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Ecological Research Series

PHYTOPLANKTON SAMPLING IN QUANTITATIVE BASELINE AND MONITORING PROGRAMS

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PHYTOPLANKTON SAMPLING IN QUANTITATIVE BASELINE AND MONITORING PROGRAMS[1/]

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FOREWORD

Effective regulatory and enforcement actions by the Environmental Protection Agency would be virtually impossible without sound scientific data on pollutants and their impact on environmental stability and human health. Responsibility for building this data base has been assigned to EPA's Office of Research and Development and its 15 major field installations, one of which is the Corvallis Environmental Research Laboratory (CERL).

The primary mission of the Corvallis Laboratory is research on the effects of environmental pollutants on terrestrial, freshwater, and marine ecosystems; the behavior, effects and control of pollutants in lake systems; and the development of predictive models on the movement of pollutants in the biosphere.

This report presents a review of methods for sampling and analyzing marine phytoplankton communities. These quantitative techniques can be used to establish ecological baselines or to conduct surveys of the impact of pollution on phytoplankton dynamics.

A. F. Bartsch
Director, CERL

ABSTRACT

An overview of phytoplankton sampling and analysis methods as they apply to quantitative baseline and monitoring surveys is provided. A need for inclusion of a preliminary field survey of the area under investigation and of flexibility in sampling design is stressed. An extensive bibliography pertinent to phytoplankton sampling and analysis is included in the report.

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SECTION 1

CONCLUSIONS

The complexity of dynamic oceanic and estuarine ecosystems prevent the preparation of a detailed and concise survey outline. The current status of phytoplankton surveys was determined through searches of data storage banks, National Oceanographic Data Center (NODC), Biological Abstracts (BA), Biological Information Retrieval System (BIRS), Bioresearch Index (BRI), Ocean Abstracts (OA), Selected Water Resources Abstracts (SWRA), Smithsonian Science Information Exchange (SSIE) and Environmental Data Index (ENDEX). The results of these searches illustrated the need for survey design flexibility to accommodate the array of phytoplankton investigative procedures. The wide selection of methodology has arisen from complexities within the marine ecosystems. The development of a specific survey design is directly a function of both the character of the marine system to be surveyed and the purposes for developing the survey. However, some general recommendations can be made.

The early developmental stages of a survey design are considered crucial, since appropriate planning is necessary to develop a general understanding of the anticipated complexities of the system under consideration. The selection of station locations and sampling frequency can dramatically influence the statistical validity of the obtained information. Where applicable, the investigative area should be divided into its discernible hydrographic sub-areas. These sub-areas should be overlaid with a grid, which provides a statistically valid basis for sampling within each sub-area. The sampling frequency must be short enough to reveal major temporal community changes. The frequency of sampling in an estuarine system should be biweekly or monthly during periods of relatively stable environmental conditions and weekly during periods of hydrographic instability. Because oceanic hydrography changes less rapidly, the sampling may vary from bimonthly to biweekly. Any perturbation of the ecosystem should be followed by intensified sampling to ascertain the resultant community alterations.

The selection of sampling devices and depths is largely dependent on the nature of the desired information. Generally, sub-surface bottle sampling is most efficient in eutrophic waters having high phytoplankton densities, while net or pump sampling may be desired for oligotrophic oceanic regions. A survey is not complete without delineation of phytoplankton vertical heterogeneity and its analysis. The screen sampler is well suited for surface sampling. The deployment of a skiff at sea is often discouraged but is the only reliable method of avoiding the "hull-effect" of a larger vessel. A significantly less satisfactory surface sampler is the modified Zaitsev neuston net.

Enumeration procedures must be carefully considered because each method has its own intrinsic deficiencies and the Utermohl method appears to be the most generally used and accepted procedure because it provides a wide range of adaptability at medium cost. Conventional live analysis should be simultaneously performed on sample aliquots.

The analysis of phytoplankton in a general baseline survey should be accompanied by a wide array of ancillary data. These data include the delineation of zooplankton, ichthyoplankton and benthic communities, in addition to standard hydrographic analyses. The Carbon ^{14} productivity estimate is the most widely used and whenever possible should be included in baseline surveys. Chlorophyll a estimates of standing stock may yield pertinent correlative information with identification, enumeration and productivity measurements and, thereby, contribute to a comprehensive phytoplankton community survey.

SECTION 2

INTRODUCTION

Baseline and monitoring surveys of estuarine, coastal and oceanic waters have been widely employed as a means of obtaining biological and ancillary data needed in assessing environmental impacts of various human activities. The size, cost and comprehensiveness of these surveys vary with the type of action and the expected impact, e.g. from small, localized additions of effluent to development of a major offshore oil field. Such surveys originate in response to enacted law, requiring that industry provide an environmental assessment before and after an event of perturbation so as to protect the public. The scientists charged with designing and carrying out these surveys are responsible, therefore, for assuring that meaningful data are collected in a manner that will allow pertinent assessment of environmental changes. The cost must be held to a level commensurate with the probable impact, and all needed observations should be taken.

Measurements and observations included in baseline surveys are usually similar but may vary somewhat with the type of environmental alteration under consideration, e.g. emphasis on hydrocarbon measurements in offshore oil fields, benthic organisms and turbidity in channel dredging, or mortality of fish eggs and larvae in power plant installations. Most surveys include standard hydrographic observations, an inclusion of lower trophic levels, and studies of the benthos and fishes. Somewhat surprising is the omission of phytoplankton studies from many of the major baseline surveys. In view of their critical role in the food web and productivity of marine waters and of their rapid response to environmental perturbations, phytoplankton should certainly be included in any survey designed to measure environmental impact. It is our experience that omission of phytoplankton studies from broad baseline surveys is usually a result of the inability of phytoplanktologists to agree on what constitutes meaningful observations.

This report is intended as an overview of methods used for phytoplankton sampling, sample treatment and analysis. It is also intended primarily for the agency personnel requesting proposals and the survey designer, rather than for practicing phytoplanktologists.

SECTION 3

PHYTOPLANKTON ECOLOGY

An accurate evaluation of the phytoplankton community in any given body of water requires an extensive knowledge of general phytoplankton biology and ecology. The following brief perusal of some of the major considerations is intended to provide insight into this dynamic system and thereby aid the researcher in both the design of an investigation and the evaluation of obtained data.

The role of phytoplankton in the marine and estuarine environment is essentially the capture of radiant energy and the metabolic concentration of dissolved inorganic chemicals. The cell uses the captured radiant energy to combine the inorganics, through photometabolism, into complex organic molecules.[1-2] These molecules are then used in cellular maintenance, growth, and reproduction. The phytoplankton community is the principal producer in the world's oceans and thus it serves as the major energy source for higher marine trophic levels. Furthermore, numerous secondary influences are produced by phytoplankton induced alterations of the aquatic environment.[3] These include uptake of carbon dioxide and the release of oxygen during periods of photometabolism. Phytoplankton were historically instrumental in the stabilization of oxygen in the atmosphere and thereby provided the aerobic environment of consumers. Waste products and other metabolites released by phytoplankton can act as regulatory agents in the succession of species. Although usually unobserved, these agents occasionally produce pronounced effects as evidenced by red tides.[4-6] Additionally, the excretion products and dead cells are functional in the determination and maintenance of the heterotrophic community.[7]

Limiting and controlling factors on the phytoplankton community generally include the availability of nutrients, solar radiation, salinity and temperature.[8-17] The activities of man have clearly altered these factors in the continental inland waters and estuaries. In addition, biotic changes on the continental shelf have been observed, as evidenced by the summer 1976, east coast bloom of *Ceratium tripos*. The decline of this bloom and its associated oxygen deficit resulted in massive finfish and shellfish mortality.[18]

The addition of inorganic and organic compounds from effluents and run-off stimulates phytoplankton growth whereas the addition of toxic compounds like DDT, PCB, PBB and Kepone may limit growth.[19-20] Excessive phytoplankton growth is usually observed as a logarithmic population increase of a very few algal species. These species completely dominate the community until some environmental factor becomes limiting. The bloom species may not be directly toxic as with the red tide dinoflagellates, but may produce secondary effects, including radically fluctuating oxygen levels between light periods of photometabolism and dark periods of respiration. Bloom species may have a deleterious effect on filter-feeding consumers by radically reducing the abundance of more desirable phytoplankton species selectively filtered as foodstuffs. The addition of trace amounts of toxic compounds to the aquatic system often, through bio-concentration mechanisms, results in highly toxic levels in the tissues of the entire associated food web. The mechanisms for the degree of bio-concentration by the algae vary with each compound and with each species.

An accurate assessment of the phytoplankton community also requires an understanding of the spatial and temporal characteristics of algae.[21-29] Spatially, the phytoplankton inhabit a three-dimensional world with accordingly complex distributional anomalies. Vertically the organisms may stratify into a surface community occupying the surface film and immediate underlying waters, a near surface community from one to approximately ten meters deep, a deep community near the compensation depth, as well as an aphotic zone community. The exact number of identifiable communities and their depth will vary in accordance with incident light, turbidity, vertical mixing and with the phytoplankton species occupying the water mass. Horizontal heterogeneity has long been observed in the patchiness of visual blooms. Windrows and down-wellings often increase cell densities of certain locations while decreasing them in the adjacent water. Patches or high density communities vary from only a few meters in width to many miles. Spatial heterogeneity is further complicated by the movements of the water. Currents are usually relatively stable in oceanic environments, more complex in continental shelf and slope waters, and often highly complicated in estuaries. Rarely is the researcher able to use a simple up-stream down-stream approach and is, therefore, required to know, either through the literature or through measurement, the circulation patterns to be encountered in the region under study.

In addition to observed spatial variations, temporal changes in the phytoplankton density and species composition must be anticipated. Seasonally the communities will proceed through compositional changes in a fairly predictable manner, the primary influencing factors usually being temperature, incident radiation, nutrients and salinity.[30-33] The particular seasonal pattern for a given water mass or area must be determined through seasonal sampling. Fluctuations within a community may occur slowly, requiring a month or more, or may be very abrupt, occurring in a week or less.[34] Once the seasonal pattern has been determined, its changes are then often predictable from knowledge of the hydrography of the water.

Sampling of the phytoplankton communities is therefore complicated by both vertical and horizontal patchiness, and abrupt or slow seasonal changes, in addition to water circulation patterns, grazing, circadian migration and radiation fluctuations.

The dynamics of the marine aquatic environments are so variable that sample design should be flexible, allowing adaptation to observed peculiarities of the system under investigation. The open seas with their relatively uniform hydrography provide an area suitable for observation and description of circadian patterns. Drogue buoys can be employed to identify water mass movements and allow repeated sampling from the same communities. In contrast, the estuarine system, each with its unique form of heterogeneity, presents extreme problems of relating replicated and sequential samples. In attempting any circadian investigations in an estuary, a prime consideration is determining what water mass is being sampled and at what time. The significance of the circadian pattern at a point location has not been accurately compared with the circadian pattern of a discreet water parcel. Also, the problems of dissimilarity of replicate samples in the estuary is more pronounced than that encountered in oceanic replication. It is evident that the more heterogeneous and dynamic a system, the greater the problems are in accurately delineating the biotic events.

SECTION 4

SAMPLING DESIGN FOR ENVIRONMENTAL ASSESSMENT

The location and number of stations and the depths to be sampled determines both the value and the cost of the obtained information. The primary consideration in a sampling scheme should be to obtain the amount of information about the phytoplankton community required to answer the questions of investigators. The correct framing of these questions is thus of the utmost importance to all that follows. An investigation of the literature pertinent to the general area and a detailed preliminary field sampling can often result in a reduction of samples required. It is desirable to determine the characteristic annual cycles and the distributional patterns in the first year or two of sampling. Once these patterns are determined with the accompanying hydrography, sampling can then be reduced to a monitoring basis. The important preliminary field sampling has often been neglected because most investigative work is limited to a fixed number of stations and samples predetermined by budgetary limitations.

4.1 Oceanic Sampling

For a proposed investigation of the continental shelf or slope, the locations and characteristics of the water masses can usually be determined from published studies on hydrography, current analysis, and biotic community analysis. The neritic, near-shore waters are usually clearly discernible from deeper shelf waters and oceanic waters. Charts of the sub-areas should be marked with a grid and random sampling should be performed within each sub-area. This type of sampling scheme provides the most statistically valid description of the communities within each sub-area. Seasonal station replication is not completely relevant since the water masses are in a state of continuous dynamic fluctuation.[28,35-40] In situations where results of an investigation are to be correlated with other biotic and hydrographic data, it may be necessary to sample on a fixed station scheme. This is often the case with inlet and discharge monitoring or when sampling is coupled with more stationary benthic community analysis. In selecting such fixed stations it is essential to locate them within each of the above mentioned sub-areas. The specific station locations within each sub-area should sample each micro-climate or each recognizable water type of that sub-area. Because of the high costs of offshore operations, it is customary to locate stations along transects to reduce the between-station time and thereby reduce the cost per sample.

4.2 Estuarine Sampling

For an investigation in an estuary the problems of station location are similar to those in the offshore environment. However, both spatial and temporal changes become more pronounced within the estuarine environment. Water movement becomes highly complicated due to interactions of tidal currents with seasonally fluctuating upstream fresh water discharge, in addition to the wind driven currents and mixing. The complexity in the composition of estuarine waters is increased by the input of agricultural, industrial and domestic impurities. As the chemical and silt laden fresh water mixes with the high saline tidal input, a wide range of temperatures, nutrients and salinities may be encountered over very short distances. Accordingly, estuarine investigations should be one of two types. A general evaluation of the entire estuary requires dividing the estuary into basic hydrographic strata followed by multiple sampling within each stratum. The number of samples needed in each stratum should be determined from variations observed in the preliminary field study. Such a sampling scheme resembles that described for the shelf and slope investigative procedures. The alternative sampling scheme is appropriate for an estuarine system with a specific localized environmental problem. The investigation is usually associated with answering specific questions of water quality alteration from some land-based operational discharge. This entails the delineation of the phytoplankton communities prior to the construction and operation of the discharge and a monitoring program after the installation. The degree of detail in the sampling scheme is directly dependent on the magnitude of the proposed discharge and its subsequent extent of alteration of hydrography and biology.

SECTION 5

PHYTOPLANKTON VERTICAL HETEROGENEITY

Once the stations have been selected it is necessary to determine the degree of specificity that the investigation requires. This is a major determinant of the number of samples to be collected at each station, the various types of collecting apparatus to be utilized, and the resultant cost of the study. If a general water quality investigation is required, it is necessary to sample the vertically stratified communities: surface micro-layer, near-surface photic zone, compensation depth and aphotic zone. These depths should reveal what complexities will be encountered at each station and between stations.

5.1 Surface Microlayer Sampling

The surface micro-layer is efficiently sampled with the Garrett-type screen sampler.[41-42] The frame and screen should be constructed of some non-toxic non-contaminating substance if the samples are to be used for anything other than identification and enumeration of the phytoplankton. The samples should be taken well away from the hull of any large ship, in relatively undisturbed water. The surface microlayer community has been found to maintain its integrity in wind-driven waves of one meter.[43] If the waves are strongly cresting the community has probably mixed with the near surface community and a gradient from one to the other would exist.

The screen sampler should be horizontally submerged, moved to an adjacent undisturbed area, and slowly lifted up through the surface waters while keeping it in the horizontal position.[42] The screen is then tilted so that the adhered water will drain from one corner of the screen until only a slow drip remains. The procedure is repeated until the desired volume of surface water has been collected. A two (2) foot square screen will generally yield approximately 75-100 milliliters per submergence. Large mouth plastic funnels and bottles are recommended for use at sea to reduce the danger of breakage and the loss of samples. Sample size generally should be in excess of the 1000 milliliters used for settling (see 6.0 sample treatment).

Alternate surface micro-layer sampling includes the glass plate method of removing the surface adhesion layer and use of the Harvey drum type sampler.[44-45] Both of these methods are most applicable in very calm waters. A modified Zaitsev neuston net could be used to sample various near surface layers and the surface sub-surface ecotone.[46-47]

5.2 Subsurface Sampling

Sampling of the near surface, compensation depth, and aphotic communities can be investigated from three different methodological approaches, yielding different evaluations.[22,48-51] Discreet depths can most accurately be sampled with any of various bottle or pump samplers. There are no standard sampling depths because of the variable vertical distributions of the phytoplankton. The exact depths to be sampled should be established by consulting previous studies of the research area together with a preliminary sampling of hydrographic and biotic profiles. A less sophisticated approach is to preselect arbitrary depths throughout the water column. This approach may result in an unnecessarily large number of samples to be analyzed. In tropic and sub-tropic oceanic waters, pre-selected depths should include the surface, near surface, 10, 25, 50, 75, 100, 150 and 200 meter depths. Temperate and boreal waters, typically more productive, require closer spacing of the samples: surface, near surface, 5, 10, 20, 40, 60, 80 and 100 meter depths. Coastal waters, estuaries and upwelling support denser phytoplankton communities and may require even closer spacing of sample depths: surface, 1.5, 3, 5, 10, 20, 40 and 60 meters.

Endogenous diurnal rhythms of phytoplankton photosynthesis, nutrient uptake, cellular maintenance, growth and reproduction, often result in vertical circadian migrations. The migration patterns have been amply demonstrated for many species and the net effect of these migrations may significantly distort results unless the time of sampling is accounted for. The time and magnitude of these circadian movements are species specific and may result from both positive and negative stimuli. Sampling for general survey information should usually be conducted between dawn and dusk; mid-morning and mid-afternoon are the more preferable times.

Migrations are generally believed to be a response to ambient light intensity. This theory accounts for depressed productivity values around noon. Recent demonstrations of a discrete surface microlayer phytoplankton community has elicited reconsiderations of light inhibition theories and may ultimately aid in clarifying migration related problems.

A thirty (30) liter Niskin bottle fitted with a large bore spigot provides accurate samples in both volume and depth and is most appropriate for offshore oligotrophic waters.[52] A sample can be immediately run through a ten (10) or twenty (20) micron sieve and then resuspended and preserved in 250 milliliter bottles. The closing bottle samplers have proved to be the most easily used quantitative sampling devices. A wide range of designs have been developed for specific applications. Generally, the samplers should have a streamlined flow-through design with no corners or areas to trap and hold residual water while being moved to the desired depth. Such a feature is mot important when sampling very small phytoplankton, fungi and bacteria.[53] Bottle construction should be of non-contaminating materials because samples are often split into aliquots for various ancillary analyses. The bottles should be designed to be used in series enabling sequential closing. Specially designed samplers have been developed for the elimination of contamination.

A bottle type sample does not account for the microdistributional anomalies so frequently encountered in phytoplankton communities. Repeated sampling at a given depth using a smaller volume bottle will provide an approximation of an integrated sample. When patchiness is a concern, an integrative sampler should be considered. Discreet depth integration can also be accomplished using a pump to bring the water to the surface[54-57] Various types of pumping systems have been employed since the late 19th century. The discharge can be run through a sieve for concentration and the filtrate collected until any desired volume has been filtered. The filtrate may be used to wash the phytoplankton from the sieve. The rate of flow in the hose and pump must be controlled to keep the cells from adhering to the surfaces and to keep cell damage to a minimum. An alternate and less desirable method of integrative sampling is to use a vertical or oblique net tow. Both types of net tows integrate not only the horizontally micro-distributed phytoplankters but also integrate the vertically stratified communities. Furthermore, there is no accurate method of determining the amount of water filtered through fine-meshed nets; only gross quantitative approximations of the population densities can be made. Dense zooplankton communities further compound the problems of quantification by clogging fine meshed nets, terminating or reducing filtration efficiency. Ctenophora, jellyfish and salps add to the difficulty by entangling organisms within a mass of tentacles and jelly, thus making samples unmanageable. However, net tows can be useful in providing large numbers of cells for morphological and taxonomic studies.

Various modifications of net type continuous samplers have been designed.[58-61] Generally, they provide a continuous sample while being towed through the water. A small aperture allows a stream of water to be filtered through a spool mounted net. The netted phytoplankton are automatically preserved in place on the net for later microscopic analysis. The continuous samples are useful in determining the horizontal distribution of phytoplankton communities over large areas of ocean. This type of sampling device has a variable spool speed which can be adjusted in accordance to the plankton density and thereby has application in both oligotrophic and eutrophic waters.

5.3 Aphotic Zone Sampling

Viable deep-water phytoplankton have frequently been reported from deep oceanic waters. The grouping of these phytoplankton as a community is still under investigation.[62] Bottle sampling of aphotic zone phytoplankton should proceed with preliminary sampling at predetermined depths between the bottom and the compensation depth. Phytoplankton concentrations often correlate with inflection points of temperature and salinity, which are usually indicative of boundaries between different water masses. Immediate live sample analysis may establish the presence of aphotic phytoplankton and define their approximate vertical position so that an intensive sampling array may be initiated at such locations.

SECTION 6

SAMPLE TREATMENT

Nearly all samples require some form of concentration prior to enumeration. The enumerative method to be used usually involves its own particular manipulative procedures. Early decisions include the volume of sample to be enumerated, the number of aliquots necessary for accurate subsampling, and the number of replicate counts to assure an accurate estimate of the actual community. The wide range of cell densities and the abundance of particulate detrital material make general recommendations for sample volumes inadvisable. Additional factors which must be considered include the size range of the community components, the resultant magnification requirements for their positive identification, the dispersal characteristics of the selected counting chambers, the minimum numbers of cells needed for satisfactory abundance estimates, and for assured observation of the rare and moderately rare species. Each counting method has pertinent publications dealing with its own unique statistical considerations. The complexities of these problems are illustrated by comparisons between various aliquot sizes and the resultant disproportionate changes within the various phytoplankton groups. A number of these methods are discussed in section 8.0 phytoplankton enumeration.

6.1 Sample Volume

The required sample volume will vary with the type of sampling gear employed, the type of analysis to be performed, and the density of the phytoplankton community. The most important consideration is cell density, which may range from only a few cells per liter in oligotrophic oceanic waters to a billion or more cells per liter in estuarine bloom conditions. A field estimate of cell densities is often the most effective means of determining appropriate sample volume; an investigator must develop the ability to make such an estimate. Erroneous estimates, however, may result from an abundance of microscopic zooplankton. Accordingly, it is usually prudent to make a preliminary shipboard microscopic examination of samples to determine the abundance of the main biotic components. This may prevent the collection of water samples that are biologically sparse, resulting in enumerations of questionable statistical validity. Biologically sparse samples usually contain insufficient numbers of the rarer species, and thereby produce erroneous community evaluations.

6.2 Live Sample Analysis

Identification and enumeration of natural communities should be performed on live material as the addition of preservatives renders many forms morphologically indistinguishable from detrital material. Rapid ship-board analysis of aliquots for enumeration of fragile forms is recommended because community alteration occurs quickly in confined containers. Sample aliquots should be chilled to about 4°C and examined within twelve (12) hours. Pre-screening with 333 micron netting to remove the larger filter feeding herbivores is advisable if aliquots are to be chilled and held.

6.3 Sample Fixation

After removal of the aliquots for live analysis and other ancillary tests, the sample should be immediately treated with an appropriate preservative, in order to stop all biological activity and fix the phytoplankton morphology and cytology for later identification or examination. In practice, no universal fixative has been developed. The selection of an appropriate fixative to stabilize the desired cell characteristics often results in preservation of two or more aliquots with different fixatives. For general identification and enumeration, investigators commonly use neutral or slightly basic formaldehyde (40%), neutralized with borax, sodium tetraborate or sodium carbonate to pH 7.0 - 7.3. Various concentrations of formaldehyde are used. If the samples are to be analyzed within a few days slightly lower concentrations may be used. The appropriate concentration range is from two to five milliliters of neutral formaldehyde per one hundred milliliters of seawater. Concentrations of fixative in excess of five percent produce progressive cellular distortion and shrinkage. Formaldehyde causes flagellated forms to shed flagella and radically distorts or ruptures the naked forms. It is generally used for diatoms, thecate dinoflagellates, and cocolithophores. The addition of sucrose improves fixation of the cellular fine structure.[63] A few drops of saturated cupric sulfate solution per liter of fixed sample retards the loss of chloroplast color and thereby aids in distinguishing phytoplankton from detritus. Clumping of the settled material frequently interferes with analysis. The addition of an emulsifying agent helps in dispersal of clumps. Detergents, however, interfere with the preparation of slides and should be used only when essential for clump dispersion.

A second commonly used fixative, Lugol's solution (2 gm potassium iodide, 1 gm iodine in 200 ml distilled water), appears to be more effective at flagella retention for many species. Enough Lugol's solution is added to sample to yield a weak tea color. If the organisms are stained too darkly the color intensity may be decreased with the addition of sodium thiosulfate. Lugol's solution is not recommended for long term preservation because it slowly decomposes; when the sample is faded the addition of fresh fixative is required.

Many other fixatives have been developed for use with particular plant groups. Some investigators fix two or more aliquots of the same sample with different fixatives to take advantage of the special features of the various fixatives. Chilling of the sample before and during fixation seems to give better results in preservation. The proper buffering of fixatives is of considerable importance in preventing the cellular structural components from dissolving. Some of the more specialized fixatives are Bouin's, Allen's (PFA), Schaudinn's and von Rath's fixative. Those containing ethanol, acetic acid, or formic acid form precipitates when added to sea water and can be used only after a sample has been concentrated. These fixatives are often very effective in the preservation of various cytological features.[64-65]

The preparation and fixation of phytoplankton for standard and scanning election microscopy requires special care. The advantages of electron microscopy in phytoplankton identification are unquestionable.[67-69] The initial microscope cost remains the major deterrent to its widespread use. As more electron microscopes come into general use many long existing taxonomic problems may be resolved. Osmium tetroxide and glutaraldehyde are frequently used in cell fixation but present extreme exposure hazards and require extra caution.

SECTION 7

SAMPLE CONCENTRATION

There are three basic methods which are generally used for concentrating samples: settling, centrifugation, and filtration. All procedures result in the loss of cells, and the extent of loss is dependent on numerous factors. Some of these are settling time, centrifugation time and speed, filter type and mesh size, and cell morphology. The accuracy of enumeration is highly dependent upon the researchers care and skill in sample manipulation. Generally, the fewer the manipulative steps the smaller the induced error.

7.1 Settling

Concentration of the preserved community by settling is often carried out in the original storage containers. After allowing a minimum of forty eight (48) undisturbed hours the supernatant can be carefully siphoned off. Siphoning should be slow, so as not to disturb the settled material.

An alternative procedure for handling of dense samples is to resuspend all particulate material by vigorously shaking the sample and immediately pouring a predetermined aliquot volume into a graduated cylinder. After settling, the supernatant should be siphoned off with extreme caution. The final concentrate volume sample should include the liquid and cells from repeated rinses of the concentrating cylinder.

7.2 Centrifugation

Modern methods of continuous flow centrifugation have eliminated many of the early problems of cell loss. A number of problems, however, still exist. The diversity of cell size, shape and flotation mechanism results in widely differential settling times.[70-73] Some flagellates are highly resistant to centrifugation, requiring speeds in excess of 18,000 rpm, while other more fragile forms may lose flagella and other morphological appendages at considerably slower speeds. Centrifugation is satisfactory for fluorescence techniques. (See 8.4 Fluorescent Microscopy).

7.3 Filtration

The use of filtering techniques and methods including molecular filters has advanced rapidly in recent years. Procedures use either vacuum or pressure to facilitate the movement of the liquid portion of a sample through the filter. Samples may be filtered singularly or in multiple sampling manifolds filtering up to thirty simultaneous samples. Live organisms may be filtered and used for culture or productivity analysis. Preserved samples can be concentrated on a gridded filter and mounted on microscope slides for identification and enumeration. A wide selection of filter materials and pore sizes is available. Ancillary procedures requiring filtered samples include liquid scintillation counting and binding assays for proteins, enzymes, nucleic acids and other super-molecules.[64,74-80]

SECTION 8

PHYTOPLANKTON ENUMERATION

The enumeration of phytoplankton is indispensable in community analysis. Lund and Talling propose three advantages in direct optical observation: 1) evaluation of cellular variability, age, growth, shape and general physical state, 2) estimation of relative abundance of both common and rare species, and 3) determination of species present and their relative positions in the community.[81] There are many methods for microscopic enumeration, each having advantages and disadvantages. These methods employ a variety of techniques including settling chambers for inverted microscopy, counting chambers for standard microscopy, and membrane filters for both standard and scanning electron microscopy. Ancillary automated methods for size and pigment quantification are valuable for extended evaluation of community dynamics. The evaluation of individual species is aided by the use of enrichment cultures.[61-82] Examples of these various methods and techniques are presented separately to aid the investigator in selecting the appropriate procedures for his or her particular needs. The specific procedural details, advantages and limitations can easily be found in the literature and should be scrutinized to assure that the selected method will provide the desired quality of enumeration.

8.1 Utermöhl Method

Probably the best known and most widely accepted technique of phytoplankton enumeration is the Utermöhl method. According to this technique, a preserved sample is placed in a vertical chamber and allowed to settle. The chamber bottom is cover-slip thin and observation is made from beneath the chamber using an inverted microscope. This sedimentation method permits the investigator great flexibility. Variable volume settling chambers permit adjustments in aliquot size to ensure proper cell densities for accurate analysis. The inverted microscope permits a full range of magnification, allowing Kohler illumination, dark field, phase contrast, and oil immersion. When necessary, cells may be manipulated or removed from the chamber for closer examination. Disadvantages include the necessity of allowing sufficient time for all cells to settle to the bottom of the chamber. This settling time is a function of the column height and cellular morphology. Preconcentration of samples permits the use of small volume chambers and shortens the settling time. A second disadvantage results from the necessity of using preserved samples. Many community surveys are limited to the preservable phytoplankton because of the difficulties in accurate identification and enumeration of the many naked flagellates.[71,83-91]

8.2 Conventional Counting Method

The oldest method of phytoplankton enumeration that is still widely used employs a shallow-chamber slide and conventional compound microscopy. The most frequently used slides are the Sedgwick-Rafter, the Palmer-Maloney, and the haemocytometer counting chambers. Advantages of these chambers relate to their ease of use. Samples can be examined with a minimum of preparation, and the chambers are appropriate for live or preserved samples, permitting quick analysis of gross community characteristics. They also reduce the problems of detrital interference and obstruction. The primary disadvantage of the Sedwick-Rafter and the Palmer-Maloney chambers is that only low magnifications can be used. This limits the enumerations to large cells and often prevents positive identifications that are based on skeletal fine structure details. A second problem results from the tendency of the cells to form a nonrandom distribution within the chamber. If only a fraction of the chamber is to be enumerated, procedures outlined by Jackson and Williams should be used.[92] The haemocytometer may be used for small cell identification and enumeration but quickly becomes clogged if larger cells are introduced. It has received considerable use in culture studies but is not a practical chamber for natural community analysis. Lund developed an inexpensive nannoplankton counting chamber which can be used with intermediate magnification. This chamber suffices for the identification of all but the smallest phytoplankton.[81,88,92-99].

8.3 Particle Counters

The use of an electronic particle counter for the assessment of a natural phytoplankton community has met a considerable number of partially resolved problems. The Coulter Counter records the changes in conductivity caused by particles as they pass through a small sampling aperture. Phytoplankton analysis methods have been adapted from the particle counter use in hematologial analysis. Its application in algal culture analysis, where the population cell sizes all fall within relatively narrow range, has been generally accepted. In natural populations, however, problems of a very wide range of cell size and shape have only begun to be resolved. Beryozkina has proposed a theoretical classification of cells based on the morphometric complexities of form, size, chromatophores, cell contour, and the number of extreme points.[100] The adaptability of this classification to the capabilities of the counter differentiation of these characters has yet to be demonstrated. Additional problems are the overlap of cell sizes of large phytoplankters and small zooplankters and the presence of detrital material.[101-103]

8.4 Fluorescence Microscopy

Investigations involving the separation of the autotrophic and heterotrophic communities often employ fluorescence microscopy techniques. Under proper lighting conditions and with the addition of acridine orange dye the chlorophyll bearing parts of the cell will produce a bright red fluorescence while the rest of the cellular material will fluoresce green. This differential fluorescence enables separate counting of the photosynthetic and the heterotrophic communities. The microscopy technique allows analysis of freshly collected samples containing live organisms or samples preserved with five percent (5%) glutaraldehyde. The technique requires a completely darkened room because any background light obscures the fluorescence. Additionally, chloroplasts of dead autotrophic organisms will fluoresce making live-cell separation difficult. Finally, the Petroff-Hausser counting chamber has a very small volume and is prone to clogging and cell distributional anomalies.[104-105]

SECTION 9

PRIMARY PRODUCTIVITY

The measurement of primary productivity by the ^{14}C light-dark bottle technique is widely accepted as the only useful open ocean survey method. Oceanic sampling usually necessitates measurements to be made from a vessel which is either underway or on station for short time periods; the ^{14}C method is well suited for these conditions. It is also highly adaptable to a wide range of experiments. These experimental uses fall into two general types: incubator experiments and in situ experiments. Adaptations of the incubator usage involve the choice between natural or artificial light. Variations of the in situ method involve a choice of sample depths and incubation depths. All methods include variations in incubation time. The in situ method is believed to best reflect natural conditions but is not easily adaptable to routine ocean surveys. The in situ method should be done concurrently with incubator experiments whenever possible, to fulfill the continuing need for comparative data. The recommended methodology by Ahlstrom and SCOR-UNESCO should be adhered to when undertaking any primary productivity investigations.[106-119]

SECTION 10

PHYTOPIGMENT FLUORESCENCE

The investigation of phytoplankton productivity in the oceans has developed along two separate lines. One is the estimation of primary productivity as measured by photosynthetic rates. The most widely used method for this is the ^{14}C light-dark bottle technique described in section 9.0 primary productivity. An alternate approach is the estimation of phytoplankton standing stock. In addition to identification and enumeration procedures, the measurement of pigment concentrations provides an estimate of the size of the phytoplankton community. Concentrations of chlorophyll are measurable through their ability to fluoresce. Fluorescence is the capacity of a pigment to absorb light at one wavelength and to emit that energy at a lower wavelength. The intensity of this fluorescence may be used to estimate the amount of chlorophyll, which in turn may be used to estimate the phytoplankton standing stock. Many procedural variations have been developed for the extraction of chlorophyll from the cells and for the measurement of fluorescence using filter fluorometers. Measurements of fluorescence are highly sensitive, permitting the use of small sample volumes and minimal light scattering interference. With the use of selective filters chlorophyll _a_ can be distinguished from detrital sources. However, chlorophyll _a_ fluorescence emission ranges over a rather broad band and overlaps with the fluorescence of other chlorophylls.[105,106,114,120,125]

A method of _in vivo_ chlorophyll fluorescence measurment was developed by Lorenzen. This method is approximately only one tenth (1/10) as sensitive as extraction procedures, but its continuous-flow features present obvious advantages. The _in vivo_ measurements exhibit large inconsistencies which partially arise from the community's general physiological condition, its recent incident radiation history, and the variable ratios of chlorophylls and ancillary pigments. This variability requires extreme caution in the treatment of fluorescence data. A linear relationship between the _in vivo_ fluorescence and extracted fluorescence has been reported.[106,114,124,126-130]

SECTION 11

SOLAR RADIATION

The measurement of radiation is broken into two basic divisions; incident solar radiation and solar radiation in the water column. Incident solar radiation is the total sea level radiant energy of both the sun and the sky. A broad spectrum radiant energy detector or pyranometer is equally sensitive to the total wavelength span, including ultraviolet and infrared along with the visible spectrum. Thus these measurements do not necessarily correlate directly with biological activities below the sea surface. The spectral distribution of the incident solar radiation has been determined and it is possible to estimate the quanta and attenuation coefficients in specified spectral regions. The radiant energy within a particular spectral region at any particular depth can then be estimated. These data are then correlated with other measurements of standing stock and primary productivity in attempting to qualify the exchange of radiant energy in the ocean.

The second aspect of solar radiation is that portion of energy available at various depths below the sea surface. The problems associated with subsurface radiant energy measurement are unresolved because of inherent difficulties in distinguishing between available energy input and the energy that is utilized in photosynthesis. There is a need for further studies involving correlations between photosynthetic productivity determinations, absolute irradiance below the surface and the relative incident radiation.[106,108,114,131-133]

SECTION 12

ANCILLARY HYDROGRAPHY

All marine and estuarine surveys should include rather extensive hydrological analysis. Most of the parameters regularly included in a hydrographic survey are measurements of substances which effect organic production. In addition, most of the procedures are adaptable to conditions encountered at sea while maintaining a relatively high degree of accuracy. Generally, procedures are available which require fairly simply apparatus and can be quickly mastered by inexperienced technicians. Automated sample analyses have been developed that, when budgetary conditions permit, improve efficiency.

Common relevant hydrographic measurements can include salinity, pH, carbonates, inorganic micronutrients, dissolved oxygen, and dissolved organic compounds. The specific procedures to be followed are thoroughly covered in Strickland and Parsons' handbook.[114,134]

SECTION 13

STATISTICAL CONSIDERATIONS

The estimation of a marine or estuarine phytoplankton community that is inhabiting a particular body of water is subject to extensive extrapolations. The identification and enumeration of phytoplankton involves the analysis of a small fraction of an aliquot which was taken from a specific water parcel, perhaps taken from a heterogeneous larger water mass. The need for using appropriate statistical analysis is therefore clearly evident. Accordingly, the development of a useful monitoring or baseline survey demands an adequate awareness of the encountered statistical problems. These problems fall into three general groups. First, the development of the field sampling design which will result in the accumulation of samples representative of the area to be investigated. The problems of nonrandom distribution of organisms into clumps or patches, which themselves may be randomly or non-randomly distributed, was discussed in the sections on ecology and sampling design. Second, the method of sample treatment and the method of sample analysis present their own statistical pecularities. Each manipulation of a sample results in an error factor. These errors are cumulative in nature and must be both accounted for and held to a minimum. Third, there are a wide variety of manipulative procedures that can be applied to the obtained sample values. Multivariate factor analysis for marine systems is in the development stages with a continual barrage of new literature on various models and theories.[26,38,135-148] The phytoplankton community is commonly regarded from a variety of perspectives including cell density, evenness, equitability, diversity, ordination, recurrent group analysis, associations, and cluster analysis.[40,150-162] Standard texts such as Boesch, Cochran, Deming, Fisher, Fryer, Hirsch, Pielou and Siegel should be consulted for general theory and methodology.[35,36,143,162-166] The abundance of literature on population analysis and the interrelationships of various parameters prohibit specific reference. A close examination of the statistical bibliography will serve as a convenient starting point for the novice statistician.

SECTION 14

REFERENCES

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16. ABSTRACT

An overview of phytoplankton sampling and analysis methods as they apply to quantitative baseline and monitoring surveys is provided. A need for inclusion of a preliminary field survey of the area under investigation and of flexibility in sampling design is stressed. An extensive bibliography pertinent to phytoplankton sampling and analysis is included in the report.

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