indiana) Photoperiod
For Fathead Minnow Chronic

Dawn to Dusk Time	Date		Day-length (hour and minute)	
6:00 - 4:45)	DEC.	1	10:45)	
6:00 -4:30)		15	10:30)	
			)	
6:00 - 4:30)	JAN.	1	10:30)	
6:00 - 4:45)		15	10:45)	
			)	
6:00 - 5:15)	FEB.	1	11:15)	5-month pre-spawning
6:00 - 5:45)		15	11:45)	growth period
			)	
6:00 - 6:15)	MAR.	1	12:15)	
6:00 - 7:00)		15	13:00)	
			)	
6:00 - 7:30)	APR.	1	13:30)	
6:00 - 8:15)		15	14:15)	
6:00 - 8:45)	MAY	1	14:45)	
6:00 - 9:15)		15	15:15)	
			)	
6:00 - 9:30)	JUNE	1	15:30)	
6:00 - 9:45)		15	15:45)	4-month spawning
			)	period
6:00 - 9:45)	JULY	1	15:45)	
6:00 - 9:30)		15	15:30)	
			)	
6:00 - 9:00)	AUG.	1	15:00)	
6:00 - 8:30)		15	14:30)	
6:00 - 8:00)	SEPT.	1	14:00)	
6:00 - 7:30)		15	13:30)	
			)	
6:00 - 6:45)	OCT.	1	12:45)	post spawning period
6:00 - 6:15)		15	12:15)	
			)	
6:00 - 5:30)	NOV.	1	11:30)	
6:00 - 5:00)		15	11:00)	

Brook Trout *Salvelinus fontinales*
(Mitchill) Partial Chronic Tests
April, 1971
(Revised January, 1972)

1.0 PHYSICAL SYSTEM

1.1 Diluter

Proportional diluters (Mount and Brungs, 1967) should be employed for all long-term exposures. Check the operation of the diluter daily, either directly or through the measurement of toxicant concentrations. A minimum of five toxicant concentrations and one control should be used for each test with a dilution factor of not less than 0.30. An automatically triggered emergency aeration and alarm system must be installed to alert staff in case of diluter, temperature control or water supply failure.

1.2 Toxicant Mixing

A container to promote mixing of toxicant-bearing and w-cell water should be used between diluter and tanks for each concentration. Separate delivery tubes should run from this container to each duplicate tank. Check to see that the same amount of water goes to duplicate tanks and that the toxicant concentration is the same in both.

1.3 Tank

Each duplicate spawning tank (preferably stainless steel) should measure 1.3 X 3 X 1 ft. wide with a water depth of 1 foot and alevin-juvenile growth chambers (glass or stainless steel with glass bottom) 7 X 15 X 5 in. wide with a water depth of 5 inches. Growth chambers can be supplied test water by either separate delivery tubes from the mixing cells described in Step 2 above or from test water delivered from the mixing cell to each duplicate spawning tank. In the second choice, test water must always flow through growth chambers before entering the spawning tank. Each growth chamber should be designed so that the test water can be drained down to 1 inch and the chamber transferred over a fluorescent light box for photographing the fish (see 2.10).

1.4 Flow Rate

Flow rates for each duplicate spawning tank and growth chamber should be 6-10 tank volumes/24 hr.

1.5 Aeration

Brook trout tanks and growth chambers must be aerated with oil free air unless there are no flow limitations and 60% of saturation can be maintained. Total dissolved oxygen levels should *never* be allowed to drop below 60% of saturation.

1.6 Cleaning

All tanks and chambers must be siphoned daily and brushed at least once per week. When spawning commences, gravel baskets must be removed and cleaned daily.

1.7 Spawning Substrates

Use two spawning substrates per duplicate made of plastic or stainless steel which measure at least 6 X 10 X 12 in. with 2 inches of .25 to .50 inch stream gravel covering the bottom and 20 mesh stainless steel or nylon screen attached to the ends for circulation of water.

1.8 Egg Cup

Egg incubation cups are made from 4-oz. 2-inch OD round glass jars with the bottoms cut off and replaced with stainless steel or nylon screen (40 meshes per inch). Cups are oscillated in the test water by means of a rocker arm apparatus driven by a 2 r.p.m. electric motor (Mount, 1968).

1.9 Light

The lights used should simulate sunlight as nearly as possible. A combination of Duro-Test (Optima FS)[1,2] and wide spectrum Gro-lux[3] fluorescent tubes has proved satisfactory at the NWQL.

[1]Mention of trade names does not constitute endorsement.
[2]Duro-Test, Inc., Hammond, Ind.
[3]Sylvania, Inc., New York, N. Y.

1.10 Photoperiod

The photoperiods to be used (Appendix A) simulate the dawn to dusk times of Evansville, Indiana. Evansville dates must correspond to actual dates in order to avoid putting natural reproductive cycles out of phase. Adjustments in photoperiod are to be made on the first and fifteenth of every Evansville test month. The table is arranged so that adjustments need be made only in the dusk times. The dawn and dusk times listed in the table (Evansville test time) need *not* correspond to the actual test times where the test is being conducted. To illustrate this point, a test started on March first would require the use of the photoperiod for Evansville test date March first, and the lights could go on any time on that day just so long as they remained on for twelve hours and fifteen minutes. Fifteen days later the photoperiod would be changed to thirteen hours. Gradual changes in light intensity at dawn and dusk (Drummond and Dawson, 1970), may be included within the photoperiods shown, and should not last for more than 1/2 hour from full on to full off and vice versa.

1.11 Temperature

Utilize the attached temperature regime (see Appendix B). Temperatures should not deviate instantaneously from the specified test temperature by more than 2°C and should not remain outside the specified temperature ±1°C for more than 48 hours at a time.

1.12 Disturbance

Spawning tanks and growth chambers must be covered with a screen to confine the fish and concealed in such a way that the fish will not be disturbed by persons continually walking past the system. Tanks and chambers must also be shielded from extraneous light which can affect the intended photoperiod or damage light-sensitive eggs and alevins.

1.13 Construction Materials

Construction materials which contact the diluent water should not contain leachable substances and should not sorb significant amounts of substances from the water. Stainless steel is probably the preferred construction material. Glass absorbs some trace organics significantly. Rubber should not be used. Plastic containing fillers, additives, stabilizers, plasticizers, etc., should not be used. Teflon, nylon, and their equivalents should not contain leachable materials and should not sorb significant amounts of most substances. Unplasticized polyethylene and polypropylene should not contain leachable substances, but may sorb very significant amounts of trace organic compounds.

1.14 Water

The water used should be from a well or spring if at all possible, or alternatively from a surface water source. Only as a last resort should water from a chlorinated municipal water supply be used. If it is thought that the water supply could be conceivably contaminated with fish pathogens, the water should be passed through an ultraviolet or similar sterilizer immediately before it enters the test system.

2.0 BIOLOGICAL SYSTEM

2.1 Test Animals

Yearling fish should be collected no later than March 1 and acclimated in the laboratory to test temperature and water quality for at least one month before the test is initiated. Suitability of fish for testing should be judged on the basis of acceptance of food, apparent lack of diseases, and 2% or less mortality during acclimation with no mortality two weeks prior to test. Set aside enough fish to supply an adequate number for short-term bioassay exposures used in determining application factors.

2.2 Beginning Test

Begin exposure no later than April 1 by distributing 12 acclimated yearling brook trout per duplicate using a stratified random assignment (see 4.3). This allows about a four-month exposure to the toxicant before the onset of secondary or rapid growth phase of the gonads.

Extra test animals may be added at the beginning so that fish can be removed periodically for special examinations (see 2.13), or for residue analysis (see 3.4).

2.3 Food

Use a good frozen trout food (e.g., Oregon Moist). Fish should be fed the largest pellet they will take a minimum of two times daily. The amount should be based on a reliable hatchery feeding schedule. Alevins and early juveniles should be fed trout starter a minimum of five times daily. Each batch of prepared food should be checked for pesticides (including DDT, TDE, dieldrin, endrin, aldrin, BHC, chlordane, toxaphene, 2,4-D, and PCBs), and the kinds and amounts should be reported to the project officer or recorded.

2.4 Disease

Handle disease outbreaks according to their nature, with all tanks receiving the same treatment whether there seems to be sick fish in all of them or not. The frequency of treatment should be held to a minimum.

2.5 Measuring Fish

Record mortalities daily, and measure fish directly at initiation of test, after three months and at thinning (see 2.6) (total length and weight). Fish should not be fed 24 hours before weighing and lightly anesthetized with MS-222 to facilitate measuring (100 mg MS-222/liter water).

2.6 Thinning

When secondary sexual characteristics are well developed (approximately two weeks prior to expected spawning), separate males, females and undeveloped fish in each duplicate and randomly reduce sexually mature fish (see 4.4) to the desired number of 2 males and 4 females, and discard undeveloped fish after examination. Place two spawning substrates (described earlier) in each duplicate. Record the number of mature, immature, deformed and injured males and females in each tank and the number from each category discarded. Measure total length and weight of all fish in each category before any are discarded and note which ones were discarded.

2.7 Removing Eggs

Remove eggs from the redd at a fixed time each day (preferably after 1:00 p.m. Evansville time, so the fish are not disturbed during the morning).

2.8 Egg Incubation and Viability

Impartially select 50 eggs from the first eight spawnings of 50 eggs or more in each duplicate and place them in an egg incubator cup for hatch. The remaining eggs from the first eight spawnings (>50 eggs) and all subsequent eggs from spawnings should be counted and placed in separate egg incubator cups for determining viability (formation of neural keel after 11-12 days at 9°C). The number of dead eggs from each spawn removed from the nest should be recorded and discarded. Never place more than 250 eggs in one egg incubator cup. All eggs incubated for viability are discarded after 12 days. Discarded eggs can be used for residue analysis and physiological measurements of toxicant-related effects.

2.9 Progeny Transfer

Additional important information on hatchability and alevin survival can be gained by transferring control eggs immediately after spawning to concentrations where spawning is reduced or absent, or to where an affect is seen on survival of eggs or alevin, and by transferring eggs from these concentrations to the control tanks. Two growth chambers for each duplicate spawning tank should always be reserved for eggs produced in that tank.

2.10 Hatch and Alevin Thinning

Remove dead eggs daily from the hatchability cups described in Step 2.8 above. When hatching commences, record the number hatched daily in each cup. Upon completion of hatch in any cup, randomly (see 4.4) select 25 alevins from that cup. Dead or deformed alevins must not be included in the random selection but should be counted as being dead or deformed upon hatch. Measure total lengths of the 25 selected and discarded alevins. Total lengths are measured by the photographic method used by McKim and Benoit (1971). The fish are transferred to a glass box containing 1 inch of test water. They should be moved to and from this box in a water filled container, rather than by netting them. The glass box is placed on a translucent millimeter grid over a fluorescent light box which provides background illumination. Photos are then taken of the fish over the millimeter grid and are enlarged into 8 X 10 inch prints. The length of each fish is subsequently determined by comparing it to the grid. Keep lengths of discarded alevins separate from those which are kept. Place the 25 selected alevins back into the incubator cup and preserve the discarded ones for initial weights.

2.11 Alevin-Juvenile Exposure

Randomly (see 4.4) select from the incubation cups two groups of 25 alevins each per duplicate for 90-day growth and survival exposures in the growth chambers. Hatching from one spawn may be spread out over a 3-to 6-day period; therefore, the median-hatch date should be used to establish the 90-day growth and survival period for each of the two groups of alevins. If it is determined that the median-hatch dates for the five groups per duplicate will be more than three weeks apart, then the two groups of 25 alevins must be selected from those which are less than three weeks old. The remaining groups in the duplicate which do not hatch during the three-week period are used only for hatchability results and then photographed for lengths and preserved for initial weights. In order to equalize the effects of the incubation cups on growth, all groups selected for the 90-day exposure must remain in the incubation cups three weeks before they are released into the growth chambers. Each of the two groups selected per duplicate must be kept separate during the 90-day period. Record mortalities daily, along with total lengths 30 and 60 days post-hatch, and total length and weight at 90 days post-hatch. Alevins and early juveniles should not be fed 24 hours before weighing. Total lengths are measured by transferring the growth chambers described earlier to a translucent millimeter grid over a fluorescent light box for photographing as described in Step 2.10 above. Survival and growth studies should be terminated after three months. Terminated fish can be used for tissue residue analysis and physiological measurements of toxicant-related effects.

2.12 Parental Termination

All parental fish should be terminated when a three-week period passes in which no spawning occurs in any of the spawning tanks. Record mortality and weigh and measure total length of parental fish, check sex and condition of gonads (e.g., reabsorption, degree of maturation, spent ovaries, etc.) (see 3.4).

2.13 Special Examinations

Fish and eggs obtained from the test should be considered for physiological, biochemical, and histological investigations which may indicate certain toxicant-related effects.

2.14 Necessary Data

Data that must be reported for each tank of a chronic test are:

a. Number and individual weights and total lengths of normal, deformed, and injured mature and immature males and females at initiation of test, three months after test commences, at thinning and at the end of test.
b. Mortality during the test.
c. Number of spawns and eggs. A mean incubation time should be calculated using date of spawning and the median-hatch dates.
d. Hatchability.
e. Fry survival, growth and deformities.

3.0 CHEMICAL SYSTEM

3.1 Preparing a Stock Solution

If a toxicant cannot be introduced into the test water as is, a stock solution should be prepared by dissolving the toxicant in water or an organic solvent. Acetone has been the most widely used solvent, but dimethylformanide (DMF) and triethylene glycol may be preferred in many cases. If none of these solvents are acceptable, other water-miscible solvents such as methanol, ethanol, isopropanol, acetonitrile, dimethylacetamide (DMAC), 2-ethoxyethanol, glyme (dimethylether of ethylene glycol), diglyme (dimethyl ether of diethylene glycol) and propylene glycol should be considered. However, dimethyl sulfoxide (DMSO) should not be used if at all possible because of its biological properties.

Problems of rate of solubilization or solubility limit should be solved by mechanical means if at all possible. Solvents, or as a last resort, surfactants, can be used for this purpose only after they have been proven to be necessary in the actual test system. The suggested surfactant is p-tert-octylphenoxynonaethoxyethanol (p-1, 1, 3, 3-tetramethylbutylphenoxynonaethoxyethanol, OPE_{10}) (Triton X-100, a product of the Rohm and Haas Company, or equivalent).

The use of solvents, surfactants, or other additives should be avoided whenever possible. If an additive is necessary, reagent grade or better should be used. The amount of an additive used should be kept to a minimum, but the calculated concentration of a solvent to which any test organisms are exposed must never exceed one one-thousandth of the 96-hr. LC50 for test species under the test conditions and must never exceed one gram per liter of water. The calculated concentration of surfactant or other additive to which any test organisms are exposed must never exceed one-twentieth of the concentration of the toxicant and must never exceed one-tenth gram per liter of water. If any additive is used, two sets of controls must be used, one exposed to no additives and one exposed to the highest level of additives to which any other organisms in the test are exposed.

3.2 Measurement of Toxicant Concentration

As a minimum, the concentration of toxicant must be measured in one tank at each toxicant concentration every week for each set of duplicate tanks, alternating tanks at each concentration from week to week. Water samples should be taken about midway between the top and bottom and the sides of the tank and should not include any surface scum or material stirred up from the bottom or sides of the tank. Equivolume daily grab samples can be composited for a week if it has been shown that the results of the analysis are not affected by storage of the sample.

Enough grouped grab samples should be analyzed periodically throughout the test to determine whether or not the concentation of toxicant is reasonably constant from day to day in one tank and from one tank to its duplicate. If not, enough samples must be analyzed weekly throughout the test to show the variability of the toxicant concentration.

3.3 Measurement of Other Variables

Temperature must be recorded continuously (see 1.11).

Dissolved oxygen must be measured in the tanks daily at least five days a week on an alternating basis, so that each tank is analyzed once each week. However, if the toxicant or an additive causes a depression in dissolved oxygen, the toxicant concentration with the lowest dissolved oxygen concentration must be analyzed daily in addition to the above requirement.

A control and one test concentration must be analyzed weekly for pH, alkalinity, hardness, acidity, and conductance, or more often, if necessary, to show the variability in the test water. However, if any of these characteristics are affected by the toxicant, the tanks must be analyzed for that characteristic daily, at least five days a week, on an alternating basis, so that each tank is analyzed once every other week.

At a minimum, the test water must be analyzed at the beginning and near the middle of the chronic test for calcium, magnesium, sodium, potassium, chloride, sulfate, conductance, total solid, and total dissolved solids.

3.4 Residue Analysis

When possible and deemed necessary, mature fish, and possibly eggs, larvae, and juveniles, obtained from the test, should be analyzed for toxicant residues. For fish, muscle should be analyzed, and gill, blood, brain, liver, bone, kidney, GI tract, gonad, and skin should be considered for analysis. Analyses of whole organisms may be done in addition to, but should not be done in place of, analyses of individual tissues, especially muscle.

3.5 Methods

When they will provide the desired information with acceptable precision and accuracy, methods described in Methods for Chemical Analysis of Water and Wastes (EPA, 1971) should be used unless there is another method which requires much less time and can provide the desired information with the same or better precision and accuracy. At a minimum, accuracy should be measured using the method of known additions for all analytical methods for toxicants. If available, reference samples should be analyzed periodically for each analytical method.

4.0 STATISTICS

4.1 Duplicates

Use true duplicates for each level of the toxic agent, i.e., no water connections between duplicate tanks.

4.2 Distribution of Tanks

The tanks should be assigned to locations by stratified random assignment (random assignment of one tank for each level of the toxic agent in a row, followed by random assignment of the second tank for each level of the toxic agent in another or an extension of the same row).

4.3 Distribution of Test Organisms

The test organisms should be assigned to tanks by stratified random assignment (random assignment of one test organism to each tank, random assignment of a second test organism to each tank, etc.).

4.4 Selection and Thinning Test Organisms

At time of selection or thinning of test organisms the choice must be random (random, as defined statistically).

5.0 MISCELLANEOUS

5.1 Additional Information

All routine bioassay flow-through methods not covered in this procedure (e.g., physical and chemical determinations, handling of fish) should be followed as described in Standard Methods for the Examination of Water and Wastewater (American Public Health Association, 1971).

5.2 Acknowledgments

These procedures for the brook trout were compiled by J. M. McKim and D. A. Benoit for the Committee on Aquatic Bioassays. The participating members of this committee are: Robert Andrew, John Arthur, Duane Benoit, Gerald Bouck, William Brungs, Gary Chapman, John Eaton, John Hale, Kenneth Hokanson, James McKim, Quentin Pickering, Wesley Smith, Charles Stephan, and James Tucker.

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Appendix A
Test (Evansville, Indiana) Photoperiod
For Brook Trout Partial Chronic

Dawn to Dusk Time	Date		Day-length (hour and minute)	
6:00 - 6:15)	MAR.	1	12:15)	
6:00 - 7:00)		15	13:00)	
			)	
6:00 - 7:30)	APR.	1	13:30)	
6:00 - 8:15)		15	14:15)	
			)	
6:00 - 8:45)	MAY	1	14:45)	
6:00 - 9:15)		15	15:15)	
			)	
6:00 - 9:30)	JUNE	1	15:30)	Juvenile-adult exposure
6:00 - 9:45)		15	15:45)	
			)	
6:00 - 9:45)	JULY	1	15:45)	
6:00 - 9:30)		15	15:30)	
			)	
6:00 - 9:00)	AUG.	1	15:00)	
6:00 - 8:30)		15	14:30)	
			)	
6:00 - 8:00)	SEPT.	1	14:00)	
6:00 - 7:30)		15	13:30)	
6:00 - 6:45)	OCT.	1	12:45)	
6:00 - 6:15)		15	12:15)	
			)	Spawning and egg incubation
6:00 - 5:30)	NOV.	1	11:30)	
6:00 - 5:00)		15	11:00)	
6:00 - 4:45)	DEC.	1	10:45)	
6:00 - 4:30)		15	10:30)	
			)	
6:00 - 4:30)	JAN.	1	10:30)	Alevin-juvenile exposure
6:00 - 4:45)		15	10:45)	
			)	
6:00 - 5:15)	FEB.	1	11:15)	
6:00 - 5:45)		15	11:45)	

Appendix B
Temperature Regime for Brook Trout Partial Chronic

Months		Temperature ° C	
Mar.	Juvenile-adult exposure	9	
Apr.		12	
May		14	
June		15	
July		15	
Aug.		15	
Sept.		12	
Oct.	Spawning and egg incubation	9	A constant temperature must be established just prior to spawning and egg incubation, and maintained throughout the 3-month alevin-juvenile exposure.
Nov.		9	
Dec.	Alevin-juvenile exposure	9	
Jan.		9	
Feb.		9	
Mar.		9	

APPENDIX

APPENDIX

1.0 BENCH SHEETS

1.1 Phytoplankton Sedgwick-Rafter Count

Phytoplankton Sedgwick-Rafter Count

River or Lake__________ Date Analyzed__________ Station No.__________

Station__________ Analyzed by __________ Date Collected __________

State __________

CODE	ORGANISM		TALLY	C/ML.	TOTALS
	Total coccoid blue-green algae per ml.				
	Total filamentous blue-green algae				
	Total coccoid green algae				
	Total filamentous green algae				
	Total green flagellates				
	Total other pigmented flagellates				

Centrics			c/ml.

Pennates			c/ml.

Most Abundant Algae

Diatoms

Centric	Melos.	Others	Totals	c/ml
Shells				
Live				

Total live centric diatoms

	c/ml.
Pennate Shells	
Live Pennates	

Total live pennate diatoms

S-R Factor:

TOTAL LIVE ALGAE (c/ml) __________

Remarks:

First check__________ Wash. sheet__________

Recorded __________ Wash. sheet checked__________

1.2 Zooplankton Count

Zooplankton Count

CODE	ORGANISM	TALLY	C/LITER
	ROTIFERA		
	Keratella		
	Brachionus		
	Polyarthra		
	Synchaeta		
	Trichocera		
		Total Rotifers per liter	
	CLADOCERA		
	Bosmina		
	Daphnia		
	Moina		
	Ceriodaphnia		
	COPEPODA		
	Nauplii		
	Cyclops & related genera		
	Diaptomus		
		Total Crustacea per liter	
	NEMATODES (per liter)		
	OTHER INVERTEBRATES: (per liter)		

Most Abundant Rotifers	

Most Abundant Crustacea	

Factor ______________

Analyzed by ______________

Date Analyzed ______________

1.3 Plankton and Periphyton Diatom Analysis

PLANKTON AND PERIPHYTON
DIATOM ANALYSIS

River ____________ Station ____________ State ____________

Live Centrics ________ Dead Centrics ________

Live Pennates ________ Dead Pennates ________

Total Live ________ Total Dead ________

S-R Count ________

Station Number ________

Date Collected ________

Analyzed by ________

Date Analyzed ________

Counting Time

Species	Total	%
Coscinodiscus		
Cyclotella		
Meneghiniana		
Melosira		
ambigua		
granulata		
distans		
Rhizosolenia		
Stephanodiscus		
hantzschii		
invisitatus		
astrea minutula		
Other centrics		
Achnanthes		
Amphiprora		
Amphora		
Asterionella formosa		
Caloneis		
Cocconeis		
Cymatopleura		
Cymbella		
Diatoma vulgare		
Diploneis smithii		
Epithemia		
Eunotia		

Species	Total	%
Fragilaria crotonensis		
construens		
Frustulia		
Gomphonema		
Gomphoneis		
Gyrosigma		
Meridion circulare		
Navicula		
Nitzschia		
Pinnularia		
Pleurosigma		
Rhoicosphenia curvata		
Stauroneis		
Rhopalodia		
Surirella		
Synedra		
ulna		
acus		
Tabellaria		
fenestrata		
flocculosa		

	FIRST	SECOND	THIRD	FOURTH	Percent others
Code					
%					

No. species ____________

Remarks:

Total count

1.4 Periphyton Sedgwick-Rafter Count

PERIPHYTON SEDGWICK-RAFTER COUNT

River or Lake______________ Inclusive Dates______________

Station______________ Date Analyzed______________

State______________ Analyzed by______________

CODE	ORGANISM	Tally	c/mm^2
	Total coccoid blue-green algae		
	Total filamentous blue-green algae		
	Total coccoid green algae		
	Total filamentous green algae		
	Total green flagellates		
	Other coccoid algae		
	Other pigmented flagellates		
	Filamentous bacteria and fungi		
	Protozoa		

Centrics	c/mm^2

Pennates	c/mm^2

Diatoms		
Centric Shells		
Live Centrics		
Total live centric diatoms		
Pennate Shells		
Live Pennates		
Total live pennate diatoms		

Preservative______________
No. Slides______________
Area Scraped______________
Scrapings diluted to ____mls.

S-R Factor__________

Remarks:

TOTAL (cells/mm^2) [ ]

First check__Recorded __

1.5 Plankton and Periphyton Pigment and Biomass

PLANKTON AND PERIPHYTON
CHLOROPHYLL AND BIOMASS DATA

I. IDENTIFYING INFORMATION:

A. Station: ______________________

B. Date: ______________________

C. Method of Sample Collection and Handling: ______________________

II. SPECTROPHOTOMETER DATA:

A. OPTICAL DENSITY MEASUREMENTS:
Instrument Used: ______________________

Rep.	Extract Volume	Dilution Factor	Optical Density Readings 750	663b*	645	630	663a*	663 b/a
1.								
2.								
3.								
4.								

*(b = before acidification; a = after acidification)

B. CHLOROPHYLL CALCULATIONS:

Rep.	Concentration of Chlorophyll in Extract (mg/l) Chl a	Chl b	Chl c	Sample area or volume (liters; m^2)	Chlorophyll content of sample (ug/l; mg/m^2) Chl a	Chl b	Chl c
1.							
2.							
3.							
4.							

III. FLUOROMETER DATA:

Instrument Used: ______________________

Rep.	Dilution Factor	Reading Before (b) Acidification: Reading R_b	Sens. Level (S)	Reading After (a) Acidification: R_a	(S)	R_b/R_a
1.						
2.						
3.						
4.						

IV. ORGANIC MATTER (ASH-FREE WEIGHT)

Rep.	Cruc. No.	Empty Crucible Weight (A)	Weight with Dry Sample (B)	Weight After Firing (C)	Sample Dry Weight (B-A)	Ash Free Weight (B-C)	Organic Matter (gm/m^2)
1.							
2.							
3.							
4.							

V. REMARKS:

1.6 Macroinvertebrates

MACROINVERTEBRATE LAB BENCH SHEET

Name of water body ______________________ Lot No. ____________
Collected by ______________________ Station No. ____________
Sorted by ______________________ Date collected ____________

*	DIPTERA	L(N)[1]	P[1]	TOTAL #	DRY WGT (mg)	*	CRUSTACEA	TOTAL #	DRY WGT
	TRICHOPTERA						HIRUDINEA		
	PLECOPTERA						NEMATODA		
	EPHEMEROPTERA						BIVALVIA		
	ODONATA						GASTROPODA		
	NEUROPTERA						OTHER		
	HEMIPTERA								
	COLEOPTERA								

Total # of organisms ____________ Total dry weight ____________
Total # of taxa ____________ Ash-free weight ____________

* Initials of taxonomists in this column

1 L=larvae, N = nymph, P = pupae

2.0 EQUIPMENT AND SUPPLIES

This section contains an abbreviated list of equipment and supplies used for the collection and analysis of biological samples. The companies and addresses are listed alphabetically at the end of the table. Mention of commercial sources or products in this section does not constitute endorsement by the U. S. Environmental Protection Agency.

Item	Source*	Cat. No.	Unit	Approx. Cost (1973)
2.1 Plankton and Periphyton				
Sampling and field equipment		*		
Water sampler, alpha bottle, nonmetallic, transparent, 6 liter	(30)	1160TT		$ 150.00
Plankton sampler, Clarke-Bumpus, 12 inch, with No. 10 and No. 20 nets and buckets	(30)	37		400.00
Plankton towing net, No. 20 (173 mesh/inch)	(30)			41.00
Plankton net with bucket, Wisconsin style, No. 20 net (173 mesh/inch)	(30)			92.00
Submarine photometer, with deck cell	(7)			500.00
Laboratory equipment				
Balance, analytical, 100 gm capacity, accuracy 0.1 mg.				1,000.00
Balance, Harvard Trip, double beam, (to balance loaded centrifuge tubes)				50.00
Centrifuge, clinical, Centricone, 8-place				100.00
Centrifuge, IEC, model UV, Refrigerated				850.00
Centrifuge head, 8-place, 100 ml				50.00
Centrifuge shields, cups			8	30.00
Centrifuge trunnion rings			8	20.00
Centrifuge tubes, plain, round bottom, polypropylene, 100 ml			16	9.00
Blood Cell Calculator (counter), 8-Key	(24)	2944-B50		110.00
Fluorometer, Turner 111 or equivalent, equipped with:	(25)			2,000.00
Red-sensitive photomultiplier tube No. R-136				
Turner No. 110-853 blue lamp, T-5				
Turner No. 110-856, lamp adaptor for T-5 lamp				
Turner No. 110-005, Standard sample holder				
Turner No. 110- , High-Sensitivity sample holder				
Turner No. 110-871, flow-through cuvette				
Corning filter No. CS-5-60 (excitation)				
Corning filter No. CS-2-64 (emission)				
Disposable vials for fluorometer, 12 X 75 mm, 5 ml, Kahn type				
Hot-plate, Thermolyne HP-A1915B, thermostatically controlled (to dry diatoms on cover glasses), 115 volts, 750 watts.				30.00
Hot-plate, Chromalox, 230 volts, 2000 watt, AC, three heat (to incinerate diatom preparation on cover glasses).				30.00
Microscope and accessories (Americal Optical, Series 10T Trinocular Microstar, or equivalent).				1,500.00
In-base illuminator and transformer.				
Trinocular body.				
Graduated mechanical stage.				
Quadruple nose piece.				
N.A. 1.25 condenser.				
Condenser mount.				
Objective, 4X, Achromatic.				
Objective, 10X, Achromatic,				
Objective, 20X, Achromatic, standard, must have working distance greater than 1 mm for Sedgwick-Rafter counts.				
Objective, 45X, Achromatic.				
Objective, 100X, Achromatic.				
Wide field eyepieces, 10X,				

*See list of suppliers at the end of this table.

Item	Source	Cat. No.	Unit	Approx. Cost (1973)
Light meter	(29)	Model 756		100.00
Muffle furnace, 1635 Temco, Thermolyne, 240 volts				180.00
Temperature control for muffle furnace, Amplitrol Proportioning Controller, 0-2400°F, for 240 volt furnace (recommended for use with Temco 1635).				230.00
Oven, Thermozone, forced draft, double walled, three shelves, 230°C.				350.00
Pipetting machine, automatic, large, BBL. (for dispensing preservative).	(24)	7750-M10		320.00
*Spectrophotometer, double-beam, recording, resolution 2 nm or better at 663 nm; Coleman-124 or equivalent.	(1)			
Washer, mechanical, glassware, variable speed, Southern Cross, Model 300-B-2, Complete.				330.00
Supplies				
Cubitainer, 1 qt (approx 1 liter)	(8)		1 doz.	7.00
Cubitainer shipping carton, 1 qt	(8)		1 doz.	4.00
Bottles, pill, square, DURAGLAS, 3 ounce for periphyton samples. Do not use caps supplied with bottles.		clear glass	½ gross	8.00
		amber glass	½ gross	15.00
Caps, Polyseal, black, size 38, G. C.M.I. thread No. 400. Use on Duraglas bottles above.	(16)		½ gross	11.00
Crucibles, Coors, high form, porcelain. size 1, capacity 30 ml	(24)	3319-B55	Case (36)	25.00
Crucible covers for above, Size G	(24)	3319-D47	Case (72)	20.00
Desiccator, aluminum, with shelf	(24)	3747-C10		22.00
Merthiolate, powder No. 20, (Thimerosal, N.F.)	(13)		¼ ounce	2.00
			1 ounce	7.00
			1 pound	95.00
Metal plate, 5 X 10 X 1/8 inches, steel (to transfer cover glasses between hot-plates).				
Micrometer, eye-piece, whipple	(16)			18.00
Micrometer, stage (American Optical)	(16)	400		32.00
Mounting medium, HYRAX	(3)		1 ounce	10.00
Pipettes, disposable, Pasteur type, 5-3/4 inches	(23)	P5205-2	2½ gross	8.00
Sedgwick-Rafter Counting Chamber, as prescribed by "Standard Methods for the examination of Water and Wastes."	(30)	1801		9.00
Tissue grinder, glass, Duall, complete	(12)	size C		10.00
Vials, Opticlear, Owens-Illinois, 3 drams, snap caps, for diatom preparation.	(21)	SK-3	Gross	11.00
2.2 Macroinvertebrates				
Boat, flat bottom, 14-16 feet, Arkansas Traveler or Boston Whaler with winch and davit, snatch-block meter wheel, and trailer, 18 hp Outboard motor, Life jackets, other accessories	(17)			3,000.00
Cable fastening tools:	(20)			
Cable clamps, 1/8 inch			25	3.00
Nicro-press sleeves, 1/8 inch			100	6.00
Nicro-press tool, 1/8 inch			1	32.00
Wire cutter, Felco		7	1	7.00
Wire thimbles, 1/8 inch			25	2.00
Cable, 1/8 inch, galvanized steel			1000 feet	89.00
Large capacity, metal wash tubs			1	3.00
Core sampler, K. B., multiple, and gravity corers	(30)	2400	1	225.00
Hardboard multiplate sampler	(30)		1	7.50
Trawl net	(30)			100.00
Drift net, stream	(30)	15	2	76.00
Grabs				
Ponar	(30)	1725	1	200.00
Ekman, 6 X 6 inch	(30)	196B	1	78.00
Petersen, 100 square inch	(30)	1750	1	200.00
Weights for Petersen	(30)	1751	1 pair	25.00

Item	Source	Cat. No.	Unit	Approx. Cost (1973)
Basket, Bar-B-Q, (RB-75) Tumbler	(22)	1	12	25.00
Sieve, US standard No. 30 (0.595 mm opening) and others as needed	(26)	V 73250 L	1 each	10.00
Flow meter, TSK, (propeller type)	(10)	313 T.S.		200.00
Flow meter, electromagnetic, two-axis	(15)			2,600.00
Mounting media, CMC-9AF	(6)		4 ounce	2.00
Mounting media, CMC-S	(6)		4 ounce	2.00
Low-temp bath	(31)	94370	1	500.00
Water pump, epoxy-encapsulated, submersible and open air.	(14)	1A-MD	2	50.00
Sounding equipment and specialized gear	(7,9,11)			
Large, constant temperature holding tanks with 1/3 hp water chiller, charcoal	(5)	MT-700	1	540.00
Polyethylene bottles, dark bottles, tubing	(18)			
Cahn electrobalance	(27)	DTL	1	1,000.00
Porcelain balls for baskets (2-inch diameter)	(4)	unlapped	1 pound	0.30
Porcelain multiplates	(4)		1	7.50
Counter, differential, 9 unit, Clay-Adams	(23)	B 4120-4	1	105.00
Counter, hand tally	(24)	3297-H10	2	11.00
Magnifier, Dazor, 2X, floating, with illuminator and base.	(6)	375 A 95	1	50.00
Microscope, compound, trinocular, equipped for bright-field and phase microscopy with 10X and 15X wide-field oculars, 4.0 X, 10X, 20X, 45X, and 100X bright-field objectives, and 45X and 100X phase objectives.			1	2,000.00
Stereoscopic dissecting Microscope	(32)		1	1,000.00
Tessovar photomacrographic Zoom System		49-65-01	1	1,779.00
Camera body, 35 mm Zeiss Contarex, for Tessovar	(32)	10-2611	1	600.00
Stirrer, magnetic	(6)	375AA4514	1	42.50
Aquaria (of various sizes)	(6)			
Aquatic dip nets	(6)			
Microscope Slides and Cover slips, Standard square, 15 mm	(6)	320A 10	10 gross	31.00
		320A210	1 ounce	3.50
Vials, specimen, glass, 1 dram, 15 mm X 45 mm	(6)	315A 57	10 gross	78.00
Petri dish, ruled grid, 150 mm X 15 mm	(2)	315AA4094	12	24.00
Freeze dryer with freezing shelf	(28)	10-800	1	4,000.00
Vacuum oven	(19)	5831	1	300.00

Sources of equipment and supplies for plankton, periphyton, and macroinvertebrates

1. Coleman Instruments
   42 Madison St.
   Maywood, IL 60153
2. Corning Glass Works
   1470 Merchandise Mart
   Chicago, IL 60654
3. Custom Research and Development Company, Inc.
   Mt. Vernon Rd., Route 1, Box 1586
   Auburn, CA 95603
4. Ferro Corporation
   P. O. Box 20
   East Liverpool, OH 43920
5. Frigid Units, Inc.
   3214 Sylvania Ave.
   Toledo, OH 43613
6. General Biological Inc.
   8200 S. Hoyne Ave.
   Chicago, IL 60620
7. G-M Manufacturing & Instrument Company
   2417 Third Ave.
   New York, NY 10451
8. Hedwin Corporation
   1209 E. Lincolnway
   Laporte, IN 46350
9. Hydro Products
   11777 Sorrento Valley Rd.
   San Diego, CA 92121
10. Inter Ocean, Inc.
    3446 Kurtz St.
    San Diego, CA 92110
11. Kahl Scientific Instruments
    P. O. Box 1166
    El Cajon, CA 92022
12. Kontes Glass Company
    Vineland, NJ 08360
13. Eli Lilly Company
    307 E. McCarty St.
    Indianapolis, IN 46206
14. March Manufacturing Company
    Glenview, IL 60025
15. Marsh-McBirney, Inc.
    2281 Lewis Ave.
    Rockville, MD 20851
16. Matheson Scientific
    1850 Greenleaf Ave.
    Elk Grove Village, IL 60007
17. MonArk Boat Company
    Monticello, AK 71655
18. Nalge Corporation
    Rochester, NY 14602
19. National Appliance Company
    P. O. Box 23008
    Portland, OR 97223
20. National Telephone Supply Company
    3100 Superior St.
    Cleveland, OH 44114
21. Owens-Illinois
    P. O. Box 1035
    Toledo, OH 43666
22. Paramont Wire, Inc.
    1035 Westminster Ave.
    Alhambra, CA 91803
23. Scientific Products
    1210 Leon Place
    Evanston, IL 60201
24. Arthur H. Thomas Company
    Vine Street at Third
    P. O. Box 779
    Philadelphia, PA 19105
25. G. K. Turner, Assoc.
    2524 Pulgas Ave.
    Palo Alto, CA 94303
26. W. S. Tyler Company
    Mentor, OH 44060
27. Ventron Instrument Corporation
    7500 Jefferson St.
    Paramont, CA 90723
28. Virtis Company
    Gardiner, NY 12525
29. Weston Instruments, Inc.
    614 Frelinghuysen Ave.
    Newark, NJ 07114
30. Wildlife Supply Company
    301 Cass St.
    Saginaw, MI 48602
31. Wilkens-Anderson Company
    4525 W. Division St.
    Chicago, IL 60651
32. Carl Zeiss, Inc.
    444 Fifth Ave.
    New York, NY 10018

2.3 Fish

Sources of information on fishery sampling equipment.

American Association for the Advancement of Science. Annual guide to scientific instruments (Published in Science).

American Society of Limnology and Oceanography. 1964. Sources of limnological and oceanographic apparatus and supplies. Special Publ. No. 1. IX:i-xxxii.

Oceanology International Yearbook/Directory.

Sinha, E. Z., and C. L. Kuehne. 1963. Bibliography on oceanographic instruments. I. General. II. Waves, currents, and other geophysical parameters. Meteorol. Geoastrophys. Abst. Amer. Meterol. Soc. 14:1242-1298; 1589-1637.

U.S. Fish and Wildlife Service. 1959. Partial list of manufacturers of fishing gear and accessories and vessel equipment. Fishery Leaflet 195. 27 pp.

Water Pollution Control Federation Yearbook.

UNITS OF MEASUREMENT

Conversion Factors and Special Tables

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International (Metric) and U.S. Customary

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CONTENTS

(Reprint includes only those items asterisked)

Chisholm, L.J., Units of Weight and Measure. International (Metric) and U.S. Customary. (NBS Miscellaneous Publication 286). For sale by Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402. Price $2.25.

Units of Weight and Measure

International (Metric) and U.S. Customary

L. J. Chisholm

The primary purpose of this publication is to make available the most often needed weights and measures conversion tables—conversions between the U. S. Customary System and International (Metric) System. A secondary purpose is to present a brief historical outline of the International (Metric) System—following it from its country of origin, France, through its progress in the United States.

Key Words: Conversion tables, International System (SI), Metric System, U. S. Customary System, weights and measures, weights and measures abbreviations, weights and measures systems, weights and measures units.

Introduction

Two systems of weights and measures exist side by side in the United States today, with roughly equal but separate legislative sanction: the U. S. Customary System and the International (Metric) System. Throughout U. S. history, the Customary System (inherited from, but now different from, the British Imperial System) has been, as its name implies, customarily used; a plethora of Federal and State legislation has given it, through implication, standing as our primary weights and measures system. However, the Metric System (incorporated in the scientists' new SI or Système International d'Unités) is the only system that has ever received specific legislative sanction by Congress. The "Law of 1866" reads:

> It shall be lawful throughout the United States of America to employ the weights and measures of the metric system; and no contract or dealing, or pleading in any court, shall be deemed invalid or liable to objection because the weights or measures expressed or referred to therein are weights or measures of the metric system.[1]

Over the last 100 years, the Metric System has seen slow, steadily increasing use in the United States and, today, is of importance nearly equal to the Customary System.

The International System *

* For up-to-date information on the international metric system, see current edition of The International System of Units (SI), Editors: Chester Page and Paul Vigoureux (NBS Special Publication 330). For sale by Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402. Price 30 cents. For NBS policy on the usage of SI, see NBS Technical News Bulletin Vol. 55 No. 1, pp. 18-20, January 1971.

[1] Act of 28 July 1866 (14 Stat. 339)—An Act to authorize the use of the Metric System of Weights and Measures.

Six units have been adopted to serve as the base for the International System: *

Length	meter
Mass	kilogram
Time	second
Electric current	ampere
Thermodynamic temperature	kelvin
Light intensity	candela

Some of the other more frequently used units of the SI and their symbols and, where applicable, their derivations are listed below.

SUPPLEMENTARY UNITS

Quantity	*Unit*	*Symbol*	*Derivation*
Plane angle	radian	rad	
Solid angle	steradian	sr	

DERIVED UNITS

Quantity	*Unit*	*Symbol*	*Derivation*
Area	square meter	m^2	
Volume	cubic meter	m^3	
Frequency	hertz	Hz	(s^{-1})
Density	kilogram per cubic meter	kg/m^3	
Velocity	meter per second	m/s	
Angular velocity	radian per second	rad/s	
Acceleration	meter per second squared	m/s^2	
Angular acceleration	radian per second squared	rad/s^2	
Force	newton	N	$(kg \cdot m/s^2)$
Pressure	newton per square meter	N/m^2	
Kinematic viscosity	square meter per second	m^2/s	
Dynamic viscosity	newton-second per square meter	$N \cdot s/m^2$	
Work, energy, quantity of heat	joule	J	$(N \cdot m)$
Power	watt	W	(J/s)
Electric charge	coulomb	C	$(A \cdot s)$
Voltage, potential difference, electromotive force	volt	V	(W/A)
Electric field strength	volt per meter	V/m	
Electric resistance	ohm	Ω	(V/A)
Electric capacitance	farad	F	$(A \cdot s/V)$
Magnetic flux	weber	Wb	$(V \cdot s)$
Inductance	henry	H	$(V \cdot s/A)$
Magnetic flux density	tesla	T	(Wb/m^2)
Magnetic field strength	ampere per meter	A/m	
Magnetomotive force	ampere	A	
Flux of light	lumen	lm	$(cd \cdot sr)$
Luminance	candela per square meter	cd/m^2	
Illumination	lux	lx	(lm/m^2)

* Recent (1971) addition of the mole as the unit for amount of substance brings the total to seven units. See asterisked footnote on page 1.

Definitions

In its original conception, the meter was the fundamental unit of the Metric System, and all units of length and capacity were to be derived directly from the meter which was intended to be equal to one ten-millionth of the earth's quadrant. Furthermore, it was originally planned that the unit of mass, the kilogram, should be identical with the mass of a cubic decimeter of water at its maximum density. The units of length and mass are now defined independently of these conceptions.

In October 1960 the Eleventh General (International) Conference on Weights and Measures redefined the meter as equal to 1 650 763.73 wavelengths of the orange-red radiation in vacuum of krypton 86 corresponding to the unperturbed transition between the $2p_{10}$ and $5d_5$ levels.

The kilogram is independently defined as the mass of a particular platinum-iridium standard, the International Prototype Kilogram, which is kept at the International Bureau of Weights and Measures in Sèvres, France.

The liter has been defined, since October 1964, as being equal to a cubic decimeter. The meter is thus a unit on which is based all metric standards and measurements of length, area, and volume.

Definitions of Units

Length

A *meter* is a unit of length equal to 1 650 763.73 wavelengths in a vacuum of the orange-red radiation of krypton 86.

A *yard* is a unit of length equal to 0.914 4 meter.

Mass

A *kilogram* is a unit of mass equal to the mass of the International Prototype Kilogram.

An *avoirdupois pound* is a unit of mass equal to 0.453 592 37 kilogram.

Capacity, or Volume

A *cubic meter* is a unit of volume equal to a cube the edges of which are 1 meter.

A *liter* is a unit of volume equal to a cubic decimeter.

A *cubic yard* is a unit of volume equal to a cube the edges of which are 1 yard.

A *gallon* is a unit of volume equal to 231 cubic inches. It is used for measuring liquids only.

A *bushel* is a unit of volume equal to 2 150.42 cubic inches. It is used for measuring dry commodities only.

Area

A *square meter* is a unit of area equal to the area of a square the sides of which are 1 meter.

A *square yard* is a unit of area equal to the area of a square the sides of which are 1 yard.

Spelling and Symbols for Units

The spelling of the names of units as adopted by the National Bureau of Standards is that given in the list below. The spelling of the metric units is in accordance with that given in the law of July 28, 1866, legalizing the Metric System in the United States.

Following the name of each unit in the list below is given the symbol that the Bureau has adopted. Attention is particularly called to the following principles:

1. No period is used with symbols for units. Whenever "in" for inch might be confused with the preposition "in", "inch" should be spelled out.
2. The exponents "^{2}" and "^{3}" are used to signify "square" and "cubic," respectively, instead of the symbols "sq" or "cu," which are, however, frequently used in technical literature for the U. S. Customary units.
3. The same symbol is used for both singular and plural.

Some Units and Their Symbols

Unit	Symbol	Unit	Symbol	Unit	Symbol
acre	acre	fathom	fath	millimeter	mm
are	a	foot	ft	minim	minim
barrel	bbl	furlong	furlong	ounce	oz
board foot	fbm	gallon	gal	ounce, avoirdupois	oz avdp
bushel	bu	grain	grain	ounce, liquid	liq oz
carat	c	gram	g	ounce, troy	oz tr
Celsius, degree	°C	hectare	ha	peck	peck
centare	ca	hectogram	hg	pennyweight	dwt
centigram	cg	hectoliter	hl	pint, liquid	liq pt
centiliter	cl	hectometer	hm	pound	lb
centimeter	cm	hogshead	hhd	pound, avoirdupois	lb avdp
chain	ch	hundredweight	cwt	pound, troy	lb tr
cubic centimeter	cm^3	inch	in	quart, liquid	liq qt
cubic decimeter	dm^3	International		rod	rod
cubic dekameter	dam^3	Nautical Mile	INM	second	s
cubic foot	ft^3	kelvin	K	square centimeter	cm^2
cubic hectometer	hm^3	kilogram	kg	square decimeter	dm^2
cubic inch	in^3	kiloliter	kl	square dekameter	dam^2
cubic kilometer	km^3	kilometer	km	square foot	ft^2
cubic meter	m^3	link	link	square hectometer	hm^2
cubic mile	mi^3	liquid	liq	square inch	in^2
cubic millimeter	mm^3	liter	liter	square kilometer	km^2
cubic yard	yd^3	meter	m	square meter	m^2
decigram	dg	microgram	µg	square mile	mi^2
deciliter	dl	microinch	µin	square millimeter	mm^2
decimeter	dm	microliter	µl	square yard	yd^2
dekagram	dag			stere	stere
dekaliter	dal	mile	mi	ton, long	long ton
dekameter	dam	milligram	mg	ton, metric	t
dram, avoirdupois	dr avdp	milliliter	ml	ton, short	short ton
				yard	yd

Units of Measurement—Conversion Factors*

Units of Length

To Convert from **Centimeters** To	Multiply by
Inches	0.393 700 8
Feet	0.032 808 40
Yards	0.010 936 13
Meters	**0.01**

To Convert from **Meters** To	Multiply by
Inches	39.370 08
Feet	3.280 840
Yards	1.093 613
Miles	0.000 621 37
Millimeters	**1 000**
Centimeters	**100**
Kilometers	**0.001**

To Convert from **Inches** To	Multiply by
Feet	0.083 333 33
Yards	0.027 777 78
Centimeters	**2.54**
Meters	**0.025 4**

To Convert from **Feet** To	Multiply by
Inches	**12**
Yards	0.333 333 3
Miles	0.000 1[illegible] 3[illegible]
Centimeters	**30.48**
Meters	**0.304 8**
Kilometers	**0.000 304 [illegible]**

* All boldface figures are exact; the others generally are given to seven significant figures.

In using conversion factors, it is possible to perform division as well as the multiplication process shown here. Division may be particularly advantageous where more than the significant figures published here are required. Division may be performed in lieu of multiplication by using the reciprocal of any indicated multiplier as divisor. For example, to convert from centimeters to inches by division, refer to the table headed "To Convert from *Inches*" and use the factor listed at "centimeters" (*2.54*) as divisor.

To Convert from **Yards** To	Multiply by
Inches	**36**
Feet	**3**
Miles	0.000 568 18
Centimeters	**91.44**
Meters	**0.914 4**

To Convert from **Miles** To	Multiply by
Inches	**63 360**
Feet	**5 280**
Yards	**1 760**
Centimeters	**160 934.4**
Meters	**1 609.344**
Kilometers	**1.609 344**

Units of Mass

To Convert from Grams

To	Multiply by
Grains	15.432 36
Avoirdupois Drams	0.564 383 4
Avoirdupois Ounces	0.035 273 96
Troy Ounces	0.032 150 75
Troy Pounds	0.002 679 23
Avoirdupois Pounds	0.002 204 62
Milligrams	**1 000**
Kilograms	**0.001**

To Convert from Metric Tons

To	Multiply by
Avoirdupois Pounds	2 204.623
Short Hundredweights	22.046 23
Short Tons	1.102 311 3
Long Tons	0.984 206 5
Kilograms	**1 000**

To Convert from Grains

To	Multiply by
Avoirdupois Drams	0.036 571 43
Avoirdupois Ounces	0.002 285 71
Troy Ounces	0.002 083 33
Troy Pounds	0.000 173 61
Avoirdupois Pounds	0.000 142 86
Milligrams	**64.798 91**
Grams	**0.064 798 91**
Kilograms	**0.000 064 798 91**

To Convert from Avoirdupois Pounds

To	Multiply by
Grains	**7 000**
Avoirdupois Drams	**256**
Avoirdupois Ounces	**16**
Troy Ounces	14.583 33
Troy Pounds	1.215 278
Grams	**453.592 37**
Kilograms	**0.453 592 37**
Short Hundredweights	**0.01**
Short Tons	**0.000 5**
Long Tons	0.000 446 428 6
Metric Tons	**0.000 453 592 37**

To Convert from Kilograms

To	Multiply by
Grains	**15 432.36**
Avoirdupois Drams	**564.383 4**
Avoirdupois Ounces	**35.273 96**
Troy Ounces	**32.150 75**
Troy Pounds	2.679 229
Avoirdupois Pounds	2.204 623
Grams	**1 000**
Short Hundredweights	0.022 046 23
Short Tons	0.001 102 31
Long Tons	0.000 984 2
Metric Tons	**0.001**

To Convert from Avoirdupois Ounces

To	Multiply by
Grains	**437.5**
Avoirdupois Drams	**16**
Troy Ounces	0.911 458 3
Troy Pounds	0.075 954 86
Avoirdupois Pounds	**0.062 5**
Grams	**28.349 523 125**
Kilograms	**0.028 349 523 125**

To Convert from Short Hundredweights

To	Multiply by
Avoirdupois Pounds	**100**
Short Tons	**0.05**
Long Tons	0.044 642 86
Kilograms	**45.359 237**
Metric Tons	**0.045 359 237**

To	To Convert from **Short Tons** Multiply by
Avoirdupois Pounds	**2 000**
Short Hundredweights	**20**
Long Tons	0.892 857 1
Kilograms	**907.184 74**
Metric Tons	**0.907 184 74**

To	To Convert from **Long Tons** Multiply by
Avoirdupois Ounces	**35 840**
Avoirdupois Pounds	**2 240**
Short Hundredweights	**22.4**
Short Tons	**1.12**
Kilograms	**1 016.046 908 8**
Metric Tons	**1.016 046 908 8**

To	To Convert from **Troy Ounces** Multiply by
Grains	**480**
Avoirdupois Drams	17.554 29
Avoirdupois Ounces	1.097 143
Troy Pounds	0.083 333 3
Avoirdupois Pounds	0.068 571 43
Grams	**31.103 476 8**

To	To Convert from **Troy Pounds** Multiply by
Grains	**5 760**
Avoirdupois Drams	210.651 4
Avoirdupois Ounces	13.165 71
Troy Ounces	**12**
Avoirdupois Pounds	0.822 857 1
Grams	**373.241 721 6**

Units of Capacity, or Volume, Liquid Measure

To	To Convert from **Milliliters** Multiply by
Minims	16.230 73
Liquid Ounces	0.033 814 02
Gills	0.008 453 5
Liquid Pints	0.002 113 4
Liquid Quarts	0.001 056 7
Gallons	0.000 264 17
Cubic Inches	0.061 023 74
Liters	**0.001**

To	To Convert from **Liters** Multiply by
Liquid Ounces	33.814 02
Gills	8.453 506
Liquid Pints	2.113 376
Liquid Quarts	1.056 688
Gallons	0.264 172 05
Cubic Inches	61.023 74
Cubic Feet	0.035 314 67
Milliliters	**1 000**
Cubic Meters	**0.001**
Cubic Yards	0.001 307 95

To	To Convert from **Cubic Meters** Multiply by
Gallons	264.172 05
Cubic Inches	61 023.74
Cubic Feet	35.314 67
Liters	**1 000**
Cubic Yards	1.307 950 6

To	To Convert from **Minims** Multiply by
Liquid Ounces	0.002 083 33
Gills	0.000 520 83
Cubic Inches	0.003 759 77
Milliliters	0.061 611 52

To Convert from Gills

To	Multiply by
Minims	**1 920**
Liquid Ounces	**4**
Liquid Pints	**0.25**
Liquid Quarts	**0.125**
Gallons	**0.031 25**
Cubic Inches	**7.218 75**
Cubic Feet	0.004 177 517
Milliliters	**118.294 118 25**
Liters	**0.118 294 118 25**

To Convert from Liquid Pints

To	Multiply by
Minims	**7 680**
Liquid Ounces	**16**
Gills	**4**
Liquid Quarts	**0.5**
Gallons	**0.125**
Cubic Inches	**28.875**
Cubic Feet	0.016 710 07
Milliliters	**473.176 473**
Liters	**0.473 176 473**

To Convert from Liquid Ounces

To	Multiply by
Minims	**480**
Gills	**0.25**
Liquid Pints	**0.062 5**
Liquid Quarts	**0.031 25**
Gallons	**0.007 812 5**
Cubic Inches	1.804 687 5
Cubic Feet	0.001 044 38
Milliliters	29.573 53
Liters	0.029 573 53

To Convert from Cubic Feet

To	Multiply by
Liquid Ounces	957.506 5
Gills	239.376 6
Liquid Pints	59.844 16
Liquid Quarts	29.922 08
Gallons	7.480 519
Cubic Inches	**1 728**
Liters	**28.316 846 592**
Cubic Meters	**0.028 316 846 592**
Cubic Yards	0.037 037 04

To Convert from Cubic Inches

To	Multiply by
Minims	265.974 0
Liquid Ounces	0.554 112 6
Gills	0.138 528 1
Liquid Pints	0.034 632 03
Liquid Quarts	0.017 316 02
Gallons	0.004 329 0
Cubic Feet	0.000 578 7
Milliliters	**16.387 064**
Liters	**0.016 387 064**
Cubic Meters	**0.000 016 387 064**
Cubic Yards	0.000 021 43

To Convert from Cubic Yards

To	Multiply by
Gallons	201.974 0
Cubic Inches	**46 656**
Cubic Feet	**27**
Liters	**764.554 857 984**
Cubic Meters	**0.764 554 857 984**

To Convert from **Liquid Quarts** To	Multiply by
Minims	**15 360**
Liquid Ounces	**32**
Gills	**8**
Liquid Pints	**2**
Gallons	**0.25**
Cubic Inches	**57.75**
Cubic Feet	0.033 420 14
Milliliters	**946.352 946**
Liters	**0.946 352 946**

To Convert from **Gallons** To	Multiply by
Minims	**61 440**
Liquid Ounces	**128**
Gills	**32**
Liquid Pints	**8**
Liquid Quarts	**4**
Cubic Inches	**231**
Cubic Feet	0.133 680 6
Milliliters	**3 785.411 784**
Liters	**3.785 411 784**
Cubic Meters	**0.003 785 411 784**
Cubic Yards	0.004 951 13

Units of Capacity, or Volume, Dry Measure

To Convert from **Liters** To	Multiply by
Dry Pints	1.816 166
Dry Quarts	0.908 082 98
Pecks	0.113 510 4
Bushels	0.028 377 59
Dekaliters	**0.1**

To Convert from **Cubic Meters** To	Multiply by
Pecks	113.510 4
Bushels	28.377 59

To Convert from **Dekaliters** To	Multiply by
Dry Pints	18.161 66
Dry Quarts	9.080 829 8
Pecks	1.135 104
Bushels	0.283 775 9
Cubic Inches	610.237 4
Cubic Feet	0.353 146 7
Liters	**10**

To Convert from **Dry Pints** To	Multiply by
Dry Quarts	**0.5**
Pecks	**0.062 5**
Bushels	**0.015 625**
Cubic Inches	**33.600 312 5**
Cubic Feet	0.019 444 63
Liters	0.550 610 47
Dekaliters	0.055 061 05

To Convert from **Dry Quarts** To	Multiply by
Dry Pints	**2**
Pecks	**0.125**
Bushels	**0.031 25**
Cubic Inches	**67.200 625**
Cubic Feet	0.038 889 25
Liters	1.101 221
Dekaliters	0 110 122 1

To Convert from **Pecks** To	Multiply by
Dry Pints	**16**
Dry Quarts	**8**
Bushels	**0.25**
Cubic Inches	**537.605**
Cubic Feet	0.311 114
Liters	8.809 767 5
Dekaliters	0.880 976 75
Cubic Meters	0.008 809 77
Cubic Yards	0.011 522 74

To Convert from **Bushels** To	Multiply by
Dry Pints	**64**
Dry Quarts	**32**
Pecks	**4**
Cubic Inches	**2 150.42**
Cubic Feet	1.244 456
Liters	35.239 07
Dekaliters	3 523 907
Cubic Meters	0.035 239 07
Cubic Yards	0.046 090 96

To Convert from **Cubic Inches** To	Multiply by
Dry Pints	0.029 761 6
Dry Quarts	0.014 880 8
Pecks	0.001 860 10
Bushels	0.000 465 025

To Convert from **Cubic Feet** To	Multiply by
Dry Pints	51.428 09
Dry Quarts	25.714 05
Pecks	3.214 256
Bushels	0.803 563 95

To Convert from **Cubic Yards** To	Multiply by
Pecks	86.784 91
Bushels	21.696 227

Units of Area

To Convert from Square Centimeters

To	Multiply by
Square Inches	0.155 000 3
Square Feet	0.001 076 39
Square Yards	0.000 119 599
Square Meters	**0.000 1**

To Convert from Hectares

To	Multiply by
Square Feet	107 639.1
Square Yards	11 959.90
Acres	2.471 054
Square Miles	0.003 861 02
Square Meters	**10 000**

To Convert from Square Feet

To	Multiply by
Square Inches	**144**
Square Yards	0.111 111 1
Acres	0.000 022 957
Square Centimeters	**929.030 4**
Square Meters	**0.092 903 04**

To Convert from Acres

To	Multiply by
Square Feet	**43 560**
Square Yards	**4 840**
Square Miles	**0.001 562 5**
Square Meters	**4 046.856 422 4**
Hectares	**0.404 685 642 24**

To Convert from Square Meters

To	Multiply by
Square Inches	1 550.003
Square Feet	10.763 91
Square Yards	1.195 990
Acres	0.000 247 105
Square Centimeters	**10 000**
Hectares	**0.000 1**

To Convert from Square Inches

To	Multiply by
Square Feet	0.006 944 44
Square Yards	0.000 771 605
Square Centimeters	**6.451 6**
Square Meters	**0.000 645 16**

To Convert from Square Yards

To	Multiply by
Square Inches	**1 296**
Square Feet	**9**
Acres	0.000 206 611 6
Square Miles	0.000 000 322 830 6
Square Centimeters	**8 361.273 6**
Square Meters	**0.836 127 36**
Hectares	**0.000 083 612 736**

To Convert from Square Miles

To	Multiply by
Square Feet	**27 878 400**
Square Yards	**3 097 600**
Acres	**640**
Square Meters	**2 589 988.110 336**
Hectares	**258.998 811 033 6**

Special Tables

Length—Inches and Millimeters—Equivalents of Decimal and Binary Fractions of an Inch in Millimeters

From 1/64 to 1 Inch

½'s	¼'s	8ths	16ths	32ds	64ths	Milli-meters	Decimals of an inch	Inch	½'s	¼'s	8ths	16ths	32ds	64ths	Milli-meters	Decimals of an inch
					1	= 0.397	0.015625							33	=13.097	0.515625
				1	2	= .794	.03125						17	34	=13.494	.53125
					3	= 1.191	.046875							35	=13.891	.546875
			1	2	4	= 1.588	.0625					9	18	36	=14.288	.5625
					5	= 1.984	.078125							37	=14.684	.578125
				3	6	= 2.381	.09375						19	38	=15.081	.59375
					7	= 2.778	.109375							39	=15.478	.609375
		1	2	4	8	= 3.175	.1250				5	10	20	40	=15.875	.625
					9	= 3.572	.140625							41	=16.272	.640625
				5	10	= 3.969	.15625						21	42	=16.669	.65625
					11	= 4.366	.171875							43	=17.066	.671875
			3	6	12	= 4.762	.1875					11	22	44	=17.462	.6875
					13	= 5.159	.203125							45	=17.859	.703125
				7	14	= 5.556	.21875						23	46	=18.256	.71875
					15	= 5.953	.234375							47	=18.653	.734375
	1	2	4	8	16	= 6.350	.2500			3	6	12	24	48	=19.050	.75
					17	= 6.747	.265625							49	=19.447	.765625
				9	18	= 7.144	.28125						25	50	=19.844	.78125
					19	= 7.541	.296875							51	=20.241	.796875
			5	10	20	= 7.938	.3125					13	26	52	=20.638	.8125
					21	= 8.334	.328125							53	=21.034	.828125
				11	22	= 8.731	.34375						27	54	=21.431	.84375
					23	= 9.128	.359375							55	=21.828	.859375
		3	6	12	24	= 9.525	.3750				7	14	28	56	=22.225	.875
					25	= 9.922	.390625							57	=22.622	.890625
				13	26	=10.319	.40625						29	58	=23.019	.90625
					27	=10.716	.421875							59	=23.416	.921875
			7	14	28	=11.112	.4375					15	30	60	=23.812	.9375
					29	=11.509	.453125							61	=24.209	.953125
				15	30	=11.906	.46875						31	62	=24.606	.96875
					31	=12.303	.484375							63	=25.003	.984375
1	2	4	8	16	32	=12.700	.5	1	2	4	8	16	32	64	=25.400	1.000

Length—International Nautical Miles and Kilometers

Basic relation: *International Nautical Mile = 1.852 kilometers.*

Int. nautical miles	Kilometers	Int. nautical miles	Kilometers	Kilometers	Int. nautical miles	Kilometers	Int. nautical miles
0		50	92.600	0		50	26.9978
1	1.852	1	94.452	1	0.5400	1	27.5378
2	3.704	2	96.304	2	1.0799	2	28.0778
3	5.556	3	98.156	3	1.6199	3	28.6177
4	7.408	4	100.008	4	2.1598	4	29.1577
5	9.260	5	101.860	5	2.6998	5	29.6976
6	11.112	6	103.712	6	3.2397	6	30.2376
7	12.964	7	105.564	7	3.7797	7	30.7775
8	14.816	8	107.416	8	4.3197	8	31.3175
9	16.668	9	109.268	9	4.8596	9	31.8575
10	18.520	60	111.120	10	5.3996	60	32.3974
1	20.372	1	112.972	1	5.9395	1	32.9374
2	22.224	2	114.824	2	6.4795	2	33.4773
3	24.076	3	116.676	3	7.0194	3	34.0173
4	25.928	4	118.528	4	7.5594	4	34.5572
5	27.780	5	120.380	5	8.0994	5	35.0972
6	29.632	6	122.232	6	8.6393	6	35.6371
7	31.484	7	124.084	7	9.1793	7	36.1771
8	33.336	8	125.936	8	9.7192	8	36.7171
9	35.188	9	127.788	9	10.2592	9	37.2570
20	37.040	70	129.640	20	10.7991	70	37.7970
1	38.892	1	131.492	1	11.3391	1	38 3369
2	40.744	2	133.344	2	11.8790	2	38.8769
3	42.596	3	135.196	3	12.4190	3	39.4168
4	44.448	4	137.048	4	12.9590	4	39.9568
5	46.300	5	138.900	5	13.4989	5	40.4968
6	48.152	6	140.752	6	14.0389	6	41.0367
7	50.004	7	142.604	7	14.5788	7	41.5767
8	51.856	8	144.456	8	15.1188	8	42.1166
9	53.708	9	146.308	9	15.6587	9	42.6566
30	55.560	80	148.160	30	16.1987	80	43.1965
1	57.412	1	150.012	1	16.7387	1	43.7365
2	59.264	2	151.864	2	17.2786	2	44.2765
3	61.116	3	153.716	3	17.8186	3	44.8164
4	62.968	4	155.568	4	18.3585	4	45.3564
5	64.820	5	157.420	5	18.8985	5	45.8963
6	66.672	6	159.272	6	19.4384	6	46.4363
7	68.524	7	161.124	7	19.9784	7	46.9762
8	70.376	8	162.976	8	20.5184	8	47.5162
9	72.228	9	164.828	9	21.0583	9	48.0562
40	74.080	90	166.680	40	21.5983	90	48.5961
1	75.932	1	168.532	1	22.1382	1	49.1361
2	77.784	2	170.384	2	22.6782	2	49.6760
3	79.636	3	172.236	3	23.2181	3	50.2160
4	81.488	4	174.088	4	23.7581	4	50.7559
5	83.340	5	175.940	5	24.2981	5	51.2959
6	85.192	6	177.792	6	24.8380	6	51.8359
7	87.044	7	179.644	7	25.3780	7	52.3758
8	88.896	8	181.496	8	25.9179	8	52.9158
9	90.748	9	183.348	9	26.4579	9	53.4557
		100	185.200			100	53.9957

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