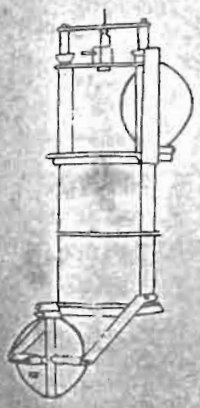
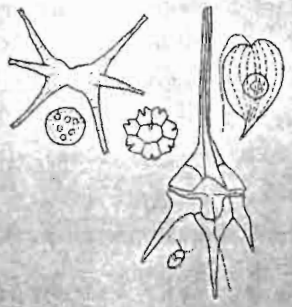
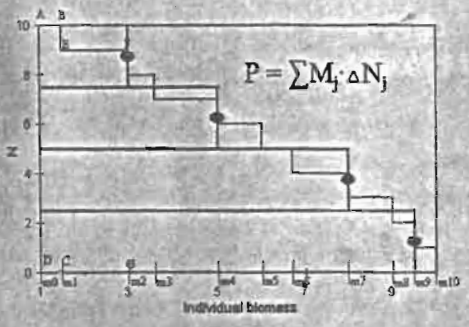
CURRENT METHODS IN AQUATIC SCIENCE

A guide to fundamental quantities and the methods for their quantitative determination













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for
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INTRODUCTION

Aquatic science is a broad and interdisciplinary field that draws on elements of biology, chemistry, geology and physics to explain and predict the nature of aquatic ecosystems. The objective of this manual is to introduce fundamental concepts and methods of aquatic science from an ecological perspective, emphasizing the role and importance of aquatic organisms. A brief introduction to important quantities and processes is combined with detailed descriptions of methods for their measurement. The methods have been chosen to be feasible for use in a well-equipped undergraduate laboratory or a basic research laboratory. The reference list offers sources for alternative methods as well as more detailed explanation of the methods and rationale for the approaches described in the manual.

The methods described here are all current, in the sense that they are still widely-used and accepted by researchers and managers. They are not necessarily of recent invention, however. Some date back to the 19th century, but are still standard techniques! The degree of acceptance of the methods emphasized here may vary depending on the specialized interests of the reader. Water quality and general ecology studies may be done quite satisfactorily with the pigment methods described here, for example, but specialists in the study of aquatic plants might see the need for a more demanding technique, such as HPLC. An attempt has been made to make the reader aware of the existence of more sophisticated methods wherever a simpler procedure has been adopted for detailed description.

In studying a new subject, the usual practise is to start from general principles and work down to the details of the subject, the approach taken by most university courses. This manual and the course it was written for represents exactly the oppposite approach, because it is concerned primarily with how the observations, measurements, and calculations should be done rather than why. This may cause some problems for those who have not studied aquatic science previously. An attempt has been made to explain the fundamental ideas about the organization and function of aquatic systems so that the importance and relevance of the methods can be appreciated, but the accounts are very brief. It is therefore quite desireable that one or more of the textbooks cited in the reference list (e.g. Wetzel 1983, Moss 1988, Horne & Goldman 1994) be consulted for additional background

It has been assumed that the reader has taken at least one course in statistics, and is familiar with descriptive statistics, ideas about sample vs population characteristics, and basic tests for comparing sample means and the distribution of data. It is also assumed that the reader knows enough about linear regression to fit a line using a spreadsheet or other numerical software package. The biological preparation is assumed to include survey courses in the plant and animal kingdoms and some introduction to ecological principles.

C. PRIMARY PRODUCERS

1. Sampling, enumeration and biomass determinations for algae

1.1 Phytoplankton

Microscopic algae are important and even dominant primary producers in many aquatic systems, especially large lakes and the open ocean. Macrophytes, both algae and flowering plants, are important or dominant primary producers in many aquatic systems too. The methods for enumerating macrophytes and determining their biomass are essentially similar to those employed for terrestrial plants and depend on identifying, and often harvesting, individual plants or groups of plants. By contrast, enumeration and biomass determination for microscopic algae require techniques that are quite specific to aquatic ecology and it is these methods that are stressed here.

Phytoplankton commonly range in size from approximately 1 μm in dimension up to 200 μm. Terms often attached to phytoplankton of different sizes are picoplankton (0-2 μm), nanoplankton (2-20 μm) and net plankton (20-200 μm). The terminology and size definitions may vary somewhat among workers. It should be remembered that in a sample from any natural water each size range will include organisms other than algae (bacteria, fungi, protozoa, and various small metazoa). The prevalence of non-algal organisms in all size ranges is one reason that the relatively laborious enumeration and biomass determination methods for algae described here are still necessary. No chemical analysis will reliably separate the algae from the "contaminating" organisms.

Historically, phytoplankton were sampled using plankton nets, with mesh size of 20-30 μm, or sometimes even larger. Nanoplankton were greatly under-estimated in such collections and picoplankton were scarcely recognized at all; not only sampling but microscopic methods were inadequate to reveal such tiny cells. In the past 20 years it has become accepted that probably the majority of natural waters are dominated, in terms of phytoplankton biomass, by nanoplankton and picoplankton. As a result, all quantitative measurements of phytoplankton biomass and productivity nowadays use whole water samples (section B1) rather than net samples. Nonetheless, net samples can be useful as a relatively quick way to learn something about community composition and are still collected for some applications.

Phytoplankton nets are usually conical with a circular opening made rigid by a metal hoop. The material is nylon of relatively specified mesh opening. A serious problem with phytoplankton nets is that the small mesh openings present a significant barrier to flow through the net. As a result, the volume of water actually filtered by the net is difficult to determine, to the extent that many workers do not recommend nets even for qualitative work (e.g. Wetzel & Likens 1991). The sample volume problem can be solved by taking a whole water sample and pouring it through the net but at the cost of some inconvenience. Commonly, nets are dragged for a known distance in the vertical or hoizontal to concentrate organisms from an approximately known volume of water (=area of net opening X distance dragged). It is important to wash the net before and after use. Before use, the bottle at the end of the net should be removed and the net submerged, raised and drained several times. The bottle is then replaced and the net tow made. At the end of the tow, the organisms should be concentrated into the bottle by repeatedly lowering the net bottle-first into the water until the opening is near the water surface, then raising

it again. It is good practice to wash the net again at the end of the sampling, when the bottle has been removed to dispense the sample. With all these precautions, net samples are only the most approximate and selective indicators of phytoplankton abundance and composition. However, they do give a pre-concentrated sample that is easy to examine with readily-available microscope equipment.

1.2 Periphyton

Periphyton are algae that live attached to surfaces, which may be the lake or stream bed or larger plants. In many streams and rivers they are the dominant primary producers, and they are also important in many lakes. Because of their association with substrates that can be physically complex and dynamic, their sampling can be relatively difficult and specialized to the system of interest. Methods that work for algae growing on rocks, for example, may not work for those growing on plants or on sand.

Where representative portions of the substrate, such as cobble or robust rooted vegetation, can be removed from the stream, reasonable samples can be obtained by scrubbing. In one approach, a template of known area (e.g. 5 cm^2) is applied to the surface of the substrate and the area around it scrubbed free of observable periphyton. The known area is then scrubbed into a container with sufficient water to make a convenient suspension. Variants of this approach have been developed for use underwater. If a pigment-based measure of algal biomass is desired, then substrates can sometimes be extracted by an apropriate solvent.

It can be readily imagined that many substrates are difficult to sample reproducibly or quantitatively. A variety of **artificial substrate** methods have been developed in an attempt to obtain more reproducible results. The substrates may be glass slides, ceramic tiles, or even artificial plants obtained from aquarium supply stores. The substrates are deployed in the system of interest and left for a period of time (often several weeks or more) to allow colonization to occur, and are then harvested by scrubbing or other methods. The obvious problem is that there is no guarantee that the community on the artificial substrate will resemble the natural one, and if the natural substrate is hard to sample then comparative samples will not be readily available. Nonetheless, artificial substrates do typically support the development of algal communities that have many of the features of those on natural substrates and they have found wide use in ecological studies. Standard methods for assessing stream water quality have been developed based on the abundance and types of algae, especially diatoms, that colonize glass slides.

1.3 Preservation and microscopic examination methods

For identification purposes, it is always desireable examine live material, yet this is often not possible. More often, samples must be preserved and wait days or months before examination. Many preservatives have been used, but a 0.5 to 2% concentration of buffered formalin was, and still is, a common choice. This preservative works well for many species but can make smaller and more delicate organisms difficult to identify (or even recognize) because of deformation, rupture, loss of flagella, etc. Particularly if nanoplankton are of interest, a better preservative is Lugol's iodine (10 g I_2 and 20 g KI dissolved in 200 ml distilled water plus 20 ml concentrated glacial acetic acid). Lugol's is a convenient preservative because the appropriate final concentration (approximately 1%) can be judged visually by adding preservative until the sample is a reddish-brown colour. The absorption of iodine by the preserved cells also increases their density and accelerates their sedimentation rate, an advantage when using the Utermöhl

method. Lugol's iodine does have the disadvantage that the staining of the cells may conceal some intracellular structures and will quench the fluorescence of chlorophyll or added fluorochromes, so Lugol's is not a good choice if the sample is to be analyzed by fluorescence microscopy (section E). A good choice in the latter case is to use glutareldehyde, neutralized to pH 7, to a final concentration of 1-3%. If samples are kept refrigerated, glutaraldehyde will give excellent preservation of fluorescence and cell form. **Like formaldehyde, glutaraldehyde is highly toxic and is to be handled with care.** Lugol's iodine is much less hazardous to human health.

Net samples of phytoplankton are strongly concentrated by the nature of the collection method, and can be easily examined by a variety of microscopic techniques. The subject organisms are also relatively large, easing the demands on the microscope system. Even dissecting microscopes can often reveal many of the net phytoplankton, and a low power compound microscope will suffice for quantitative work. If nanoplankton and picoplankton are of interest then life is not so easy. The first problem is how to concentrate the whole water sample, because there are too few phytoplankton in most natural waters to allow any meaningful analysis simply by examing a drop of sample on a slide. Generally, 5 to 50 ml of sample must be concentrated onto some kind of slide or microscope chamber to allow analysis of nanoplankton and picoplankton. There are two main ways of doing this.

The first concentration method is used in the **Uttermöhl method** of phytoplankton analysis (Lund 1951). The Uttermöhl method uses a removable tower mounted over a special kind of well slide, or counting chamber. The tower is completely filled with preserved sample, sealed with a cover plate, and left to allow cells to sediment into the counting chamber. A safe rule of thumb for sedimentation time in hours, for Lugol-preserved samples, is to multiply the height of the tower in cm by 3. A 10 cm tower would then need 30 hours of sedimentation time to ensure that all cells have fallen into the counting chamber before the tower is removed and replaced with a cover slip. An advantage of this technique is that it is gentle and gives a uniform distribution of organisms in the counting chamber. Disadvantages are the sedimentation time and, importantly, the need for a special kind of microscope. Because Uttermöhl counting chambers are unavoidably quite thick, a conventional compound microscope that examines the sample from above will not be able to examine the sample at high magnification. The thickness of the chamber is too great compared to the working distance of high power objectives. Instead, an inverted microscope that places the objective beneath the chamber is needed. Although common in well-equipped research labs, inverted microscopes are relatively expensive. The sedimentation and counting chambers are also quite expensive. Nonetheless, the Uttermöhl method has become the standard for quantitative assessment of total phytoplankton because it can allow recognition and enumeration of phytoplankton of all sizes.

For picoplankton analysis, even the Uttermöhl method may not be adequate. If the inverted microscope is very good and the sample is obliging then pico-phytoplankton can be recognized, counted and sized using an oil-immersion (100X) objective on an inverted microscope. This requires counting chambers with special, very thin, bottom plates that are expensive and fragile. In many samples, however, it is hard to reliably detect the tiny picoplankton that may be hidden by detritus or other organisms. There may also be enough overlap in size and shape between pico-phytoplankton and the bacterioplankton that it is hard to

classify the organisms encountered. This is a serious problem in the open ocean and many large lakes where much or most of the phytoplankton biomass is in the picoplankton. Here, **fluorescence microscopy** can help. The concentration method most commonly employed for fluorescence microscopy in aquatic ecology is filtration. Further description will be given in section E.

Guides for the identification of phytoplankton are specialized literature, and many are in French or German and not readily-available. Wetzel & Likens (1991) list a number of works, mainly in English. Prescott (1978) and Findlay & Kling (1979) are particularly useful to the study of Canadian fresh waters.

1.4 Quantitative abundance and biomass by microscopy

Regardless of the exact type of examination method used, quantitative estimates of abundance follow certain rules. It is unusual to enumerate all the organisms in the counting chamber or slide because there are usually far too many and it would take too much time to scan the entire area of the chamber. Instead, a known area of the chamber is surveyed and the concentration of organisms in the original sample is calculated as

1.1 Concentration (#/l) = $[N \cdot A_t]/[A_c \cdot V]$

where N is the number of organisms seen, A_t is the total area of the counting chamber (or slide), A_c is the area surveyed, and V is the volume of sample added or concentrated into the chamber (in liters). A_t is known from the dimensions of any particular counting chamber, while A_c must be calculated. In one approach, randomly-selected fields are surveyed and the total number of fields for the total N is recorded. A_c is then calculated as the product of the area of view at the magnification used and the number of fields. The area of the field is determined using an ocular micrometer. In a variation of this approach, an ocular grid in one of the eyepieces superimposes a grid over the field of view, and the area of the grid or its components may be used as counting units. It is then necessary to calibrate the area of the grid (or its components) using an ocular micrometer. Alternatively, one or more transects across the chamber may be surveyed, and A_c determined from the known length of the transect and the width of the field of view, again determined with an ocular micrometer.

Regardless of the specific method used to arrive at the application of equation 1.1, an obvious question is how large N should be for reliable results. If the chamber is used properly then the organisms should be randomly distributed within it. If so, the probability of encounter in any given counting unit (field of view, grid, etc.) follows a Poisson distribution. Then, the variance is proportional to the mean and the precision of the count can be calculated from N, as exemplified in Table C1.

Table C1. The precision of phytoplankton counts as a function of sample size (N) assuming random distribution of organisms in the counting chamber

Number of organisms (N)	95% C.I. as % of count	95% C.I. as range
4	±100%	0-8
16	±50%	8-24
100	±20%	80-120
400	±10%	360-440
1,600	±5%	1,520-1,680
10,000	±2%	9,800-10,200

It can probably be imagined that very few investigations can afford the luxury (or drudgery) of counting samples to a precision much better than ±10% on a routine basis. Fortunately, such precision is more than adequate for many purposes. For example, phytoplankton populations typically wax and wane by more than an order of magnitude on a seasonal basis, so counts with even a ±20% precision would be quite useful to describe such variations.

A complication encountered in many samples is that many of the phytoplankton are colonial organisms. If the total cell number (N) is obtained by multiplying the number of colonies by the mean number of cells per colony then the precision of the estimate will be overestimated. In most such cases, a realistic estimate of precision can be obtained if estimated from the total number of independent particles (colonies or unicells). A more rigorous estimate of precision encounters much more complicated statistics but is not usually necessary in practice.

A great variety of chambers other than the Uttermohl exists for quantitative phytoplankton enumeration. The Sedgewick-Rafter cell is one common type and is suitable for relatively low-magnification examination. Heamocytometers and variants such as the Palmer-Maloney cell can be used where the phytoplankton suspension is fairly concentrated. A practical counting chamber can also be made inexpensively from plexyglass slides. The chambers vary in the volume of sample they can accomodate and the total viewing area afforded; choice is based mainly on the anticipated size and concentration range of the phytoplankton to be encountered.

Phytoplankton vary widely in size so that an estimate of total concentration as numbers of organisms per volume is not a very good estimate of **biomass per volume**. To calculate total biomass, it is necessary to estimate the individual biomass of the various organisms encountered. This is done by estimating the **biovolume** of the organism from its dimensions and then converting the biovolume into a corrresponding mass, the biomass. The conversion factor from biovolume to biomass varies among species and the most careful work uses factors determined from laboratory studies of algal cultures, where biomass can be more directly measured. An approximate conversion can be made by assuming that algae are on average slightly denser than water (roughly 1.05-1.1 mg/ml), but this will lead to serious errors in organisms such as diatoms, which have large intracellular vacuoles that are largely water.

Determining the biovolume of organisms seen and measured under the microscope is not without its challenges. While dimensions may be measureable, it is still necessary to assume some sort of geometric shape for which the volume can be calculated. Phytoplankton are not all simple shapes such as spheres and cylinders, although many of them can be so approximated and lead to simple formulae, e.g.:
sphere: $V = \pi D^3/6$; rod: $V = \pi D^2 L/4$; cone: $V = \pi D^2 L/12$; ellipsoid: $V = \pi D^2 L/6$
where D is diameter and L is length. Many common phytoplankton shapes are relatively elaborate and must be approximated as a combination of various geometric shapes such as cones and ellipsoids. Wetzel & Likens (1991) provide illustrations and formulas to deal with most common shapes encountered.

Clearly, the labour and book-keeping involved in total phytoplankton biomass determinations by microscopic methods is substantial. The next section briefly discusses alternatives and improvements.

1.5 Instrumental and automated methods for enumeration and biomass

Three main classes of instrument have been developed and applied to plankton enumeration problems. **Electronic particle analyzers**, exemplified by the Coulter Counter, have been around for decades. They induce a flow of sample through an aperture across which an electrical potential is maintained. When a particle (e.g. a phytoplankton cell) passes through the aperture it displaces some of the electrolyte solution in which it is suspended and this changes the potential across the aperture. The instrument counts the number and size of perturbations to the potential and interprets these as particles of various sizes. Unfortunately, any particle in the size range that the aperture can detect (and this is selectable from about 1 μm to 200 μm) will register as a particle so the instrument cannot by itself discriminate between phytoplankton and other particles. With some prior knowledge of the samples (e.g. whether there are certain size ranges that will be almost entirely phytoplankton) electronic counters can be used to some effect. They are extremely useful for laboratory cultures.

Flow cytometers also induce a flow of sample through an aperture but they use optical information obtained by aiming a laser at the particles as they pass through the inspection point. The scattering and fluorescence properties of the particle can be assessed, and this gives flow cytometers the great advantage that they can discriminate phytoplankton from other particles. The chlorophyll fluorescence given off by phytoplankton (see section C2.2) is readily detected and sets phytoplankton apart from other organisms and detritus. The size of particles (phytoplankton) can be estimated from the scattering properties. Flow cytometers have found much application in oceanography because they are a very effective way to size, enumerate and characterize the tiny picoplankton that dominate much of the open ocean. Unfortunately they are not at present very effective at analyzing net phytoplankton or the larger nanoplankton, partly because the sampling rate of flow cytometers is so low (only a μl per second or so) that they cannot easily get statistically-meaningful measures of the larger, and rarer, phytoplankton. The more complicated shapes typical in the larger phytoplankton are also a problem for flow cytometers (and particle counters). The geometric assumptions used to infer particle size may be quite wrong when shapes depart widely from that of a sphere, and physical problems of clogging may arise in the tiny and delicately-calibrated plumbing of the instrument.

Image analysis systems can now be purchased and used to capture, digitize and analyze microscope images of phytoplankton samples, such as the typical field of view in an Uttermohl chamber. Such systems can be used as "semi-automatic" devices in which the operator points to particles (phytoplankton) on the screen and enters identity and size information, or as fully-automated systems that use pattern-recognition algorithms to determine the number, size, and even identity of organisms in the image. The latter goal is still not a practical reality for general application, although some systems have been "taught" enough to analyze some phytoplankton community samples. The success of such systems depends very much on the quality of the optical components, including the camera.

There is at present no universally-applicable, automated way to get total phytoplankton community biomass or to identify all the species in the community. Human-guided microscopic analysis is still the benchmark method. Computers have helped, however, and there are now programs that can greatly speed the volume and biomass calculations. One such program is COUNT/PHYTO, developed at the Canadian Museum of Nature. COUNT/PHYTO "knows" a large number of phytoplankton by name. Given the number and, in some cases, dimensions of the various species of phytoplankton encountered the program will total the biovolumes and biomass by species and for the total community. This is a great saving in effort for the analyst.

2. Plant pigments

2.1 Variety and distribution of plant pigments

The principal photosynthetic pigment of the Anthophyta (higher plants) and Bryophyta (mosses) is chlorophyll *a*, with lesser amounts of chlorophyll *b* and carotenoid pigments occurring in most species. Chlorophyll *a* (Chl *a* for short) is also the single most important and widespread pigment among the algae. The algae display, however, a far wider array of photosynthetic pigments than do the Anthophyta. The pigment content of algae is in fact one of the characteristics used to classify them Table C2).

All plants, whether Anthophyta or algae, contain Chl *a*. The concentration of Chl *a* is therefore widely used as an approximate index of plant biomass. The Chl *a* content of plants varies depending on their physiological condition and their taxonomic status, so the index is only approximate and must be subject to careful interpretation. It is nonetheless one of the most useful plant-specific measurements one can make in plankton samples, where the particulate matter always contains detritus and non-plant organisms.

Careful analysis of plant pigments in samples of phytoplankton can give useful information about the types of algae present, while quantification of certain break-down products can be used to infer rates of grazing activity in the community. Among algae, at least four different forms of chlorophyll (a, b, c_1 and c_2) are known. The carotenoid pigments, particularly the xanthophylls, are quite specific in their occurrence among the major algal groups. The phycobilin pigments, of which there are three major types, occur only in red algae, blue green algae and cryptomonads. All the non-Chl *a* photosynthetic pigments are known as accessory pigments because their physiological role is to transfer light energy to a Chl *a*-protein complex. Accessory pigments help algae harvest a wider range of wavelengths of light than higher plants, with their simpler pigment composition, can generally manage.

Table C2. The principal pigments in some major groups of algae and in the higher plants. Occurence is an approximate indication of where the group is most likely to be found.

Plant Group	Chlorophylls	Carotenoids	Phycobilins	Occurence
Anthophyta (flowering plants)	Chl *a*, *b*	β-carotene	none	benthic, littoral
Cyanophyta (= cyanobacteria, blue-green algae)	Chl *a*	β-carotene	c-phycoerythrin, allophycocyanin, c-phycocyanin	ubiquitous
Chlorophyta (green algae)	Chl *a*, *b*	α, β, and γ-carotene, lutein, siphonoxanthin siphonein	none	ubiquitous in freshwater, benthic in marine systems
Chrysophyta (diatoms and their chromophyte relatives)	Chl *a*, *c*, c_1, c_2	β-carotene fucoxanthin diatoxanthin diadinoxanthin	none	ubiquitous
Dinophyta (dinoflagellates)	Chl a, c_2	β-carotene peridinin neoperidinin	none	planktonic, marine and freshwater
Phaeophyta (brown algae)	Chl *a*, c_1, c_2	β-carotene fucoxanthin	none	benthic, marine
Rhodophyta (red algae)	Chl *a*	α and β-carotene	r-phycocyanin allophycocyanin c-phycoerythrin	benthic, especially marine
Cryptophyta (cryptomonads)	Chl *a*, c_2	α-carotene diatoxanthin	phycoerythrin phycocyanin	planktonic, especially freshwater
Euglenophyta (euglenoids)	Chl *a*, *b*	β-carotene astaxanthin antheraxanthin diadinoxanthin neoxanthin	none	planktonic, freshwater
Prochlorophyta (prochlorophytes)	Chl *a*, *b*	β-carotene zeaxanthin cryptoxanthin	none	marine, recently discovered

2.2 Detection methods

Quantification of plant pigments can be done using measurements of either fluorescence or light absorption. Fluorescence measurements exploit the fact that molecules such as Chl *a* can be excited to a triplet state by absorption of photons, and a significant proportion of the triplet molecules will return to the ground, or singlet, state by emitting a photon of light of wavelength longer than that originally absorbed. For Chl *a,* for example, blue light is absorbed and excites the molecule, which then emits red light by fluorescence (which is measured by the fluorometer). Other pigments, Chl *b* for example, would be optimally excited at a different wavelength and would also emit light of a different wavelength from that of Chl *a*, so fluorescence measurements can be quite specific for pigments of interest. Fluorescence is a highly sensitive method for measuring pigments but the instrumentation is relatively expensive, especially if excitation and emission spectra are needed (useful for pigment identification). Absorption measurements can be done even with fairly basic, and affordable, spectrophotometers but are less sensitive. In measuring pigments by absorption, the object is to select wavelengths that correspond to the absorption maxima for the pigments of interest. For Chl*a*, for example, there are well-defined absorption maxima both in the blue and red parts of the spectrum. The absorption of a sample can be compared to a standard curve or, in many cases, to conversion equations published in various standard methods handbooks (e.g. Strickland & Parsons 1972). The availability of such equations is a convenience in the approximate discrimination of different pigments in a sample (e.g. Chl *a* vs Chl *b*) and is an advantage of absorbance measurements. For critical separation and identification, High Performance Liquid Chromatography (HPLC) is necessary but the methods are expensive, time-consuming, and unsuitable for routine application to aquatic samples. In this course, both fluorescence and absorption measurements, but not HPLC, will be used.

Both fluorescence and absorption can be measured directly on whole water samples to determine phytoplankton pigment concentrations, but only fluorescence is sensitive enough to be applicable to most natural waters. Almost all fluorometers for routine use in aquatic science are designed to measure Chl *a* rather than other pigments. The *in vivo* fluorescence of Chl *a* measured on whole water is influenced considerably by the physiological condition and light exposure of the phytoplankton, and is only an approximate index of Chl *a* concentration. It is nonetheless a very useful quantity because it can be measured in automated or semi-automated ways to obtain surveys of phytoplankton abundance that are too laborious or expensive to obtain any other way. For example, submersible fluorometers are available that can be lowered through the water column, recording Chl *a* fluorescence as they go and either transmitting the data back to the ship or storing it in memory for later downloading. Such instruments, widely available but still quite expensive, allow the distribution of plant biomass to be assessed throughout the water column in a matter of minutes. Other configurations include moored instruments that monitor Chl *a* fluorescence over time at fixed locations, and flow-through instruments that monitor phytoplankton abundance in processes such as aquaculture.

2.3 Concentration and extraction of pigments

More accurate measurements of pigment concentrations generally require the pigments to be extracted into solvents, in which either fluorescence or absorption can be measured under

standardized conditions. Most often, and almost invariably for absorption methods, some prior concentration of the sample is necessary when dealing with phytoplankton or periphyton. The concentration is most often done by filtration. In general, filters capable of retaining even the smallest of phytoplankton (the picoplankton) should be used, with a nominal pore size of 1.0 μm or less. Suitable filters include Whatman glass fiber filters, grade GF/F, and Millipore HA cellulose acetate (0.45 μm pore). The filters are then extracted in the solvent(s) of choice. In waters with very low phytoplankton concentrations, such as the open sub-tropical oceans and some very oligotrophic lakes (e.g. L. Superior) it may be necessary to filter up to 200 ml for a good fluorometric measurement of Chl *a*. Absorbance measurements in such systems would require filtering several liters of sample. For more typical fresh waters, samples of less than 1 liter will usually be enough for absorbance measurements.

Different pigments often call for different solvents. For example, the chlorophylls are all highly soluble in relatively non-polar solvents such as acetone, but the proteinaceous phycobilin pigments require a detergent extraction under conditions of basic pH. Even for a given type of pigment, however, a variety of methods using different solvents and/or associated treatments can be found. A comprehensive survey of the different approaches that have been developed even for one pigment, Chl *a*, is beyond our scope in this course. The main consideration is always whether a given solvent can be expected to succeed with the plant material in hand; some algae and pigments are definitely harder to extract than others. An additional consideration is that, if published conversion equations are to be used to convert absorbance to pigment concentration, then the solvent system for which the equations were developed must be used. Besides choosing a solvent system, the analyst must also decide whether a mechanical treatment (e.g. grinding or sonication) is necessary to acheive a satisfactory extraction efficiency. The literature on pigment analysis is large, and we will try only a few of the more generally useful methods here.

One useful and simple extension of both the fluorometric and absorbance approaches exploits the fact that acidification of pigment extracts converts chlorophyll to a breakdown product called pheophytin, in which the Mg atom has been lost from the ring structure of the parent molecule. The absorbance and fluorescence properties of pheophytin are measurably different from those of chlorophyll, and the relative abundance of the two types of pigment can be measured using absorption or fluorescence measurements before and after acidification of the sample. The resulting information on the prevalence of chlorophyll breakdown product in the sample can be used to infer the health of the plants and the activity of grazing animals (which also convert chlorophyll to pheophytin). For critical applications, discrimination of chlorophyll from pheophytin should be done using HPLC but the simpler methods described here are often useful.

In all pigment work, it should be remembered that the compounds of interest are designed to absorb light. They all have the potential to be damaged by excess light energy if exposed to bright light, especially when extracted into solvents, and samples at all stages of preparation should be suitably protected. Many of the pigments, especially Chl *a*, are also very sensitive to acids and corresponding care must be taken. This even includes ensuring that filters used in the analysis are never touched by fingers (unless perhaps gloved) since acids naturally present on the skin can induce significant degradation of Chl *a*.

2.4 Procedures for chlorophyll and carotenoid pigments

a) *In vivo* fluorescence of Chl *a* in discrete samples

1. Ensure that the fluorometer has been on for at least 15 minutes before measurements are made. The sample should be kept in darkness for 15 minutes before measurement. Read the fluorescence of a blank tube (de-ionized water is a suitable blank), then read the sample. The Turner Designs fluorometer will automatically find the optimal range for the sample. Record the fluorescence value, which is in arbitrary units, and proceed to the next sample.
2. When a number of samples have been analyzed by this method and by one or more of the following methods, a relationship can be established between chlorophyll *a* concentration and the *in vivo* fluorescence. For samples of uniform type (e.g. samples of phytoplankton collected from the surface mixed layer of a lake over the course of a day) the relationship will usually be quite close, and *in vivo* fluorescence values (which are simple to collect and can even be done *in situ*) can be used to predict actual chlorophyll *a* concentration in samples not analyzed by more demanding methods.

b) Extracted pigments by absorption (spectrophotometric method)

1. Filter the water sample through a suitable filter, keeping filtration pressure below 0.3 atm to minimize damage to the phytoplankton. Consult the demonstrator for recommended volumes.
2. Transfer the filter to a centrifuge tube and add 10 ml of an alkaline 90:10 acetone-water mixture (100 ml distilled water made to 1000 ml with spectral grade acetone plus 2 drops of reagent grade ammonium hydroxide). Cap tightly, and allow to extract passively for one hour on ice and in the dark. Without mechanical disruption pigments will not be fully extracted from many types of plant material (e.g. blue-green algae), and 16-20 hours of extraction time is more standard for passive extractions, but we will get most of the pigments out with this abbreviated method.
3. When grinding or other mechanical disruption methods are used, centrifugation of the extract is essential. With passive extraction it is often unnecessary, but if required (i.e. if extracts are obviously turbid) then spin the tubes at maximum setting (4,00-5,000 rpm) on a bench-top (clinical) centrifuge for 5 minutes.
4. Decant the extract to a spectrophotometer cuvette and measure absorbance at 750 (for turbidity correction), 665, 664, 663, 647, 630 and 480 nm. At each wavelength, the spectrophotometer should be adjusted to 100% transmission using a matched cuvette containing only the solvent mixture, before measuring the sample absorbance.
5. Add 1 or 2 drops of 1N HCl to the cuvette, mix thoroughly, and repeat the absorbance measurements at 750, 665 and 663 nm.
6. Rinse the cuvette thoroughly with alkaline acetone and shake dry. Proceed to the next sample.
7. Calculation of pigment concentrations can now be done by a variety of methods, each of which makes certain assumptions about the predominant types of pigments present and their absorption characteristics in the solvent. Regardless of which method is used, subtract the turbidity absorbance (750 nm) from the readings at other wavelengths before proceeding with the calculations.
8. Monochromatic method for Chl *a* and phaeopigments (Lorenzen 1967):

Chl *a* (µg/l) = $[k \cdot F \cdot (E665_b - E665_a) \cdot v]/[V \cdot Z]$

Phaeo (µg/l) = $[k \cdot F \cdot (R \cdot E665_a - E665_b) \cdot v]/[V \cdot Z]$

where
$E665_b$ = absorbance at 665 nm before acidification
$E665_a$ = absorbance at 665 nm after acidification
k = absorption coefficient of Chl *a*, =11.0
F = a calibration factor for effect of acid on k, = 2.43
R = maximum ratio of E665b:E665a in absence of phaeopigments, = 1.7
v = volume of extract, in ml
V = volume of water filtered in liters
Z = pathlength of cuvette in cm

9. Trichromatic method for chlorophylls a, b and c (Jeffrey & Humphrey 1975):

Chl *a* (µg/l) = $[C_a \cdot v]/[V \cdot Z]$
where $C_a = 11.85 \cdot E_{664} - 1.54 \cdot E_{647} - 0.08 \cdot E_{630}$

Chl *b* (µg/l) = $[C_b \cdot v]/[V \cdot Z]$
where $C_b = 21.03 \cdot E_{647} - 5.43 \cdot E_{664} - 2.66 \cdot E_{630}$

Chl *c1+2* (µg/l) = $[C_c \cdot v]/[V \cdot Z]$
where $C_c = 24.52 \cdot E_{630} - 1.67 \cdot E_{664} - 7.60 \cdot E_{647}$

V, Z and v are as defined above, and E_{630}, E_{664} and E_{647} are the values measured before acidification.

c) Extracted chlorophyll and pheophytin by fluorescence

1. Before reading your samples, consult the detailed instructions for the use of the fluorometer, and blank it with acetone solvent. The fluorometer has been set to deliver excitation light of 430-450 nm (blue) and to sense the emitted light at 650-675 nm (red), which is optimal for chlorophyll *a*.
2. Dilute some of the extract from part (b) above until the pigment colour is barely noticeable; this will require approximately a 100-fold dilution. Be sure to note the actual dilution that you used so that you can back-calculate the true concentration of pigment. Fill a fluorometer cuvette and read the fluorescence of the diluted extract (the original would be off-scale).
3. Add 2 or 3 drops of 1N HCl, mix thoroughly, and read the fluorescence again.
4. Pigment concentrations are then calculated using a calibration factor previously obtained by analyzing samples of pure chlorophyll *a* of known concentration:

Chl *a* (µg/l) = $F \cdot [\tau/(\tau-1)] \cdot (R_b - R_a) \cdot D \cdot (v/V)$

$$\text{Pheo } (\mu g/l) = F \cdot [\tau/(\tau-1)] \cdot (\tau R_a - R_b) \cdot D \cdot (v/V)$$

where F is a calibration factor (= µg/l Chl *a*·/ R_b), V is volume of water filtered (ml), v is volume of solvent used to extract the pigment (ml), and τ is the ratio of fluorescence before acidification to that after acidification in samples of pure chlorophyll *a*. Where extracts have been diluted before measurement, the concentrations must also be corrected for the dilution factor, D. If no dilution was used then D=1.0. The acid ratio, τ, varies with the sensitivity setting of the fluorometer. Consult demonstrator for appropriate values of τ and F.

5. Pigment concentrations determined by this method will not necessarily agree with those determined by absorbance measurements as conducted above. Thinking about the diversity of light-absorbing and fluorescing pigments that can be found in aquatic plants, and the sensitivity of absorption and fluorescence properties to the pigment's environment (solvent type, pH, etc.), you can perhaps understand why none of the foregoing methods is likely to be absolutely accurate for any bulk pigment sample from a natural community. Fully accurate identification and quantification of pigments in such samples is still something of a research project in itself.

3. Primary Production

3.1 Primary production defined

Primary production is the conversion of inorganic carbon to organic carbon by the photosynthetic activity of the plant community. It is the fundamental basis for most food webs on Earth, including the aquatic food webs of lakes and streams. In freshwater aquatic systems we distinguish between primary production taking place in the lake or stream (autochthonous production) and that contributed by the terrestrial part of the catchment (allochthonous). Some systems, such as small streams and lakes in heavily wooded countryside, depend largely on allochthonous production. The oceans, larger lakes and many mid-sized streams depend mainly on autochthonous production. Because of the importance of primary production, the relevant literature is huge and continually growing. Two excellent recent books (Kirk 1994, Falkowski & Raven 1997) review the current state of the field and should be consulted by anyone wishing to know more about aquatic primary production.

Aquatic plants are usually categorized based on their size and growth habit, and methods for measuring primary production vary accordingly. The phytoplankton are microscopic (usually) algae, living suspended in the water column. They are the predominant primary producers in most larger lakes and in the oceans. Benthic algae live attached to the bottom, and range in freshwater from microscopic forms to macroscopic colonies of 10 or more cm dimension. Benthic algae are often the predominant primary producers in mid-sized rivers and streams. Macrophytes are relatively large plants; in freshwaters they usually belong to the flowering plants or the bryophytes but in the oceans they are often algae. They may dominate primary production in shallower rivers and lakes, and in some marine coastal zones. The relatively large size of the macrophytes demands special and rather laborious methods for estimating their primary production. The methods emphasized in this section are designed mainly for estimating primary production by phytoplankton, but with some modification can also be applied to benthic algae.

It is important in measuring any ecological process to be quite clear about exactly what one wishes to measure. In the case of primary production, we can conceive of at least three different types of production. The first is **gross primary production**, the total rate of plant photosynthesis before any losses. However, the plants themselves have to use some of their photosynthetic products to meet their own metabolic needs, so that we might prefer to think in terms of **net primary production**, which is the excess of photosynthesis over the immediate respiratory needs of the plants. Then again, we might find it more useful to measure **net community production**, which is the excess of photosynthesis over the respiratory needs of the plants and the other organisms (including grazers) that form the local community. Most often in ecology, we would like to know the rate of net primary production, which is the best estimate of the plant production potentially available to support the local food web, and we would like to have our answer in terms of carbon or its energy equivalent. Ironically and unfortunately, it is net primary production that is most difficult to measure in natural aquatic plant communities.

3.2 The major approaches to measuring primary production

Oxygen-based methods for primary production exploit the fact that photosynthesis involves stoichiometric release of O_2 and uptake of CO_2:

(1) $nCO_2 + nH_2O \rightleftharpoons nCH_2O + nO_2$

Equation (1) runs to the right in the light (photosynthesis) and to the left in darkness (respiration). The **oxygen light and dark bottle technique** involves enclosing the plants of interest in sealed containers, some of which are exposed to illumination and some of which are kept dark. The oxygen concentration is measured at the outset and then again after a period of incubation (often 4-8 hours). If the change in oxygen concentration in an illuminated bottle is dL, and the change in a corresponding dark bottle is dD, then

(2) gross primary production = dL-dD (note dD is <0)

(3) net community production= dL

Note that equation (3) assumes that the non-plant members of the local community are represented in the bottle. If they were not, then equation (3) would define net primary production, but it is very difficult to separate aquatic plants (especially benthic algae and phytoplankton) from all the other organisms in their community. Knowing just how much of the non-plant community respiration is represented in the bottle is one of the points of uncertainty when interpreting some oxygen-based production estimates.

The light and dark bottle method can, with appropriately-sized containers, be applied to any type of aquatic plant community. It has the great strength that the quantities in equations (2) and (3) are relatively well-defined. Variations can also sometimes be used to estimate production without confining the plants at all (e.g. the **diurnal curve method**). Like any method, however, it has its drawbacks. The most serious is the relative lack of sensitivity of the method, which seriously limits its use for phytoplankton except in the richest of lakes. Another problem is that the ratio of oxygen evolved to carbon assimilated is not really constant and equal to unity, as equation (1) would have us believe. Variations result from the fact that plants assimilate things other than CO_2, and some of those other assimilation processes involve oxygen uptake or release themselves. One important example is nitrate (NO_3) assimilation. A further complication is that to use equations (2) and (3) we have to assume that respiration of plants and the animals sharing their community is the same in the light as in the dark; we now know that this is often untrue. Unfortunately there is no routine way to measure respiration in the light. Although extremely useful, oxygen-based methods do not give absolutely accurate primary production estimates in ecological applications.

Harvest methods depend on measuring the accumulated biomass of plants at intervals through the growing season. With appropriate corrections for losses between sampling times it is possible to get good estimates of net primary production for macrophytes using such methods. For benthic algae and phytoplankton, harvest methods do not work well and are seldom used because losses are so large, compared to net biomass accumulation, that even slight errors in their estimation will lead to huge errors in estimated production. Harvest methods require considerable effort and the ability to visit the sites repeatedly, if good estimates are to be obtained.

Bio-optical models are used mainly in oceanography, where a major challenge is to estimate the productivity of a dilute suspension of tiny plants (the marine phytoplankton) over a vast area (entire seas or oceans). In such situations, direct measurement of the process of interest (primary production) may have to be sacrificed in order to get at least some relevant data on the necessary time and space scales. Bio-optical models have the great advantage that they can make

use of synoptic data such as ocean surface colour (from satellite observations). A simple bio-optical model is:

$$(4)\ P = E \cdot B \cdot \theta \cdot \phi,$$

where P is the rate of photosynthesis, E is the incident radiation (PAR), B is the concentration of photosynthetic pigments (normally reported as chlorophyll *a*), θ is the efficiency of light harvest by phytoplankton pigments, and ϕ is the quantum efficiency of photosynthesis (moles C assimilated per mole of photons absorbed) for the phytoplankton in question. Pigment concentration, B, can be estimated by various remote sensing methods, while climatic values (i.e., "normal" values) for E are already known for virtually any place in the world. An active area of research involves ways to estimate θ and ϕ from remotely-sensed data on upwelling radiation, including light emitted by fluorescence of the plants themselves.

Radiocarbon methods are the remaining major category of primary production method. They provide the majority of primary production data for most of the ocean and larger lakes, where phytoplankton predominate and oxygen methods are too insensitive. They depend on introducing the long-lived radioactive isotope ^{14}C and measuring its subsequent appearance in the plants. The basic calculation is:

$$(5)\ P = [PO^{14}C(t) - PO^{14}C(0)] \cdot DIC/T^{14}C \cdot t$$

where P is primary production rate, $PO^{14}C$ is the amount of ^{14}C in particulate organic form at the beginning (0) and end (t) of the incubation, DIC is the total concentration of dissolved inorganic carbon, $T^{14}C$ is the total amount of 14C introduced, and t is the duration of the incubation. It is assumed that the added ^{14}C mingles almost instantaneously with the naturally occurring dissolved inorganic carbon, which is heavily dominated by the natural stable isotope ^{12}C. This is a good assumption, because interconversion between CO_2, HCO_3 and CO_3 is very rapid compared to any biological processes. Equation (5) also assumes that the plants do not discriminate between ^{14}C and ^{12}C. In fact, most plants have a small discrimination in favour of ^{12}C (about 6% on average) but the factor is small and discrimination is variable, so it is common practice to ignore the discrimination altogether. The method measures inorganic C assimilation by whatever process may be occurring, and it is often necessary to run controls (such as dark bottles) to correct for carbon uptake that is not due to photosynthesis.

The great strength of radiocarbon methods is sensitivity, sufficient to measure primary production in virtually any aquatic habitat that has any significant primary production. Radiocarbon measurements can also be made with relatively short incubations and small sample volumes, as the current exercise will show. This facilitates their use in calibrating and developing bio-optical models and in conducting synoptic surveys. The greatest drawback is the uncertainty as to what type of primary production is actually measured. When the experiment starts, the plants contain essentially no ^{14}C and therefore they release none in respiration; equation (5) may therefore give an estimate of gross primary production for very short incubations. As time passes, more of the substrates for respiration will become labelled with ^{14}C and the measured rate will then be less than gross primary production. One rule of thumb for phytoplankton is that net primary production is about 90% of gross under optimal conditions, and it is commonly assumed that radiocarbon methods measure something intermediate between gross and net primary production. An additional drawback to radiocarbon methods is that they require careful containment of the sample, and cannot be used at all in some jursidictions that

disallow use of radioisotopes. Nonetheless, they are the most important and widely used methods for planktonic primary production.

3.3 The P-E model approach

When estimating primary production by aquatic plants, and especially phytoplankton, we usually need some method for working out how much production would occur over the entire daylight period (or longer) and the whole depth of the water column. Otherwise we have only localized estimates of production intensity, which are of limited use. Most often, it is not feasible to incubate samples at large numbers of depths for the entire day. This is particularly true in studies of large lakes and oceans. Furthermore, it is desirable to have a method that can make use of partial data, and provide some means of extrapolating results to places and times for which full data sets are lacking. The P-E modelling approach is an answer to these needs.

The method starts by recognizing that solar irradiance diminishes in approximately exponential fashion with increasing depth in the water column, and that the irradiance at the surface of the water varies through the day in approximately sinusoidal fashion (subject to weather). If we can measure the functional relationship between the rate of primary production and irradiance, then we can calculate the primary production rate at any depth from our knowledge of irradiance. This generally entails measuring the primary production rate normalized to some index of phytoplankton biomass (almost invariably chlorophyll *a*), so that any variations in phytoplankton biomass with depth (relatively easy to measure) can be translated into variations in production rate. It is then a simple matter to integrate the production rate over depth and time to work out the primary production rate beneath a unit area of lake surface for an entire day. The accompanying figures illustrate the rationale in graphical form.

Much research has been devoted to finding the best functional form, or P-E model, to describe the relationship between primary production rate and irradiance for different plant communites, including phytoplankton. A number of different formulations seem to be acceptable in practice: the one used here is relatively simple. It assumes that primary production is an increasing and saturating function of irradiance. Although primary production rates are sometimes observed to diminish again at very high irradiances (a phenomenon termed photoinhibition) the model used here does not allow for any such decreases. The procedures outlined below include a means of testing for the presence of photoinhibition.

3.4 Application of the P-E model approach

Following are step-by-step instructions for estimating primary production rates, using stations in L. Erie as an example. The procedure includes a means of estimating the probable impact of changes in certain water quality parameters. The P-E modelling approach employed here is widely used in large freshwater and marine systems. The first 4 steps involve manipulation of water and isotopes, while the rest concern the assay of the samples and handling of the data.

1. Obtain a water sample from Lake Erie, noting the date, time and depth of sampling, and the station number.
2. Under subdued light, spike a portion of the sample with $NaH^{14}CO_3$, mix thoroughly, then dispense aliquots (5 ml) to 20 ml glass scintillation vials in a light gradient incubator,

cooled by a circulating water bath to match the *in situ* lake water temperature. Put an additional sample (also 5 ml) of the spiked lake water in a 20 ml glass vial, for measurement of time-zero activity (T0). Without delay, take the T0 sample to a fume hood and add 6N HCl sufficient to reduce the pH to 2 or less (0.25 ml of 6N Hcl works well). As soon as possible thereafter, start the incubation by turning on the incubator lights. Take a sample (0.2 ml) of the spiked lake water to measure total activity (T14C).

3. While the samples are incubating, filter aliquots of unspiked lake water sample, saving the filters in the freezer for subsequent chlorophyll *a* analysis. Save the filtrates in the refrigerator pending measurement of DIC by alkalinity (Gran) titration. Preserve a further aliquot for future determination of phytoplankton species composition.
4. After the desired incubation period (1 hour in this case), turn off the incubator lights and remove samples from the incubator under subdued light. All samples except totals should be acidified immediately as for the T0 sample and left for at least 20 hours. This treatment drives off as $^{14}CO_2$ any ^{14}C that has not been fixed into organic form, so the remaining activity represents all the primary production, including any that leaked out of the phytoplankton during the incubation or sample handling. This is termed total organic ^{14}C, or $TO^{14}C$. A scintillation "cocktail" is then added to all the samples and they are ready for analysis, or counting, with a liquid scintillation counter.
5. Given a set of samples, ready for scintillation counting and representing one primary production station in Lake Erie, gather the chlorophyll *a*, DIC and water transparency data for that station, together with information about its location, depth and thermal structure. Obtain the irradiance data corresponding to the station as well.
6. Assay ("count") your samples using the liquid scintillation counter (LSC) according the detailed operating instructions for the instrument you are using. You will get results in terms of counts per minute (cpm) and an index of counting efficiency (SQP). You must calibrate the SQP using standards of known activity but variable counting efficiency that are provided with the LSC. You can then estimate the counting efficiency for your own samples by comparing their SQP with the standard curve. The simplest method is to do a linear interpolation between the two standards whose SQP values bracket those of your sample. Your cpm values can then be corrected to disintegrations per minute (dpm) by dividing them by the efficiency. For example, a cpm value of 900 when divided by an efficiency of 0.9 (90%) would be 1000 dpm.
7. All dpm values should then be converted to dpm per ml of sample volume. Note that the sample volume is different between $T^{14}C$ and $TO^{14}C$. You can then calculate the primary production rate at each incubator irradiance using equation (5) with the difference that you are dealing with total organic, not just particulate, production. Note that your production rate will have the same units as DIC/Time.
8. The remaining steps involve the use of specialized programs for calculation and integration (over time and depth) of primary production rates (Fee 1990). Like many specialized software packages used in research, these programs are still designed to operate on the old DOS system. They will work on most modern operating systems, including Windows, but some fiddling is required. Detailed instructions are given separately. In brief, a program called PSPARMS is first used to define the photosynthesis-irradiance (P-

E) relationship, given the photosynthetic rates determined for your sample. The P-E parameters calculated by PSPARMS, and information about water clarity and chlorophyll *a* concentration, are then entered in the program DPHOTO, which will calculate rates of primary production for depths throughout the photic zone. DPHOTO will also give the vertically-integrated (i.e., areal) daily production rate for the station represented by your sample. Although not used in this course, an additional program (YPHOTO) can use output from the other programs to define seasonal or annual rates of production if sufficient measurements are available.

9. One of the benefits of the P-E modelling approach is that it allows you to assess possible sources of error, and to ask how certain types of environmental change might affect primary production. Using DPHOTO, for example, the implications of altered water clarity, phytoplankton biomass, or photosynthetic performance are readily evaluated. The lab exercises designed to illustrate this ability are described separately.