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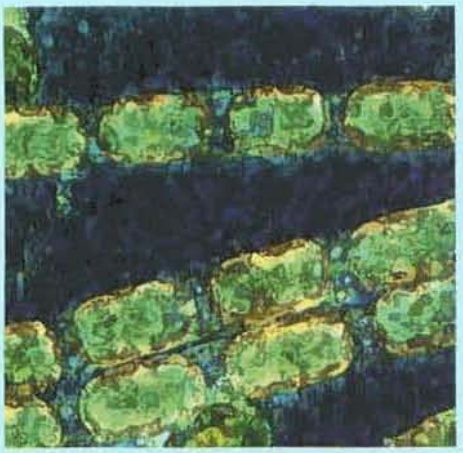

CHAPTER 19

USING CULTURES TO INVESTIGATE THE PHYSIOLOGICAL ECOLOGY OF MICROALGAE

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CONTENTS

Key Index Words: Microalgae, Experiment, Pigment, Photosynthesis, Growth, Irradiance, Spectrum, Nutrient Limitation, Nutrient Starvation, Temperature

1.0. INTRODUCTION

Cyanobacteria and their eukaryotic descendants, the single-celled algae (to which we refer collectively as *microalgae*), play a vital role in the ecology of the planet, being responsible for roughly 45% of global productivity (Field et al. 1998) and supporting food webs in waters from ponds to oceans. However, trying to quantify their contributions to ecological dynamics and biogeochemical cycles remains a daunting process for various reasons. Standing crops of microalgae (expressed as the concentration of the pigment chlorophyll *a* [Chl*a*] per unit volume) vary over more than six orders of magnitude (Fig. 19.1).

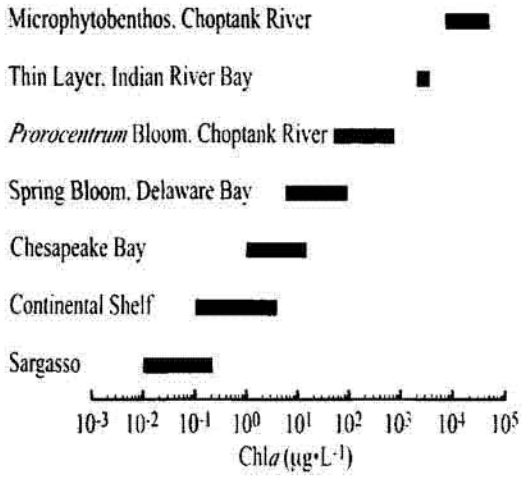

FIGURE 19.1. Variation in microalgal biomass, as chlorophyll *a* (Chl*a*) on a gradient from the oligotrophic Sargasso Sea (36° 20' N, 72° 20' W) to eutrophic estuaries in Delaware and Maryland. Note the log scale. All samples were collected in late spring to early summer. (From MacIntyre, Richard Geider and Jason Adolf, unpublished data.)

Chl*a* (see Table 19.1 for abbreviations) is used by field ecologists and modelers as an index of biomass because it is unique to photosynthetic organisms and relatively easy to measure or derive from remote sensing. However, translating Chl*a* abundance to the more useful currency of microalgal carbon is complicated by the fact that the organisms are physiologically plastic and alter their quotas of cellular carbon and pigments in response to environmental factors such as irradiance, nutrient availability, and temperature (Fig. 19.2a), thereby altering their Chl*a*:C ratios. Consequently, the ratio of cellular carbon to Chl*a* for a single species can vary by more than a factor of 10 in response to varying growth conditions (Geider and Osborne 1987). In addition to this phenotypic variability (acclimation), there is also considerable genotypic variability (Fig. 19.2b). This reflects adaptations (genetically determined differences) that are rooted in the polyphyletic evolution of taxonomic groups with a wide diversity in the structure and function of their organelles (Delwiche 1999) and inherent differences in cellular structures (Chan 1980). Physiological plasticity is not confined to the Chl*a*:C ratio but is also reflected in other indices of biomass such as the Chl*a*:N ratio (Fig. 19.2c,d). The abundance of microalgae cannot, therefore, be simply described by any single currency of biomass such as Chl*a* or N content. The uncertainties in defining ecological roles extend beyond finding a convenient parameter for describing biomass. Microalgal biomass-specific physiological responses also vary with changes in environmental characteristics such as irradiance, temperature, and nutrient availability (Fig. 19.3). Although defining the biomass of microalgae in a water body is difficult, predicting their biomass-specific rate of photosynthesis, which is a highly desirable description of their contribution to the global carbon cycle, is even more so.

The influences of environmental factors on the growth, chemical composition, and physiological responses of microalgae, though complex and time-dependent, are determined solely by biochemical processes under genetic control. The role of the microalgal physiologist is to describe these influences and to determine the role of the environment in regulating the organisms' growth and survival. However, there can be no fixed parameterization of cause and effect that applies across all groups: There are taxonomic differences in the structure and integration of such photosynthetic components as pigments, electron carriers, and enzymes. Whereas the microalgae as a group do vary widely in terms of their biochemical composition and physiological responses, the variability is ordered, reflecting genetically determined reactions to the characteristics of the environment in which they are growing. For example, microalgae differ widely in size and shape, and within the diverse taxa, their pigmentation and photosynthetic responses will to some extent scale according to allometric laws (Finkel and Irwin 2000, Finkel 2001, Raven and Kübler 2002).

Experiments with cultures are and will remain central to our understanding of microalgal responses to environmental variability. We describe here the types of responses that can be studied, the systems to use, and some of the issues that must be addressed to relate results from laboratory experiments to the growth of microalgae at large. Much of the information is general in nature and is intended to illustrate the principles rather than the specifics of culturing. For instance, we do not provide detailed step-by-step directions for use of techniques such as fluorescence or spectrometry because of variations among different models of instruments and the innumerable possibilities for experimental design. We refer you to other sections of this volume for detailed information on the preparation of media (Chapters 2–5), maintenance of cultures (Chapter 10), and enumeration of cells and determination of growth rate (Chapters 16–18).

Although most of the data that we show are from species isolated from estuarine and marine waters, the principles of culturing for examining physiological responses apply equally to species isolated from fresh waters and/or benthic habitats. The data illustrate the

TABLE 19.1 Summary of the symbols and abbreviations used in the text.

Symbol	*Description*	*Typical Unit*
a^{Chl}	Mean value of $a^{Chl}(\lambda)$ over PAR (400–700 nm)	$m^2 \cdot [mg\ Chl a]^{-1}$
$a^{Chl}(\lambda)$	Chl*a*-specific absorption at wavelength λ	$m^2 \cdot [mg\ Chl a]^{-1} \cdot nm^{-1}$
A_e	Weighting coefficient to convert PAR to PUR	dimensionless
α	Initial, light-limited rate of photosynthesis in a PE curve	$g\ C \cdot [g\ Chl a]^{-1} \cdot hr^{-1} \cdot (\mu mol\ photons \cdot m^{-2} \cdot s^{-1})^{-1}$ *or* mol O_2 or $C \cdot [g\ Chl a]^{-1} \cdot hr^{-1} \cdot (\mu mol\ photons \cdot m^{-2} \cdot s^{-1})^{-1}$
β	Parameter describing reduction in photosynthesis at high irradiance	$g\ C \cdot [g\ Chl a]^{-1} \cdot hr^{-1} \cdot (\mu mol\ photons \cdot m^{-2} \cdot s^{-1})^{-1}$ *or* mol O_2 or $C \cdot [g\ Chl a]^{-1} \cdot hr^{-1} \cdot (\mu mol\ photons \cdot m^{-2} \cdot s^{-1})^{-1}$
CFC	Ratio of variable to maximum fluorescence in the dark ± DCMU: $(F_d - F_0)/F_d$	dimensionless
Chl*a*	Chlorophyll *a*	$\mu g \cdot L^{-1}$ *or* $pg \cdot cell^{-1}$
Chl*a*:*C*	Ratio of Chl*a* to POC	$g \cdot g^{-1}$
Chl*a*:*N*	Ratio of Chl*a* to PON	$g \cdot g^{-1}$
DIC	Dissolved inorganic carbon	$g \cdot L^{-1}$ *or* M
DOC	Dissolved organic carbon	$g \cdot L^{-1}$ *or* M
E	Mean value of $E(\lambda)$ over PAR (400–700 nm)	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
$E(\lambda)$	Irradiance at wavelength λ	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1} \cdot nm^{-1}$
E'	Hypothetical reference irradiance with equal intensity between 400 and 700 nm	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
E_a	Irradiance absorbed by a cell	mol photons $\cdot [g\ Chl a]^{-1} \cdot s^{-1}$ *or* $\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
E_d	Downwelling irradiance	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
E_k	Saturating parameter in a P vs. E curve: $= P_m/\alpha$	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
F, F_s	Fluorescence in darkness or light, measured before saturating flash	arb.
F_0	Dark-adapted fluorescence without DCMU	arb.
F_d	Dark-adapted fluorescence in the presence of DCMU	arb.
F_m, F_m'	Maximum fluorescence in darkness or light, measured after a saturating light flash	arb.
F_v/F_m	Ratio of variable to maximum fluorescence in the dark: $(F_m - F)/F_m$	dimensionless
F_v'/F_m'	Ratio of variable to maximum fluorescence in the light: $(F_m' - F_s)/F_m'$	dimensionless
K_C	Compensation irradiance in μ vs. E curve, where $\mu = 0$	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
K_E	Saturating parameter in a μ vs. E curve	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
λ	Wavelength	nm
μ	Specific growth rate	d^{-1}
$\mu_{E,T}$	Nutrient-replete specific growth rate at irradiance E and temperature T	d^{-1}
μ_m	Maximum specific growth rate	d^{-1}
μ_N	Nutrient-limited specific growth rate	d^{-1}
ϕ	Quantum yield of carbon fixation	mol $C \cdot [mol\ photons]^{-1}$
ϕ_m	Maximum quantum yield of carbon fixation	mol $C \cdot [mol\ photons]^{-1}$
PAR	Photosynthetically active radiation, 400–700 nm	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
PE	Photosynthesis as a function of irradiance	
P_E	Photosynthesis at irradiance E	$g\ C \cdot [g\ Chl a]^{-1} \cdot hr^{-1}$ *or* mol O_2 or $C \cdot [g\ Chl a]^{-1} \cdot hr^{-1}$
P_m	Light-saturated rate of photosynthesis in a PE curve	$g\ C \cdot [g\ Chl a]^{-1} \cdot hr^{-1}$ *or* mol O_2 or $C \cdot [g\ Chl a]^{-1} \cdot hr^{-1}$
POC *or* C	Particulate organic carbon	$\mu g \cdot L^{-1}$ *or* $pg \cdot cell^{-1}$
PON *or* N	Particulate organic nitrogen	$\mu g \cdot L^{-1}$ *or* $pg \cdot cell^{-1}$
PQ	Photosynthetic quotient; unit oxygen evolved per unit carbon assimilated	dimensionless
PUR^{Chl}	Photosynthetically usable radiation	mol photons $\cdot [g\ Chl a]^{-1} \cdot s^{-1}$
PUR	Photosynthetically usable radiation	$\mu mol\ photons \cdot m^{-2} \cdot s^{-1}$
R_E	Respiration rate at irradiance E	$g\ C \cdot [g\ Chl a]^{-1} \cdot hr^{-1}$ *or* mol O_2 or $C \cdot [g\ Chl a]^{-1} \cdot hr^{-1}$
X	Index of biomass (cell number, Chl*a*, POC, PON, etc.)	See above

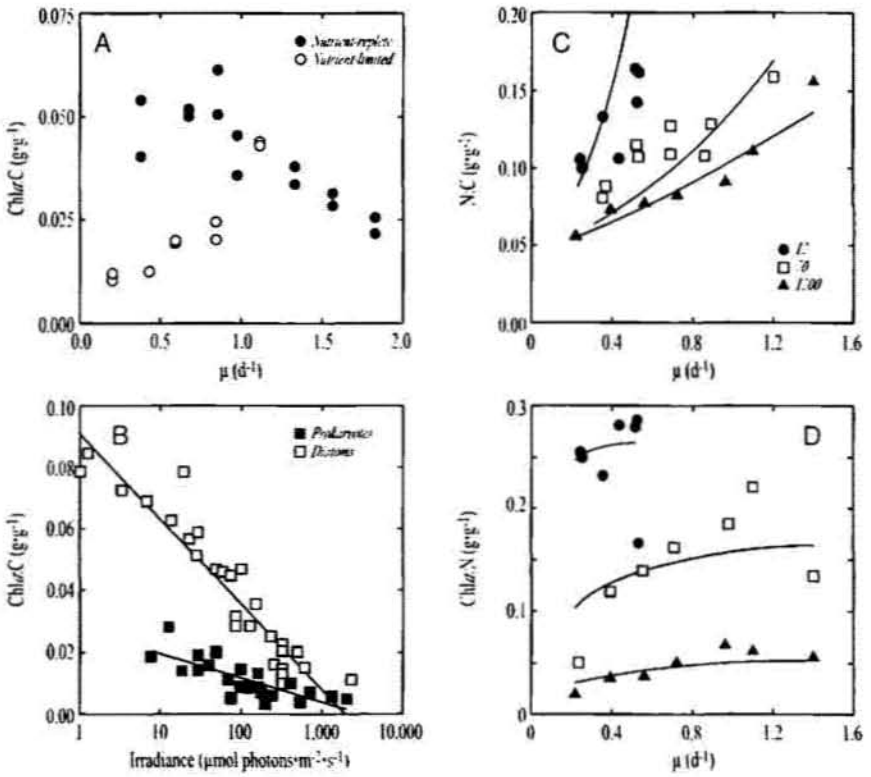

FIGURE 19.2. Variation in the chemical and physiological characteristics of microalgae with growth rate (redrawn and modified from Geider 1992, Cullen et al. 1993). **(a)** Variation in the Chl*a* : C ratio as a function of growth rate in the diatom *Thalassiosira pseudonana* (Hustedt) Hasle et Heimdal (Xiaolong Yang, MacIntyre and Cullen, unpublished data). Nutrient-replete cultures = semicontinuous culture at irradiances between 25 and 900 μmol photons · m^{-2} · s^{-1}. Nutrient-limited cultures = chemostats at 200 μmol photons · m^{-2} · s^{-1}. **(b)** Variation in the Chl*a* : C ratio as a function of growth irradiance in the diatoms *Cylindrotheca fusiformis* (Ehr.) Reimann et Lewin, *Phaeodactylum tricornutum* Bohlin, *Thalassiosira eccentrica* (Ehr.) Cleve and *Thalassiosira weissflogii* (Grun.) Fryxell et Hasle, and the prokaryotes *Microcystis aeruginosa* Kütz. em. Elenkin, *Prochlorothrix hollandica* Burger-Wiersma, and *Synechococcus* spp. Nutrient-replete conditions under continuous light (Chan 1980, Raps et al. 1983, Falkowski et al. 1985, Geider et al. 1985, 1986, Kana and Glibert 1987a, 1987b, Burger-Wiersma and Post 1989). Note the 3*x* difference in taxonomic trends. **(c)** Variations in the N : C ratio of the diatom *Skeletonema costatum* (Greville) Cleve as a function of nutrient-limited growth rate at 12, 70, and 1200 μmol photons · m^{-2} · s^{-1}. Data are from Sakshaug et al. (1989). Lines are relationships predicted by the dynamic balance model of Geider et al. (1998). **(d)** Variations in the Chl*a* : N ratio of *S. costatum* as a function of nutrient-limited growth rate at 12, 70, and 1200 μmol photons · m^{-2} · s^{-1}. Symbols and data sources as in **(c)**.

species' responses to particular growth conditions and should not be taken as representative for other species or other conditions of growth.

2.0. DESCRIBING THE DISTRIBUTIONS AND ACTIVITIES OF MICROALGAE IN NATURE

2.1. Measurements in the Field

Field studies of the microalgae have been central to our understanding of their ecology (e.g., Riley 1947, Sverdrup 1953, Redfield 1958, Margalef 1978, Reynolds and Bellinger 1992) and remain vital to our efforts to describe their dynamics. Broadly, the central objective of field studies is to describe and explain the distributions and activities of microalgae in nature. However, interpretation of data collected on natural assemblages is always subject to the following major constraints:

- Many direct measures of biomass such as particulate organic carbon (POC) are compromised by the presence of contaminants (bacteria, zooplankton and their fecal pellets, other detrital material, and the occasional charismatic vertebrate) (Eppley et al. 1977).

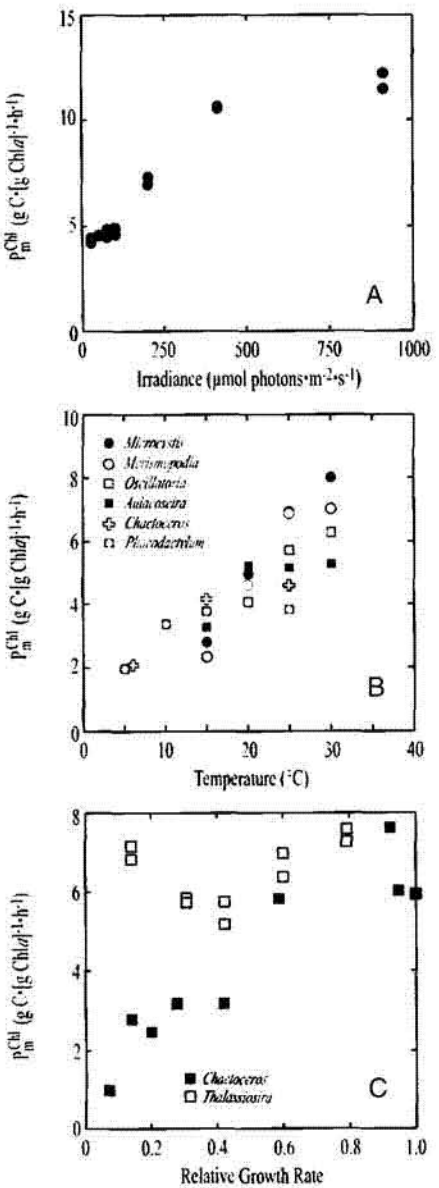

FIGURE 19.3. Variation in the chlorophyll *a* (Chl*a*)–specific light-saturated rate of photosynthesis, P_m, with irradiance, temperature, and nutrient availability. **(a)** P_m as a function of growth irradiance in nutrient-replete cultures of the diatom *Thalassiosira pseudonana* at 20° C (Xiaolong Yang, MacIntyre and Cullen, unpublished data). **(b)** P_m as a function of growth temperature in nutrient-replete cultures of the cyanobacteria *Microcystis aeruginosa*, *Merismopedia tenuissima* Lemmermann, and *Oscillatoria* spp. (Coles and Jones 2000) and the diatoms *Aulacoseira granulata* (Ehr.) Simonson (Coles and Jones 2000), *Chaetoceros calcitrans* Paulsen (Anning et al. 2001), and *Phaeodactylum tricornutum* (Li and Morris 1982). **(c)** P_m as a function of nutrient-limited growth rate in the diatoms *Chaetoceros neogracile* (Schütt) van Landingham (formerly *C. gracilis* Schütt) and *T. pseudonana*. Both were grown in N-limited continuous culture: *C. neogracile* at 160 μmol photons · m^{-2} · s^{-1} and *T. pseudonana* at 200 μmol photons · m^{-2} · s^{-1}. Data on *C. neogracile* are from Thomas and Dodson (1972). Data on *T. pseudonana* are from Xiaolong Yang, MacIntyre and Cullen; redrawn from Cullen et al. (1992b).

- The environmental history of cells before their sampling is unknown. Physiological plasticity and the time lags involved in responses to environmental influences can confound the interpretation of cause (the environmental conditions) and effect (biochemical and physiological characteristics) if the cells have been transported or have migrated from waters with different environmental conditions.
- When microalgae are collected and confined for experimental determination of rate processes, conditions in the natural turbulent environment cannot be simulated reliably (Legendre and Demers 1984, Cullen and Lewis 1995).

Thus, few measurements made in the field can be taken as accurate measures of the distributions or physiological responses of natural microalgae. The general approach is to measure all of the bulk properties of the water attributable to microalgae (e.g., the concentration of Chl*a* and other pigments, rate of photosynthesis during incubations, uptake of nutrients, nitrogen fixation, etc.) and develop models to relate the measurements that can be made (Chl*a*, irradiance, nutrient concentration, activities of enzymes) to the rates and properties of interest (e.g., microalgal biomass, instantaneous rate of photosynthesis, growth rate, or nutrient limitation). Resulting models, such as those used to calculate productivity from remotely sensed ocean color, have proved to be effective in converting large amounts of readily acquired data into estimates of a difficult-to-measure process on large scales (Platt and Sathyendranath 1993, Behrenfeld and Falkowski 1997a). The reliability of extrapolations to conditions other than those for which the models were calibrated, such as when predicting global responses to changes in atmospheric CO_2, cannot be easily assessed.

2.2. Models of Physiological Responses

An alternative approach to describing the characteristics of microalgae as a function of environmental factors is to use a first-principles model, in which the physiological response is defined in terms of light, temperature, and nutrient supply. The model can be parameterized to approximate the biochemical and physiological control points, such as the redox state of the plastoquinone pool (Escoubas et al. 1995, Maxwell et al. 1995). Extrapolating from cultures to field populations requires a detailed understanding of how environmental conditions influence microalgal growth for the species in culture and for the different algal assem-

blages in the natural environment (Flynn and Hipkin 1999, Flynn and Fasham 2003). One such dynamic model has proved to be robust in reproducing microalgal responses to conditions of fluctuating nutrient and irradiance availability when calibrated on the responses of algae fully acclimated to their environment (Taylor et al. 1997, Geider et al. 1998, Lefèvre et al. 2003). Such a calibration is possible only under the controlled conditions that can be obtained when populations of microalgae are grown in cultures.

2.3. The Need for Experiments with Cultures

The organization of pigments, end products of photosynthesis, and growth rate respond to changes in light, temperature, and nutrient availability in a dynamic way (see Figs. 19.2 and 19.3). This process must be characterized to describe the distributions, growth rates, and physiological activities of microalgae based on limited data, such as measurements of chlorophyll, irradiance, and temperature. Physiological characteristics, such as optimal temperatures for growth, maximum chlorophyll content, and capabilities to store pulses of nutrients, differ between taxa, reflecting adaptations that have evolved through natural selection. Appreciation of these adaptations can lead to a better understanding of the environmental factors that define the niches of microalgae, thereby leading the way to a description of environmental controls on community composition and ultimately on food web structure and biogeochemical cycles.

3.0. APPROACHES TO CULTURING

Microalgae can be cultured in many ways. The simplest classification of these depends on whether they are in balanced growth.

- If conditions are kept constant and within the bounds of survivability, the cells will eventually become fully acclimated to their environment; their physiological responses will be at equilibrium and their growth will be balanced.
- If cultures are responding to repeatable and predictable changes, they may be in dynamic equilibrium and their growth will be balanced, when considered over the cycle of variability.
- If cultures are subjected to step-changes in conditions or stochastic variability, they will not be at equilibrium, and thus, their responses will not be acclimated and their growth will be unbalanced.

Because microalgae respond to perturbations in their environment, and because their physiology takes a finite time to respond, fully acclimated growth occurs only after long exposure to highly stable conditions. However, long-term stability is atypical of natural environments and microalgal growth that is fully acclimated to constant conditions is a completely artificial state. Its relevance, and therefore the relevance of culturing under conditions that do not reflect the full range of natural variability, is open to some debate. The rationale for studying acclimated growth has much in common with Plato's Theory of Forms, which holds that an individual Thing is the product of the interaction between an eternal, ideal, and abstract Form and the Thing's surroundings. As a result, the abstract Form embodies a higher level of reality than its temporal imitation, the Thing. By analogy, the state of a cell (the Thing) can be viewed as the deviation from its Form (the fully acclimated state) in response to its surroundings (the environment). A critical difference from Plato's theory is that each set of environmental conditions would have its own fully acclimated condition so that the Form would be a continuum rather than a single state. However, the central relationship between Thing and Form is analogous so that the fully acclimated state remains the expression of an idealized condition from whose contours we can discern the underlying elements of the cell's expression in response to a mutable world. This approach is inherently reductionistic and, in our opinion, pragmatic: Much can be learned by observing and describing the mechanisms by which microalgae regulate their physiology to attain the acclimated state. An alternative, and incompatible, view of the cell's ecology is holistic and holds that the whole cannot be derived simply from the sum of its parts. To a holist, a description of acclimated growth is an abstraction made meaningless by its detachment from the complexity of the real world. The two opposing views have been explored extensively by Ernest Nagel and Robert Pirsig, among others, neither of whom found a resolution between them.

The approach to modeling and culturing that we describe in the following is fundamentally reductionistic, holding that the interactive effects of light, temperature, and nutrient availability on microalgal physiology can be described meaningfully in experimental studies, and that a description of physiology under artificial con-

trolled conditions can inform our understanding of dynamics in natural assemblages. We argue that acclimation can be described with response functions that are general (MacIntyre et al. 2002) and consistent with dynamic models that are parameterized with observations of cultures in balanced growth (Geider et al. 1998, Flynn and Hipkin 1999). We also argue that both the chemical composition and growth rate under acclimated conditions and the metabolic means by which they are achieved are adaptations that define in part the niche of each microalga (Cullen and MacIntyre 1998). This has been the subject of debate (e.g., Siegel 1998) since the apparent paradox of coexistence of different species was proposed by Hutchinson (1961). The holist will likely balk at this point and will find little with which to agree in what follows.

3.1. Types of Growth

The prerequisite for effective experimental work with cultures is a clear understanding of what is being asked, an informed choice of the appropriate culturing system, and an appropriate analysis of results. Methods for culturing microalgae and for using cultures to investigate their physiological ecology can be organized in terms of whether the cells are acclimated to their environmental conditions and whether their growth is nutrient replete, nutrient starved, or nutrient limited.

3.1.1. Balanced vs. Unbalanced Growth

The specific growth rate is a rate of change of biomass that is set by the relative magnitude of anabolic processes (photosynthesis) and catabolic processes (respiration):

$$\mu = P - R, \tag{1}$$

where μ is the specific growth rate, P is photosynthesis, and R is respiration. Here, all rates are expressed in units of inverse time (e.g., g C/[g C]/d = d^{-1}). Cells are described as being acclimated (i.e., in balanced growth) when internal adjustments of metabolic pathways have ceased so the rate of change of all cellular components is the same (Shuter 1979, Eppley 1981):

$$\mu = \left(\frac{1}{X}\right)\left(\frac{dX}{dt}\right), \tag{2}$$

where X is any index of biomass, such as cell number, Chl*a*, carbon, or nitrogen. (For a more detailed discussion of growth rates, see Chapter 18.) This condition can occur only when environmental conditions are held constant for many generations. Such constant conditions are highly artificial: the diel cycle of irradiance imposes a daily imbalance of photosynthesis versus respiration ensuring that unbalanced growth is the hallmark of autotrophic existence. However, microalgae can readily acclimate to environmental conditions that are repeated day to day, in that they achieve acclimated balanced growth over a photocycle (Shuter 1979, Eppley 1981). Equation 2 would apply, then, to rates averaged over a photocycle. Growth is balanced but in dynamic, rather than simple, equilibrium. This can be illustrated by a comparison of within-day and between-day variability of Chl*a*, carbohydrate, and protein levels in cells grown on a light–dark cycle (Fig. 19.4). All indices increased during the light period, although the specific rates varied by more than threefold. In the following dark period, Chl*a* increased by about 10%, protein and cell number increased by about 50%, and

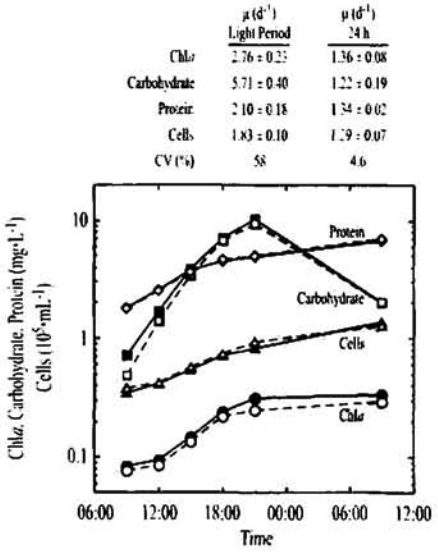

FIGURE 19.4. Variation in four indices of biomass in a semicontinuous culture of the diatom *Thalassiosira pseudonana* grown on a 12:12 L:D cycle (Xiaolong Yang, MacIntyre, and Cullen, unpublished, partially shown in Parkhill et al. 2001). The shaded bars indicate the dark period. Open and closed symbols are for replicate cultures. Exponential growth rates in the light period were determined by linear regression of ln-transformed data collected between 09:00 and 21:00 on the first day (Equation 3). Exponential growth rates over the 24-hour period were determined by linear regression of ln-transformed data collected at 09:00 on successive days. CV is the coefficient of variance (standard deviation/mean, expressed as a percentage). Note the good agreement between growth rates collected over the 24-hour period as opposed to those collected over the light period alone.

carbohydrate fell by 80%. The pattern is consistent with daytime synthesis of excess carbohydrate fueling nocturnal protein synthesis and cell division (Cuhel et al. 1985, Cullen 1985). However, when growth rates were calculated at the 24-hour interval, matching the length of the light–dark cycle, there was no significant difference between estimates based on the different indices. This is balanced growth in dynamic equilibrium.

3.1.2. *Nutrient Stress: Limitation and Starvation*

Because the process of acclimation is one of physiological and chemical change, cells that are acclimating to environmental conditions are fundamentally different from those that have already acclimated and are in balanced growth. It is important, therefore, to incorporate acclimation in the terminology for describing the effects of nutrients on microalgal growth. The various conditions under which nutrient supply imposes a level of stress on cellular physiology have been variously described as a limitation. We use the following definitions (Parkhill et al. 2001): *Limitation* refers to acclimated growth in which the growth rate is reduced as a result of nutrient availability; *starvation* refers to unbalanced growth in response to recent depletion of a nutrient; and *stress* is the inclusive generic term that covers both conditions.

Classically, *limitation* has been used to describe any restriction of growth by nutrient availability. It can refer to two very different circumstances; Liebig and Blackman limitation (Cullen et al. 1992b). Liebig's Law of the Minimum has its basis in agronomy and is properly applied to final yield, which is a measure of biomass, not the rate at which it accumulates (i.e., specific growth rate). In contrast, Blackman limitation has its basis in descriptions of kinetics and refers to the effect of a factor on a rate process, of which growth is an analog. This is illustrated in Figure 19.5, which shows the course of biomass changes in a closed system. Growth is initially exponential:

$$X_t = X_0 \exp(\mu t), \tag{3}$$

where X_0 is the initial biomass, X_t is the biomass at time t, and μ is the specific growth rate, with units of inverse time (e.g., d^{-1}). Note that this is *not* synonymous with the division rate, which is a growth rate expressed in divisions $\cdot$ d^{-1} and is calculated in Base 2 rather than Base e (Guillard 1973b). The relationship between them is

$$\textit{Division Rate} = \frac{\mu}{\ln(2)} = \frac{\mu}{0.693} \tag{4}$$

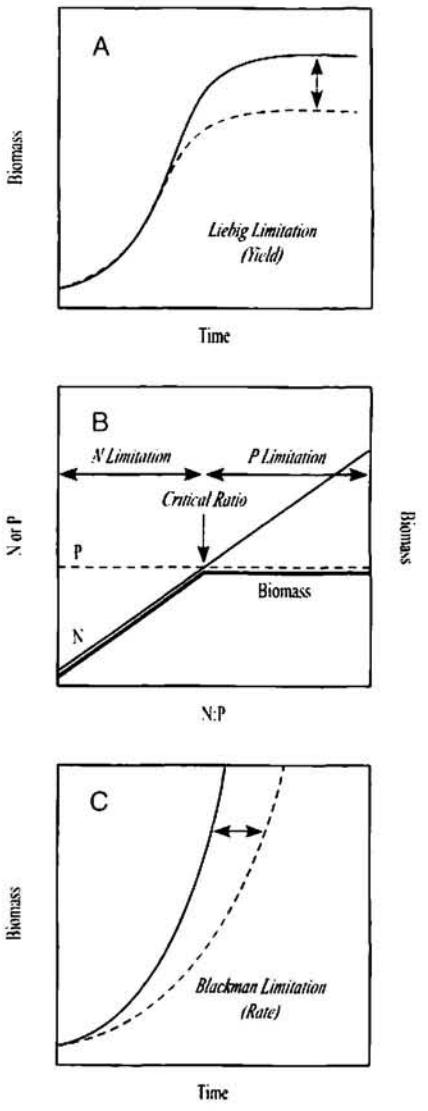

FIGURE 19.5. **(a)** Schematic representation of Liebig limitation of yield (biomass) in a culture. The final yield, which is the maximum biomass achieved, is determined by the abundance of a single factor, according to Liebig's Law of the Minimum. The divergence in final yields, caused by a difference in the abundance of the limiting nutrient, is indicated by the arrow. Note that the initial exponential growth rates are the same. **(b)** Schematic representation of Liebig limitation along a gradient of N : P, where the nutrient ratio was obtained by altering nitrogen (*light solid line*) against a fixed concentration of phosphorus (*dashed line*). The biomass at the Liebig yield (*heavy line*) is limited below the critical ratio by nitrogen and above it by phosphorus. **(c)** Schematic representation of Blackman limitation of growth in a culture. The growth rate is determined by the availability of a limiting nutrient. The divergence in growth rate, caused by a difference in the abundance of the limiting nutrient, is indicated by the arrow. Note that the exponential growth rates are different regardless of the final yield that is achieved.

As shown in Figure 19.5a, as the cells deplete the nutrients in the growth medium, the nutrient with the lowest concentration relative to the cells' requirements declines to the point at which the rate of uptake cannot match cellular demand. At this point, the cells enter *nutrient starvation*. The growth rate μ (see Equation 3) declines to zero as the limiting nutrient is exhausted and the cells enter stationary phase. The final yield is determined by the initial concentration of the nutrient that was present in the lowest availability relative to the cellular requirements. During the latter part of the growth phase, growth is neither exponential nor balanced: As the limiting nutrient is depleted, the rate of growth declines due to Blackman limitation (see following discussion), and once the limiting nutrient is gone, the yield is set by Liebig limitation. Cells do not acclimate to a particular nutrient concentration, because concentrations are always changing until the nutrient is exhausted, at which point neither growth nor acclimation can continue. The example in Figure 19.5a is appropriate for describing the transient process of nutrient starvation and is useful for exploring responses of microalgae to intermittent nutrient supply. It should be recognized, though, that the results of such nutrient starvation experiments may depend on preconditioning; for instance, the effects of nitrogen starvation on a marine diatom were delayed for nutrient-replete cultures as compared to acclimated nutrient-limited cultures (Smith et al. 1992, Parkhill et al. 2001).

The biomass yield in Liebig limitation is set by two factors: the intrinsic requirements of the cells (cell quota) and the external concentrations of nutrients. Microalgae regulate their internal nutrient quotas by reducing them in response to limited availability until they reach a minimum value (Droop 1968, 1974, Morel 1987). As they exhaust the concentration of a limiting nutrient, the cellular nutrient quota is reduced as cell division continues and the assimilation of limiting nutrient slows and then stops; that is, the quota reaches a minimum value. Ultimately, cell division stops and the population has reached the Liebig yield. The nutrient that sets the yield is the one whose quota declines to the cellular minimum first. This is illustrated for nitrogen versus phosphorus limitation in Figure 19.5b. When nitrogen is low relative to phosphorus, the cells reduce the nitrogen quota to the minimum value before the availability of phosphorus causes the phosphorus quota to decline to the minimum. That is, the yield is nitrogen limited. Conversely, when nitrogen is high relative to phosphorus, cellular phosphorus is reduced to the minimum before nitrogen is exhausted. That is, the yield is phosphorus limited. Under balanced nutrient-limited growth (e.g., in a chemostat: see following discussion), the crossover occurs at the critical ratio, a point at which the quotas of both nitrogen and phosphorus are at the minimum value (Terry et al. 1985).

The critical ratio has been measured for N : P in four species of microalgae and for vitamin B12 : P in one. The critical N : P ratio, expressed as an atomic ratio, was about 30 in the chlorophyte *Scenedesmus* spp. (Rhee 1978); 38 to 49 in the prymnesiophyte *Pavlova* (formerly *Monochrysis*) *lutheri* (Droop) Green (Terry et al. 1985); 25 to 33 in the diatom *Phaeodactylum tricornutum* (Terry et al. 1985); and 35 to 40 for the diatom *Chaetoceros muelleri* Lemmermann (Leonardos and Geider 2004). It is expected to and does vary with growth irradiance (Terry et al. 1985, Leonardos and Geider 2004). As discussed elsewhere (Geider and La Roche 2002), this is considerably higher than the Redfield ratio of 106 : 16 : 1 C : N : P (Redfield 1958, Goldman et al. 1979), arguably one of the most abused parameters in the field of aquatic ecology. The Redfield ratio is an approximation of composition averaged over very large scales of time and space and does not describe the conditions of individual cells or populations. Even so, it can be used as a guide to which nutrient will limit growth in culture; biasing N : P concentrations by fivefold to tenfold on either side of the ratio will ensure that a culture is limited by nitrogen or phosphorus. Note that in f/2 medium with 2 mM dissolved inorganic carbon (DIC), the C : N : P ratio is 55 : 24 : 1 (Guillard 1973a) and the cells will become carbon limited before exhausting nitrogen or phosphorus.

Unless the aim is to study carbon limitation, we recommend bubbling cultures with air. It should be passed through a cartridge filled with activated carbon (Guillard 1973a) to remove the volatile organics such as pump oils and solvents that are common in laboratory air and then through a sterile 0.2-μm capsule filter to maintain sterility in the culture. For N-limited cultures, the air should be bubbled through dilute phosphoric acid and then distilled water to trap atmospheric ammonium that is released is significant quantities from many cleaners and from people who smoke. We direct the reader to Chapters 2 through 5 for other considerations of nutrient composition in medium, including the relative merits of autoclaving and microwave sterilization on CO_2, ammonium, and silicate concentrations (Guillard 1973a, Keller et al. 1988); species-specific trace metal and buffering requirements (Keller et al. 1987); and the need to add selenium to cultures grown in polycarbonate or modern Pyrex culture vessels (Price et al. 1987) to avoid inadvertent trace-metal limitation.

In contrast to limitation of yield, Blackman limitation refers to the rate constant that describes a reaction (the analog of μ in Equation 3). This is illustrated in Figure 19.5c, which shows two cultures in exponential growth where one has a lower achieved growth rate than the other. If the slower growth is due to a reduction in the efficiency of a metabolic process because of restricted availability of a nutrient, the cells are *nutrient limited*. This can be an acclimated condition, provided that the rate of supply of the nutrient matches the growth rate, and growth can be both exponential and balanced. (See Kacser and Burns [1973] and Rees and Hill [1994] for biochemical limitation of rates.) We consider both nutrient starvation and nutrient limitation as they occur in different types of cultures.

3.2. Batch Culture

One of the simplest forms of culture is a batch culture, in which growth follows multiple phases (Fig. 19.6). Typically, there is an initial lag phase during which there is minimal growth. Experience shows that the lag is short if the inoculum is large and the parent culture has been acclimated to similar conditions; the lag phase is more an artifact of transfer than an inherent stage of growth. This is followed by an exponential growth phase (Equation 3) until a nutrient or CO_2 is depleted, after which growth slows and cells enter stationary phase (Fig. 19.6a). If the concentrations of nutrients are high and the incident irradiance relatively low, there is also the potential for the culture to shade itself and become light limited as cell density becomes very high.

It is very difficult to define a period of acclimated growth in batch cultures, because the progressive accumulation of biomass changes the availability of nutrients and light (Fig. 19.6c). Both changes drive acclimative responses in pigmentation and photosynthetic performance. The reduction in mean irradiance available to the cells as the culture starts to shade itself causes a response of increased pigmentation. As a result, even while growth can be described well by fitting to Equation 3, the apparent growth rate depends strongly on the index of biomass being used; the Chl*a*-based rate is twice that of the POC- or PON-specific rates (Fig. 19.6a). This is reflected in the variability in the biomass ratios (Fig. 19.6b) during the exponential phase; growth is clearly not balanced, as defined according to Equation 2. In this case, the nutrient setting the Liebig limit is nitrogen; after the culture has exhausted the supply

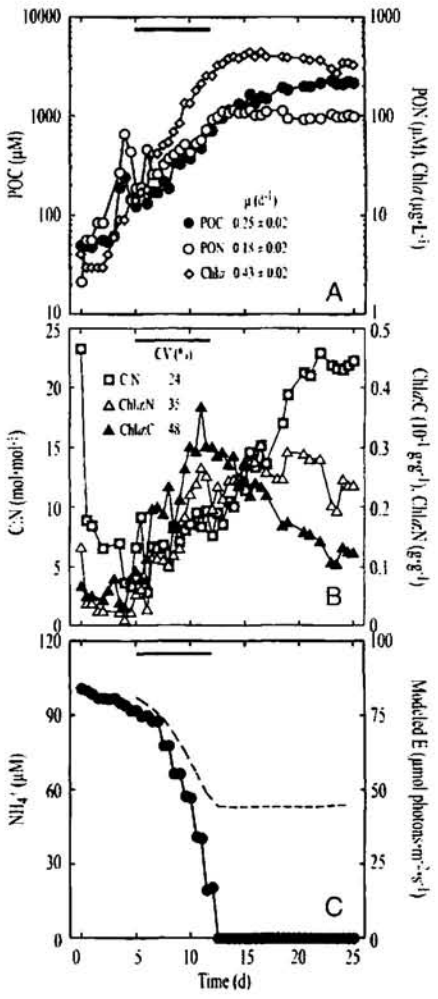

FIGURE 19.6. **(a)** Variation in the biomass of the prymnesiophyte *Isochrysis galbana* Parke during growth in batch culture. Note log scale. Exponential growth rates (Equation 3) were fitted to data collected between day 5 and day 12 (*black bar*), when ammonium concentrations were above the limit of detection **(c)**. Note that growth rates vary by 2.4 times, depending on the index of biomass used to calculate the rate. **(b)** Variation in ratios of the biomass indices over the same period. Coefficients of variance (standard deviation/mean, expressed as a percentage) are shown for these over the period over which the growth rates were calculated (*black bar*). Their variability indicates that growth was not balanced (Equation 2). **(c)** Variability in measured levels of ammonium, the nitrogen source in the medium, and in modeled light levels in the culture. (Light levels were modeled by Geider et al. 1998). (Data courtesy Kevin Flynn.)

of ammonium, on Day 12, accumulation of PON ceases. Accumulation of Chl*a* and POC does not. Batch cultures cover multiple growth phases, and growth is, at best, fleetingly acclimated, with growth rate and physiological state representative of nutrient-replete growth at the experimental irradiance. If cultures are to be maintained in balanced growth, an alternative method to batch cultures is needed.

3.3. Nutrient-Replete Growth in Semicontinuous and Turbidostat Cultures

To maintain cells in nutrient-replete exponential growth, the culture must be diluted regularly to keep cell concentrations low so that nutrient concentrations remain high and light levels are unaffected by self-shading (Beardall and Morris 1976). In semicontinuous cultures, a modification of the batch method, the culture is diluted repeatedly to restrict the range of biomass density (Fig. 19.7a). The growth rate is then calculated from Equation 3, using the change in biomass and interval between dilutions. The approach is labor intensive, because the biomass must be assayed before each dilution to maintain the culture within the desired range. (A description of means for monitoring biomass is given at right.) A more technologically complex way of maintaining the culture is to use a turbidostat, a continuous culture in which an optical sensor and automated pump are used to achieve the same effect (Myers and Clark 1944, Rhee et al. 1981). This is illustrated in Figure 19.7b, where the transparency of the culture was monitored continuously at 680 nm. This wavelength was chosen because it coincides with the red absorption peak of Chl*a* and so is a convenient means to monitor the abundance of cells containing the pigment.

A schematic representation of the turbidostat is shown in Figure 19.8a. A reservoir of fresh medium was connected to the culture via a pump, which was turned on when attenuation rose above a preset threshold and was turned off when it fell below a second. The supply of medium was, therefore, triggered on demand by growth of the culture. The preset limits that regulated pumping differed by about 3% of the dynamic range of the signal, so biomass and the apparent growth rate were maintained at a nearly constant level. In a turbidostat, the achieved growth rate, which is the nutrient-replete growth rate at the experimental irradiance and temperature $\mu_{E,T}$, is equivalent to the dilution rate, which is the inflow rate divided by the volume of the culture vessel

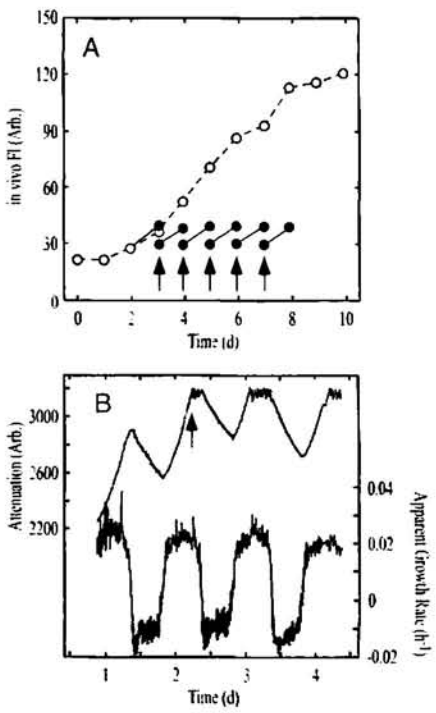

FIGURE 19.7. **(a)** Variation in the dark-adapted fluorescence of cultures of the pelagophyte *Aureococcus anophagefferens* Hargraves et Sieburth during growth in batch culture (*open symbols*) and in semicontinuous culture (closed *symbols*). Cultures were diluted daily (*arrows*) to maintain biomass (as indicated by fluorescence) within a restricted range. Note the limited variation in between-day slopes in semicontinuous culture as compared to the batch culture. (Data courtesy Fran Pustizzi.) **(b)** Variation in abundance and apparent growth rate of a culture of *A. anophagefferens* during growth in a turbidostat on a 12-12 L-D cycle (Todd Kana and MacIntyre, unpublished data.). The shaded bars indicate the dark period. Abundance was determined optically every 3 minutes, as transmission of light at 680 nm through the culture vessel. Apparent growth rate was calculated from the slope of ln-transformed data (Equation 3) over successive 9-minute intervals. Dilution with fresh medium was initiated when attenuation rose to more than 3200 units and was curtailed when it fell to 3100 units. The point at which dilution started is indicated by the arrow. Thereafter, data collected during the light period has the characteristic saw-tooth pattern of alternating intervals of growth and dilution. Declines in attenuation during the dark period are not due to dilution but to changes in the optical characteristics of the cells.

(Vol time^{-1}/Vol = time^{-1}). Because the sensor measures light attenuation across the sample vessel and initiates the supply of medium based on changes in it, it is very important that the attenuation reflects changes in the suspended culture alone. If the cells start to stick to the walls of the vessel, they will initiate dilution beyond

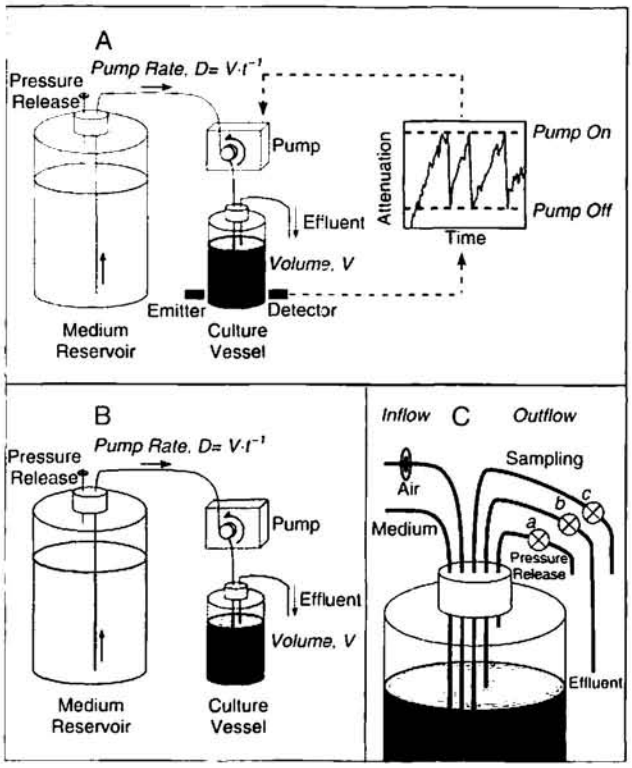

FIGURE 19.8. **(a)** Schematic representation of a turbidostat. For clarity, only the tubing through which medium and effluent from the culture flow are shown. In this configuration, an optical sensor interrogates the culture in the growth vessel. An alternative setup is to pump the culture through a separate detector loop, which has the advantage that the monitoring can be configured with alternating cleaning cycles and determination of background attenuation (Le Floc'h et al. 2002). The signal from the detector triggers an actuator that turns the pump on when attenuation exceeds a preset level. The influx of fresh sterile medium from the reservoir dilutes the culture until attenuation falls below the second preset level, at which point the pump is turned off. (The pressure relief on the medium reservoir should have an in-line sterile 0.2-μm filter to maintain sterility in the medium.) The culture is maintained at constant volume, V, by passive draining of effluent during the dilution. The growth rate is the intrinsic, nutrient-replete growth rate at the experimental irradiance and temperature, $\mu_{E,T}$. This is the rate of replacement of medium, D (the volume pumped per unit of time per volume of culture; $D = V \cdot t^{-1}/V = t^{-1}$), which is equivalent to the mean rate of increase of attenuation. **(b)** Schematic representation of a chemostat. For clarity, only the tubing through which medium and effluent from the culture flow are shown. The pump that supplies fresh sterile medium runs continuously, at a rate, D, set by the experimenter. (The pressure relief on the medium reservoir should have an in-line sterile 0.2-μm filter to maintain sterility in the medium.) The culture is maintained at constant volume, V, by passive draining of effluent during the dilution. The growth rate is a predetermined nutrient-limited growth rate at the experimental irradiance and temperature, $\mu_{N,T}$. This is the rate of replacement of medium, D (the volume pumped per unit of time per volume of culture; $D = V \cdot t^{-1}/V = t^{-1}$). **(c)** Schematic representation of the plumbing for a turbidostat or chemostat. The inflow tubes deliver fresh sterile medium and air that has been filtered through an in-line sterile 0.2-μm filter. There are three outflow tubes, each of which should have a gradient to prevent material in them flowing back into the culture and each of which has a pinch-valve (*a*, *b*, and *c*) that can completely occlude it. In normal operation, the pressure relief valve, *a*, and the effluent line, *b*, should have the valves open, and the sampling tube, *c*, should have the valve closed. Discrete samples of the culture can be taken by closing valves *a* and *b*. A large-volume syringe is then attached to the sampling tube and valve *c* is opened. When the sample has been drawn into the syringe, valve *c* is closed and valves *a* and *b* are reopened. See Chapter 5 for more information on preserving sterility during culturing.

what is needed to maintain the growth of the cultures in suspension and cause it to be washed out. If wall growth starts in a turbidostat, the culture should be decanted into a clean vessel. (Also, the light measurement must be unaffected by ambient light, for example, by gating.) If the culture is to be grown on a light–dark cycle, the light that is used to interrogate the culture should be of a long enough wavelength that it is not photosynthetically active, ideally above 750 nm (see Section 5.0) and at as low an irradiance as the signal/noise ratio will permit. This will prevent the sensor from augmenting the growth irradiance during the day

and inadvertently inducing photosynthesis and growth during the dark period.

The acclimated growth rate at the chosen temperature (and salinity) is then a function of irradiance and can be described in terms of three intrinsic characteristics of the cells. These are the maximum (i.e., light-saturated and nutrient-replete) growth rate, μ_m (d^{-1}), and two irradiances. The first, K_C, is one at which photosynthesis and respiration are equal so there is no net growth (Equation 1). The second, K_E, is the irradiance at which nutrient-replete growth becomes light saturated and is the analog of E_k, the saturating irradiance in a photosynthesis-irradiance curve (Talling 1957):

$$\mu_{E,T} = \mu_m\left(1 - \exp\left[-\frac{E - K_C}{K_E - K_C}\right]\right), \qquad (5)$$

where $\mu_{E,T}$ is the growth rate (d^{-1}) at irradiance E and temperature T. In practice, most studies omit K_C from the equation. Because balanced growth cannot be negative, Equation 5 only holds for the condition in which E is greater than K_C. The same effect can be obtained by expressing growth in terms of $(\mu + R)$ (Cullen et al. 1993), which is mathematically simpler. Both K_E and μ_m are functions of temperature and salinity (Grzebyk and Berland 1996, Underwood and Provot 2000) and vary by about an order of magnitude between species (Langdon 1987, Geider et al. 1997, MacIntyre et al. 2002).

Irradiance is often expressed as photosynthetically active radiation (PAR, defined as 400 to 700 nm) in energetic or quantum-based units. Even if the possible influences of artificial versus natural light spectra on algal physiology are ignored, it is advisable to account for the spectral quality of the light source when quantifying experimental irradiance. This is done by calculating photosynthetically utilizable radiation (PUR) (Morel 1978), a measure of the light that can be absorbed by the microalgae (see following discussion).

One caveat in dealing with turbidostats is that the optical characteristics of the small sample used to monitor the culture must reflect the culture as a whole, and cells must be homogeneously distributed when culture volume is removed. In practical terms, this means that cells must be prevented from aggregating by sinking or swimming. The sinking rates of cells vary widely as a function of their size, shape, and physiological status (Smayda 1970, Bienfang and Szyper 1982, Bienfang et al. 1983, Richardson and Cullen 1995), as does their propensity for migratory swimming (Kamykowski 1995), but all will eventually become heterogeneously dispersed in the absence of any mixing. The mixing can be episodic or continuous and can be achieved by bubbling or using a mechanical stirrer. Though necessary to satisfy the assumption of homogeneity, the increased turbulence associated with stirring can impede the growth of some species, particularly dinoflagellates (Juhl et al. 2001, Sullivan and Swift 2003). Bubbling can also be problematic, because the bubbles interfere with the measurement of attenuation if they are in the light path. Conversely, unless concentrations of microalgae are kept low, a culture that is not bubbled with air is likely to enter carbon limitation, a form of nutrient limitation that is often overlooked. The choice between automated assessment of culture concentration and replacement of medium and periodic assessment and replacement is often dictated by the sensitivities of the microalgae.

3.4. Nutrient-Limited Growth in Chemostat and Cyclostat Cultures

In a turbidostat, medium is delivered when algal concentrations exceed a threshold, so algal growth is countered with dilution, and the dilution rate, which matches the growth rate, is determined by irradiance and temperature as they influence the intrinsic nutrient-replete growth rate, $\mu_{E,T}$ (Equation 5). In contrast, chemostat cultures are maintained in balanced growth under nutrient-limited conditions at a growth rate that is set by the experimenter (Rhee et al. 1981). A schematic representation of the chemostat is shown in Figure 19.8b. The medium, formulated to be deficient in one nutrient, is delivered continuously but at a rate, D, less than the nutrient-replete growth rate, $\mu_{E,T}$. The achieved growth rate, μ_N, is equivalent to D, which is the inflow rate divided by the volume of the culture vessel (Vol $time^{-1}$/Vol = $time^{-1}$). Chemostat theory, the theoretical underpinning of the equivalence between μ_N and D, has been described by Rhee et al. (1981). A cyclostat is a chemostat grown under a repeatably varying regimen of light or (rarely) temperature. Cells in a cyclostat are also in balanced growth but are in dynamic equilibrium, rather than simple equilibrium. The growth rate is again equivalent to the dilution rate when averaged over the day, as for nutrient-replete growth on a light–dark cycle (see Fig. 19.4), although cell division can be entrained to the light–dark cycle in a diel rhythm (Chisholm 1981; see Chapter 21). Semicontinuous cultures can be run as quasi-chemostats so the culture is nutrient limited rather than nutrient replete (Sakshaug

et al. 1989, MacIntyre et al. 1997b). Instead of diluting the culture on demand to maintain its density at a constant level, a regular (usually daily) dilution is applied at a rate below μ_{ET}. When the measured growth rate is equal to the dilution rate and acclimation is com-plete, the cultures are in balanced nutrient-limited growth. The approach differs from a chemostat in that the fresh medium is added episodically rather than continuously.

The continuous supply of nutrients in the chemostat introduces elements of both Liebig and Blackman limitation on growth. At all but the highest nutrient-limited growth rates, the residual concentration of the limiting nutrient in a chemostat is a very small fraction of the initial concentration in the medium (McCarthy 1981, Garside and Glover 1991). Biomass in the culture is determined by the initial concentration of limiting nutrient (Rhee 1978), which is consistent with Liebig limitation (see Fig. 19.5b). That is, if the input medium has 50 μg-atom · L^{-1} nitrate and other nutrients are supplied in excess (and if excretion of dissolved organic nitrogen [DON] is low, which is typical), the concentration of particulate nitrogen in the culture will be close to 50 μg-atom · L^{-1}. However, the growth rate of microalgae in a chemostat is equal to the dilution rate, which is set by the experimenter, and the physiological adjustment by the cells to the supply of the limiting nutrient (see Fig. 19.2) reflects the degree of imposed Blackman limitation.

In a chemostat, the growth rate does not vary with irradiance, temperature, or the nutrient composition of the medium; rather, irradiance and temperature influence the degree of stress that is imposed at a particular nutrient-limited growth rate and the concomitant acclimative state that is achieved in response. Consequently, growth rates in chemostats are often expressed as relative to the maximal rate (i.e., μ_N/μ_{ET}), thereby eliminating much of the variability in results that is a function of growth rate alone (Goldman 1980).

The concept of relative growth rate is critical to the interpretation of experiments in nutrient-limited continuous cultures because imposition of an added stress can induce responses that are difficult to interpret. By definition, the growth rate in the chemostat, μ_N, will not vary even though an experimentally imposed stressor (e.g., a toxic compound or ultraviolet-B [UV-B] radiation) may have an adverse effect on the nutrient-limited cells. If it is sufficiently deleterious to cause a reduction in the nutrient-replete growth rate, μ_{ET}, the stressor would cause a highly counterintuitive *increase* in relative growth rate because μ_N remained constant at the experimental setting and μ_{ET} declined. In our opinion, it is difficult to resolve the inhibitory effect of a stressor if the growth rates in chemostat cultures grown with and without the stressor are the same so that the relative growth rate (μ_N/μ_{ET}) is higher for the algae under stress. We do not recommend this experimental approach.

As in a turbidostat, when a culture is grown in a chemostat, there is no net change of biomass (Fig. 19.9a). If the medium is supplied at a rate higher than μ_{ET}, the cells are unable to divide rapidly enough to maintain a stable population and are washed out of the growth vessel. As with other cultures, the cells are in steady-state growth when there are no changes in the ratios of indices of biomass (Equation 1, compare Fig. 19.9b and Fig. 19.6b) or in indices of physiological

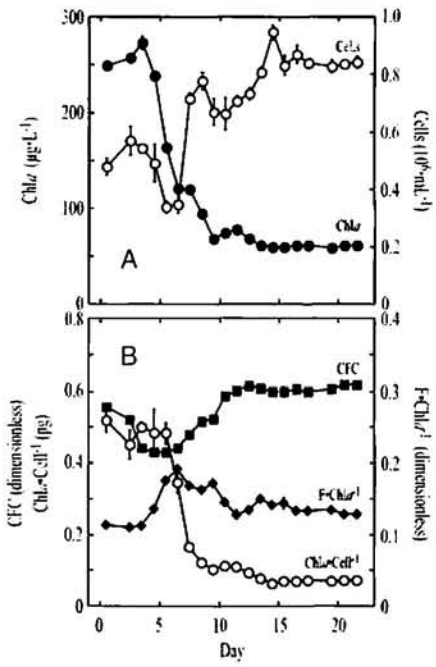

FIGURE 19.9. (a) Variation in the biomass of the diatom *Thalassiosira pseudonana* during growth in a chemostat culture (Xiaolong Yang, MacIntyre and Cullen, unpublished data). The chemostat was inoculated from a batch culture on day 0 and maintained at a dilution rate of 0.2 d^{-1}. The nutrient-replete growth rate, μ_{ET} (Equation 5), at the same irradiance was 1.33 d^{-1}. (b) Variation in the cellular chlorophyll quota (the ratio of the two biomass indices) and in two physiological indices, cellular fluorescence capacity (*CFC*) and $F \cdot Chla^{-1}$. CFC is the ratio of variable fluorescence to maximum fluorescence of dark-adapted cells, as measured in the presence of the inhibitor DCMU, and is an analog of the quantum yield of PSII. The parameter $F \cdot Chla^{-1}$ is the ratio of dark-adapted Chl*a* fluorescence of the culture *in vivo* to the fluorescence of its Chl*a* content *in vitro* (Kiefer 1973a). Constancy in these indicators after day 15 indicates that the cells were fully acclimated to their environment and that growth was balanced (Equation 2).

response that are responsive to changes in growth rate and nutrient status (see following discussion). As a rule of thumb, plan on 10 generations of growth to be assured that the cells are fully acclimated. Much time can be saved by using an inoculum that has been grown under similar conditions. For nutrient-limited chemostats, this could include subjecting the parent culture to early phases of starvation before inoculation. This also serves to eliminate carryover of limiting nutrient from the inoculum to the chemostat.

The relative concentrations of nutrients that are needed to ensure that a given nutrient is limiting in the chemostat are usually chosen by assuming that the Redfield ratio (Redfield 1958) of 106 : 16 : 1 C : N : P (Goldman et al. 1979) or an extension of the Redfield ratio that includes trace metals (see Chapter 4) is an approximation of the critical ratio (see Fig. 19.5b). The concentration of the limiting nutrient is then set so that its relative abundance is well below the Redfield ratio. The absolute concentrations of nutrients should be set so that biomass is low enough to keep the sample optically thin, to avoid second-order effects of variability in irradiance within the growth vessel. In theory this depends on the optical characteristics of the cells (their numerical abundance and specific absorption and scattering coefficients), the diameter of the vessel, its optical geometry, and so on (Droop 1985). In practice, we try to keep biomass below 100 μg Chl*a* · L^{-1}. Published relationships between nitrogen or phosphorus and Chl*a* (see Fig. 19.2) can be used as a rough guide in determining conversion factors between nutrient concentrations and Chl*a*.

3.5. Cultures in Unbalanced Growth

The types of cultures we have discussed are most frequently used for determining the physiological status of microalgae under equilibrium conditions. An alternative approach is to impose varying environmental conditions on a culture and monitor its responses. The simplest case is one in which a single variable is changed when a culture is in balanced growth. The culture is then monitored during the acclimative process to determine the time course of establishing a new equilibrium. Such experiments have been done frequently with irradiance (Post et al. 1985, Cullen and Lewis 1988, Sukenik et al. 1990, Anning et al. 2000), and less often with nutrient concentrations (Sciandra and Amara 1994, Le Floc'h et al. 2002) or temperature (Anning et al. 2001). Although informative about the rate of acclimation, such conditions are still highly artificial.

Approaches that are more realistic and correspondingly more difficult to interpret impose environmental changes on time scales that are comparable to or shorter than the response time of physiological or behavioral acclimation. These include imposing changes in irradiance (Marra 1978a, b; Kroon et al. 1992a, b; Ibelings et al. 1994), nutrients (Sciandra 1991, Bernard et al. 1996), or species composition in competition experiments (Litchman 1998, 2003) and observing the responses. In some of these (Marra 1978a, b), the light source was natural sunlight. To the extent that the conditions in the culture vessel mimic those in nature, the outcomes can then be used to predict responses to variations in nature; such observations have been analyzed extensively, for example, to predict photosynthetic responses during mixing (Marra et al. 1985, Neale and Marra 1985, Denman and Marra 1986, Pahl-Wostle and Imboden 1990, Franks and Marra 1994). The approach has the attraction of relying less heavily on what a holist would regard as 'unnatural' conditions in the culture. Even so, there is a limit to its usefulness because it can be wildly inaccurate to extrapolate from one set of conditions to another; the physiological process that limits a microalga's photosynthetic rate is determined in large part by the rate of change in the environmental cue (Lewis et al. 1984, Cullen and Lewis 1988, MacIntyre et al. 2000). For instance, regulation of Chl*a* content, accumulation and repair of photoinhibitory damage, and photosynthetic induction all have the potential to limit the rate of photosynthesis in a varying light field but have response times that vary over almost four orders of magnitude (Cullen and Lewis 1988, Baroli and Melis 1996, MacIntyre et al. 1997a, Neidhardt et al. 1998). Their relative importance as potential limiting rates for photosynthesis depends on whether the rate of change in the light field due to mixing is high (MacIntyre and Geider 1996) or low (Kamykowski et al. 1996) and applying the wrong rate constants in a simulation yields seriously inaccurate predictions.

4.0. MONITORING CULTURES

Sampling methods range from periodic analysis by hand to computer-driven continuous measurement via automated sensors. Manual analysis is much more common. A schematic representation of the plumbing required to remove small samples from a culture is shown in Figure 19.8c. Here, we recommend several techniques that we have found valuable; they are sensitive, reliable, and

relatively fast. Sensitivity is important because if the culture is to be maintained, destructive analysis must not consume material more rapidly than the culture can produce it. The other major considerations are as follows:

1. If the monitoring is designed to ensure that the culture is acclimated to its growth conditions, it is important to measure more than one parameter to satisfy the requirement of Equation 2 that the rate of change of all parameters be equal, and in the case of continuous cultures, zero.
2. When microalgae are grown under light–dark cycles, physiological responses (Prézelin et al. 1977, Harding et al. 1981), cell division (Chisholm and Brand 1981), and such behaviors as migration (Palmer and Round 1965, Weiler and Karl 1979, Cullen 1985, Serôdio et al. 1997) are entrained to the photoperiod. Consequently, if the culture is being monitored to determine whether growth is balanced, it is critical that the culture be sampled at the same point in the cycle (see Fig. 19.4).

4.1. Cell Numbers

We recommend at least two estimates of biomass and/or physiological response when monitoring cultures. One that requires relatively little material is a cell count (see Chapter 16). Cells can be counted microscopically using bright-field microscopy with a hemocytometer or another counting chamber or by using epifluorescence after filtration onto a black membrane filter (MacIsaac and Stockner 1993). The latter is preferable where the cell density is low, so that the volume contained by the hemocytometer is too small to give a statistically acceptable estimate. Microscopic enumeration can be more tedious and less precise than some other methods, but experience has shown that it is an important part of experimentation with cultures; visual inspection provides important information on the morphology of cells and the presence of contaminants that could have a strong bearing on the interpretation of results.

Rapid counts can also be made using a Coulter counter or flow cytometer calibrated with fluorescent beads of a known concentration (Olson et al. 1993, see Chapter 17). The flow cytometer can measure the statistical distribution of several parameters (fluorescence of Chl*a*, phycobilin and/or a vital stain, and side and forward-angle scattering) simultaneously and could be particularly useful when used with a stain such as 4′,6-diamidino-2-phenylindole (DAPI) (Porter and Feig 1980) or fluorescein diacetate (FDA) (Jochem 1999). The forward-angle scattering also gives a first-order estimate of cell volume and, from that, cell carbon (DuRand et al. 2002). However, caution should be used in applying volume to carbon conversion factors, because diatoms, in particular, seem to have different carbon densities (carbon to volume relationships) from other protists (Menden-Deuer and Lessard 2000).

4.2. Chlorophyll *a*

Chl*a* is ubiquitous in microalgae and the methods for measuring it are sensitive, precise, and (mostly) accurate (see Chapter 20). It is a highly useful index for monitoring growth in cultures, particularly in concert with *in vivo* fluorescence (see following discussion). For the purposes of monitoring, it is not necessary (and it would not be cost-effective) to determine Chl*a* by the most accurate and discriminating method, high-performance liquid chromatography (HPLC). Fluorometric analysis after extraction in 90% (V/V) acetone is a sensitive, precise, and generally accurate means of assessing the concentration from small aliquots, although the technique is less reliable in species that have high levels of Chl*b* and/or tough cell walls that impede pigment extraction (Holm-Hansen 1978, Welschmeyer 1994). Acceptably precise estimates (coefficients of variance of <5% between replicates) can usually be obtained from extracts with concentrations of as little as 1 to 5 μg Chl*a* · L^{-1}. As most fluorometers require only 3 to 5 mL of the extract to make a measurement, this translates to a minimum of about 10 ng of Chl*a* per aliquot (i.e., 0.1 mL of a culture with 100 μg Chl*a* · L^{-1}). Measurement of Chl*a* can be biased by the presence of Chl*b* or the degradation product chlorophyllide *a*, which has similar optical qualities (Holt and Jacobs 1954, Jeffrey et al. 1997). A correction can be made for degradation products by measuring fluorescence before and after the sample has been acidified (Holm-Hansen 1978), but the method is still unreliable in the presence of Chl*b*. An alternative is to use filters that optimize for absorption and emission by Chl*a* alone (Welschmeyer 1994). The latter is faster and acceptably accurate (Box 19.1, Fig. 19.10).

Our default technique for fluorometric assessment of Chl*a* is to filter a small volume onto a glass-fiber filter and extract the pigments passively in the dark at −20° C for 24 hours, using 90% (V/V) acetone. Note that this is not recommended for HPLC protocols (see Chapter 20) because of the possibility of some degradation of the Chl*a*. However, the primary degradation

BOX 19.1 A Comparison of Experimental Methods for Determining Chl*a*.

A comparison of estimates of the Chl*a* content in samples of the prasinophyte *Pycnococcus provasolii* Guillard guides our discussion of techniques for measuring Chl*a* in cultures (Fig. 19.10). The alga has high levels of Chl*b* and Mg-2,4-divinyl pheoporphyrin *a*$_5$ monomethyl ester (Mg DVP; see Table 19.3), both of which can compromise assessment of Chl*a*, so it is a good experimental organism to test the methods. The benchmark estimate is obtained by high-performance liquid chromatography (HPLC) (see Chapter 20), as described by van Heukelem et al. (1992). The default rapid estimate is by passive extraction in 90% (v/v) acetone for 24 hours in darkness and at −4° C. followed by fluorometric determination against a pure Chl*a* standard (Welschmeyer 1994).

Alternative methods are as follows:

- *Spectrophotometry:* The extraction technique is the same as for fluorometry, so there is no gain in speed. It is less sensitive than the fluorometric method and tends to overestimate Chl*a* (MacIntyre et al. 1996, Van Heukelem et al. 2002), even when the estimate is corrected for interference by other chlorophylls (Jeffrey and Humphrey 1975, Jeffrey et al. 1997), as shown in Fig. 19.10a.
- *Grinding (not tested):* Grinding the sample in a mortar with a Teflon pestle or in a Ten-Broek tissue homogenizer obviates the need for the long passive extraction. Our frequent observation of the splatter-line of partially ground filter on the adjacent surfaces suggests that this is not an accurate technique in inexperienced hands.
- *Freeze/thaw:* Here, the filter is frozen in one part of distilled water and thawed with nine parts of acetone to give a final concentration of 90% (v/v) acetone (Glover et al. 1986). The method greatly accelerates extraction: As the sample freezes, ice crystals can break the cells' membranes and extraction is effectively complete in the time it takes to freeze a small aliquot of water. The method gives much better extraction in species with relatively tough cell walls such as the cyanobacterium *Synechococcus* spp. (Glover et al. 1986). It may still underestimate Chl*a* (Fig. 19.10a), perhaps because of increased chlorophyllase activity in the aqueous phase during the freeze/thaw cycle: Chlorophyllase activity can increase with the aqueous content in acetone/water mixtures (Barrett and Jeffrey 1964).
- *Extraction in DMSO and acetone:* An alternative solvent is a 2 : 3 mixture of dimethyl sulfoxide (DMSO) and 90% (v/v) acetone (Shoaf and Lium 1976). Pigments are fully extracted in the mixture in 15 minutes of darkness and at room temperature, and the accuracy and precision compare favorably with HPLC (Fig. 19.10A and C). In a comparison of side-by-side fluorometer calibrations with 90% (v/v) acetone and the 90% acetone (v/v)/DMSO mixture, we found that there was no significant difference between their calibration slopes (Student's paired *t*-test: $P = 0.71$, $R^2 = 0.92$, n = 6). The same calibration can, therefore, be used for either method. Stramski and Morel (1990) report a similar result. DMSO must be handled with caution because it is readily absorbed through the skin and can transport other chemicals, such as the acetone, with it. Like acetone, it is a respiratory irritant and so should be used in a fume hood and handled with heavy gloves. Under no circumstances should either solvent be pipetted by mouth.
- *Direct injection:* An even more rapid extraction technique eliminates filtration: a small volume of culture (1% of the final extact volume) is pipetted directly into the solvent. The method compared favorably with HPLC when the solvent was 90% acetone (v/v)/DMSO and extraction was for 15 minutes in darkness at room temperature (Fig. 19.10A). When the same technique was used with 90% acetone and 24-hour extraction, a dense precipitate formed that gave an artifactually high estimate of Chl*a*. The estimate was lower and more accurate when the precipitate was removed by centrifugation (Fig. 19.10a,b).

product, chloropyllide *a*, has optical properties comparable to Chl*a*'s (Holt and Jacobs 1954), so the fluorometric technique is insensitive to minor degradation. Although it is important to keep the sample cold and in the dark during extraction to minimize degradation of Chl*a*, fluorescence yield in solvent is a function of temperature and water condenses on cold cuvettes, so the extract should be warmed to room temperature before fluorescence is measured. This can be done quickly in a darkened water bath to prevent degradation of pigment. For cultures, the fluorometric technique compares favorably with HPLC estimates of Chl*a* (see Fig.

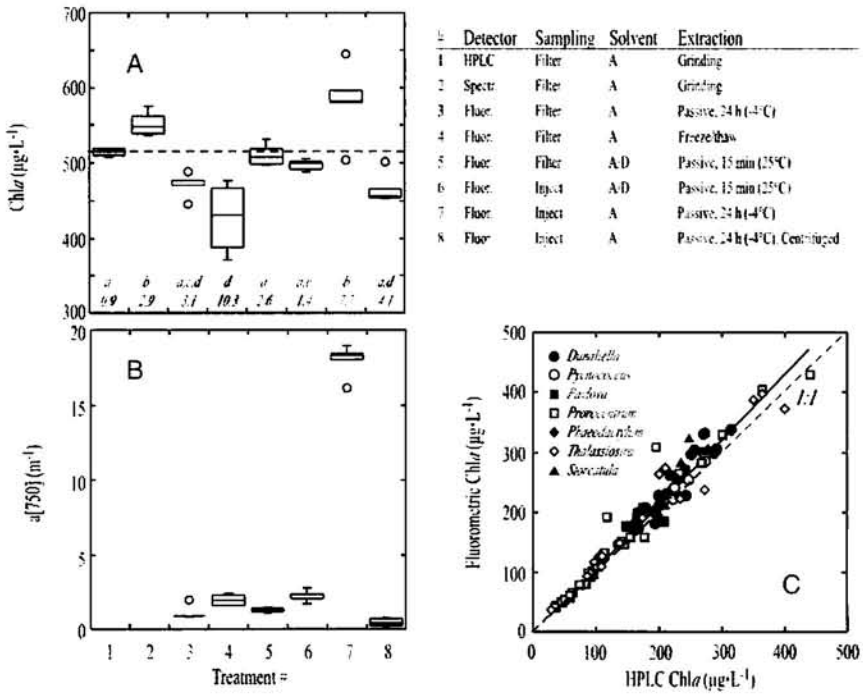

FIGURE 19.10. Comparison of chlorophyll *a* (Chl*a*) extraction by different analytical methods. **(a)** Box plot of estimated Chl*a* in a dense culture of the prymnesiophyte *Pycnococcus provasolii*, as determined by higher-performance liquid chromatography (HPLC) (Van Heukelem et al. 1992), spectrophotometry (Jeffrey and Humphrey 1975), and fluorometry (Welschmeyer 1994) (n = 6 for each treatment). Samples determined by fluorometry were either collected on Whatman GF/F filters or pipetted directly into the solvent (filter vs. injection; see key). All filtered samples had the same volume: Solvent volumes were varied over a 3*x* range to optimize for the detection method. Pigments were extracted in either 90% acetone or a 3 : 2 mixture of 90% acetone and dimethyl sulfoxide (DMSO) (Shoaf and Lium 1976). A, 90% acetone; A/D, 2 : 3 DMSO:90% acetone. The sample injected into 90% acetone (#7) was centrifuged at 3,100 *g* for 5 minutes to remove precipitates and re-read (#8). Numbers under each treatment are the coefficients of variance (%). Treatments identified with the same letter (*a–d*) were not significantly different (one-way ANOVA, Tukey's *a posteriori* test for significance, $P < 0.05$). **(b)** Scattering characteristics of the samples for fluorometric analysis in **(a)**, as determined by apparent absorption at 750 nm. Absorption was measured against 0.2 μm-filtered 90% acetone in a 1-cm cuvette at the port of an integrating sphere. Note the high value for culture injected directly into 90% acetone (#7). **(c)** Chl*a* estimated fluorometrically (Welschmeyer 1994) after 15 minutes of extraction in 3 : 2 90% acetone : DMSO and estimates made by HPLC after extraction by grinding in 90% acetone. The 100 samples were from cultures with diverse accessory pigments: Chl*b* and carotenoids (*Dunaliella tertiolecta* Butcher, chlorophyte; *P. provasolii*, prasinophyte); Chl*c* and carotenoids (*Pavlova lutheri*, prymnesiophyte; *Prorocentrum minimum* [Pavillard] Schiller, dinoflagellate; *Phaeodactylum tricornutum* and *Thalassiosira weissflogii*, diatoms); and Chl*c* and phycoerythrin (*Storeatula major* Hill, cryptophyte). The cultures were variously nutrient-replete and nutrient starved. On average, the fluorometric method overestimated Chl*a* by 5% ± 3% (*solid line*). The coefficient of determination (R^2) was 0.94.

19.10a) (MacIntyre et al. 1996), but passive extraction of pigments is too slow for decisions on whether to transfer or harvest a culture. We discuss the pros and cons of alternative techniques in Box 19.1.

4.3. Attenuation

Optical techniques in general lend themselves to noninvasive monitoring of microalgal cultures. Dedicated light detectors (e.g., Thermo Oriel, Ocean Optics) are relatively inexpensive and can be aligned with emitters for continuous measurement of transmission through the culture vessel. The turbidostat data in Figure 19.7b were collected in this way. In practice, wall growth in front of the source and detector (or on the source and detectors themselves if they are inserted into a culture through ports) biases the data record. Formation of these biofilms is particularly problematic with benthic microalgae and cyanobacteria. Accumulation or consumption of colored nonalgal material in the culture (e.g., vitamin and trace-metal solutions) also biases the record unless the data are blank-corrected frequently. Continuous monitoring using optical techniques is best

done by removal of discrete samples, as part of a cycle that includes determination of the blank and cleaning with a detergent solution followed by rinsing (Le Floc'h et al. 2002).

4.4. *In Vivo* Fluorescence

There are only a few means by which the abundance and/or physiological responses of microalgae can be measured without destructive sampling. Foremost among these is measurement of Chl*a* fluorescence. This pigment has the useful property that a proportion of absorbed light energy (excitation) is re-emitted with a longer wavelength (emission) as fluorescence. Chl*a* fluorescence can be measured from live cells (*in vivo*) or after extraction of the pigment into a solvent. The two measures, however, are not the same. In live cells, the excitation energy can be directly absorbed by the pigment itself or absorbed by accessory chlorophylls, carotenoids, or phycobilins and transferred to Chl*a*. When pigments are extracted in solvent, the disruption of the associated pigment–protein complexes ensures that only light absorbed directly by Chl*a* itself can be re-emitted.

Because fluorescence can be stimulated by relatively low light levels that induce no lasting physiological stress, *in vivo* fluorescence can be used as a nondestructive means of measuring growth rate (see Chapter 18). Monitoring is particularly easy if cultures are grown in 25-mm diameter (50-mL) tubes that can be fit into many conventional fluorometers (Brand et al. 1981). This method is particularly useful when cultures must be maintained under axenic conditions, where repeated removal of subsamples may compromise the culture. Assuming growth is balanced, changes in fluorescence correlate with changes in biomass. Some care has to be taken to ensure that this is so.

The amount of Chl*a* fluorescence depends on several factors:

$$Fl = \sum \left[E_{Ex}(\lambda) \cdot a^{Chl}(\lambda) \right] \phi_{Fl}, \qquad (6)$$

where Fl is fluorescence, $E_{Ex}(\lambda)$ is the scalar irradiance of the excitation beam at wavelength λ, and $a^{Chl}(\lambda)$ is Chl*a*-specific absorption at wavelength λ, and ϕ_{Fl} is the quantum yield of fluorescence. None of these terms is constant, so fluorescence should be used as a measure of biomass only with caution.

1. The excitation characteristics of a fluorometer, $E_{Ex}(\lambda)$ (which determines absorption and can influence quenching; see later discussion), are set by its manufacturer and by the age of the lamp. Consequently, fluorescence is a relative property and cannot be compared directly either between two fluorometers of different types (Cullen et al. 1988) or even between different fluorometers that are the same model.
2. Chl*a*-specific absorption, $a^{Chl}(\lambda)$, varies with the complement of accessory and photoprotective pigments, which in turn vary between species and within a species during acclimation to irradiance and under nutrient starvation and nutrient limitation (Sosik and Mitchell 1991, Wilhelm and Manns 1991, Latasa and Berdalet 1994, Goericke and Montoya 1998, MacIntyre et al. 2002). It also varies with the degree of pigment packaging imposed by variations in the cellular Chl*a* quota (Duysens 1956, Berner et al. 1989, Dubinsky 1992).
3. The quantum yield, ϕ_{Fl}, can increase by as much as 50% when cells enter stationary phase (Kiefer 1973b, Beeler SooHoo et al. 1986, Beutler et al. 2002). The relationship between fluorescence and Chl*a* will, therefore, vary with growth conditions (see Fig. 19.9b). There is also variability in quantum yield due to fluorescence quenching, the reduction of the yield due to utilization of absorbed photons in photochemistry (photochemical quenching) or due to an enhanced probability of heat dissipation (nonphotochemical quenching, induced in bright light). With the exception of the relatively slow repair of photoinhibitory damage, quenching processes relax in darkness with a time constant of about 5 minutes or less (Kolber et al. 1990). The effects of short-term quenching on the fluorescence–Chl*a* relationship can, thus, be reduced to a minimum by placing the sample in darkness for 20 to 30 minutes before the measurement.

Fluorescence *in vivo* can be used only as a proxy for Chl*a* if the growth conditions are stable and the cells are acclimated to them. For instance, it is not a reliable measure of Chl*a* throughout growth in a batch culture, except in the early phase where the culture is optically 'thin' and nutrients are present in excess.

Because the relationship between Chl*a* and fluorescence is so sensitive to both short-term and long-term responses of the photosynthetic apparatus, the ratio of the two is a useful index of whether cells are in balanced growth. It can be parameterized in dimensionless terms as $F \cdot \text{Chl}a^{-1}$ (Kiefer 1973b), which is the ratio of dark-adapted fluorescence *in vivo* to the fluorescence of the same concentration of Chl*a* *in vitro* after extraction in

solvent, measured with the same fluorometer. This is a sensitive indicator of acclimative state and reaches an equilibrium value when biomass-based indices also indicate that cells are in balanced growth (see Fig. 19.9). It also has the advantage that it can be compared between studies, provided that the excitation characteristics (spectral quality and intensity) of the fluorometers used to measure it are similar and that the same solvents are used in the *in vitro* measurements. Determination of Chl*a* directly to make the comparison requires destructive sampling of the culture, though.

Another dimensionless but highly sensitive fluorescence parameter is the ratio of variable to maximum fluorescence. This can be measured in the dark after destructive sampling, using the inhibitor 3-[3,4-dichlorophenyl]-1,1-dimethylurea (DCMU). We retain Vincent's (1980) description of this parameter as the cellular fluorescence capacity (CFC) (see Table 19.1, Fig. 19.9), to distinguish it from the analogous parameter, F_v/F_m, that is measured nondestructively using either a pulse-amplitude–modulated (PAM) (Schreiber et al. 1986, 1993) or a fast repetition rate (FRR) fluorometer (Falkowski et al. 1986, Kolber and Falkowski 1993). The PAM and FRR fluorometers differ in the intensity and duration of the exciting flash, so the former measures multiple PSII turnover events and the latter measures single turnover events. Both can also measure the ratio of variable to maximum fluorescence under actinic illumination, F_v'/F_m', which can be equated with the quantum yield of PSII (Genty et al. 1989, 1990). Estimates of F_v/F_m from PAM and CFC are correlated (Parkhill et al. 2001). Estimates of F_v/F_m from PAM and FRR are also correlated, but the relationship is nonlinear and the estimates differ in magnitude (Koblízek et al. 2001, Suggett et al. 2003).

The nomenclature used in describing fluorescence is confusing and notoriously inconsistent between different research groups. Kromkamp and Forster (2003) provide a review of the nomenclature and a comparison of *CFC*, F_v/F_m, and F_v'/F_m'. Both *CFC* and F_v/F_m can be measured rapidly on small amounts of biomass, making them ideal for assaying the physiological status of cultures. They are great indicators of a culture's status, being sensitive, quick, and easy to measure. F_v/F_m in continuous cultures can be high, despite strong nutrient limitation, when cells are fully acclimated (Cullen et al. 1992b, Parkhill et al. 2001). This acclimation requires more time than it takes for other properties of the culture to reach a stable value, so reductions in F_v/F_m are often cited as an indication of nutrient stress in both starved and limited cultures (Geider et al. 1993a, 1993b).

The PAM and FRR fluorometers could in theory interrogate a culture through the vessel walls and so be used to assess its physiological status noninvasively. It is easy to do with most PAMs (Lippemeier et al. 2001), which have fiberoptic emitter/detector assemblies. The configuration of commercially produced FRR fluorometers does not lend itself to this application, but modifications can be made to monitor cultures (Y. Huot, unpublished data). The idea should be treated with caution: *CFC* and F_v/F_m are calculated as the difference between minimum and maximum fluorescence, normalized to the maximum ($[F_d - F]/F_d$ and $[F_m - F_0]/F_m$), so the numerator is insensitive to background fluorescence, but the denominator is not (Cullen and Davis 2003). Overestimation of the blank leads to underestimation of the ratio and vice versa, and the bias is amplified as biomass decreases. For the cultures shown in Figure 19.2a (40 to 140 μg Chl*a* · L^{-1}), the blank was only 1 to 3% of F_d; for the lower biomass natural samples in Figure 19.1 (0.1 to 4.6 μg Chl*a* · L^{-1}), the blank was 1 to 13% of F_d (data not shown). This is high enough to warrant correction for background fluorescence. In practice, this is best done by gravity-filtering the sample and using the filtrate, whose fluorescence usually differs from both fresh medium and distilled water. Using vacuum filtration at anything above vanishingly low pressures can give an erroneously high blank, presumably through rupture of a small but significant fraction of the cells releasing fluorescing cell debris into the filtrate. Note that *CFC* can also be biased if the excitation intensity is too high when cultures are acclimated to low irradiance (Parkhill et al. 2001). This causes some photosynthetic induction, which causes F to rise and so *CFC* is underestimated. The problem can be corrected by using appropriate neutral-density filters to attenuate the excitation beam.

5.0. PHOTOSYNTHESIS AND IRRADIANCE

Photosynthesis is a fundamental property of autotrophic microalgae that must be quantified to understand the role of aquatic systems in the global carbon cycle. The spatial and temporal scales of natural variability preclude measurement, so estimates are based on models of photosynthetic response to environmental factors (Behrenfeld and Falkowski 1997a, b). Photosynthesis varies with irradiance in a nonlinear manner, described experimentally as a photosynthesis–irradiance (PE) curve. Short-term changes in pho-

tosynthetic rate during exposure to different light intensities are due to regulation of the connectivity and activity of components of the photosynthetic apparatus. Long-term changes are due to acclimative regulation of their abundance (see Fig. 19.3). Describing both the short- and the long-term variability is central to our understanding of microalgal ecology. However, an unambiguous description of the causal factors and the magnitude and rate of the resulting changes in photosynthetic response is possible only under controlled conditions, so model development will depend on careful comparison between the variation in PE responses in cultures with descriptions of natural conditions. Variability in estimates of the PE response depends on the means used to measure it, the time course of the measurement, the biomass parameter used to normalize the data, and any spectral differences between the incubator and the growth vessel. We deal with these in turn.

5.1. Reconciling Light Fields: PAR vs. PUR

Photoautotrophic algae need light for photosynthesis, but not all photons are equal. For a given light source, the nutrient-replete growth rate can be described as a function of irradiance (Equation 5), or more accurately, spectral scalar irradiance. For measurement of irradiance itself, we refer the reader to Kirk (1994). A microalga's growth rate at a given intensity of PAR (measured as the average over 400 to 700 nm) depends both on its value relative to K_E and on the spectral quality of light (Glover et al. 1987). This is largely because microalgae absorb light of different wavelengths with different efficiencies (Fig. 19.11).

Measurement of light absorption by microalgae is complicated by the fact that at all but high densities their absolute absorption is low and that, as particles, they both absorb and scatter light. Cultures can be grown to high enough densities to ensure adequate signal/noise ratios with a benchtop spectrophotometer. However, doing so inevitably produces gradients in irradiance within the culture vessel that complicate the description of growth irradiance; the least ambiguous estimates of growth irradiance are obtained when samples are optically thin. Scattering within a sample introduces two errors into estimates of absorption. The first is that the directional nature of scattering causes losses in the transmitted photon beam that are interpreted as absorption. The second is that multiple scattering at high particle densities increases the probability of subsequent absorption (absorption enhancement).

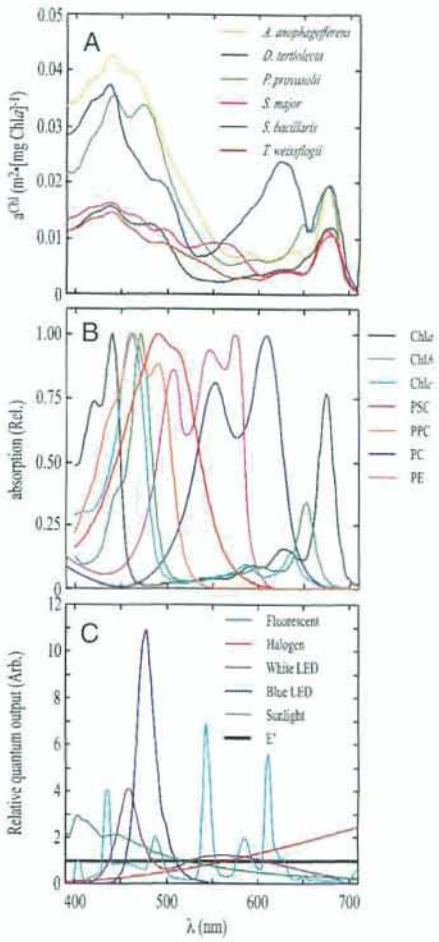

FIGURE 19.11. **(a)** Variation in pigment-specific absorption with wavelength for five species of microalgae grown at the same irradiance (80 μmol photons · m^{-2} · s^{-1} of fluorescent light). The species have diverse accessory pigments (see Table 19.3) characteristic of their taxa: *Aureococcus anophagefferens*, pelagophyte; *Dunaliella tertiolecta*, chlorophyte; *Pycnococcus provasolii*, prasinophyte; *Storeatula major*, cryptophyte; *Synechococcus bacillaris*, cyanobacterium; *Thalassiosira weissflogii*, diatom. **(b)** Variation in absorption *in vitro* for the pigments found in the species in **(a)**: chlorophylls *a*, *b*, and *c*; photosynthetic and photoprotective carotenoids (PSC and PPC); and R-phycoerythrin (PE) and R-phycocyanin (PC). Spectra are expressed relative to their maximum absorption. Spectra for chlorophylls and carotenoids are from Bidigare et al. (1990). Spectra for phycobilins are redrawn from Kirk (1994). **(c)** Spectral output of four light sources commonly used in growth and physiology experiments, sunlight, and a theoretical flat spectrum, *E'*, used to estimate the correction factor A_e (see text for details). The artificial light sources were a cool white fluorescent tube (Osram Sylvania F40T12), a quartz halogen lamp (General Electric ENH250), a white LED (Emissive Energy Inova 5X), and a blue LED (Chelsea Instruments FAST^{tracka} excitation LED). Sunlight was measured under clear sky conditions at Dauphin Island, Alabama. All sources have been scaled to the same value of PAR (the integrated output between 400 and 700 nm).

1. If cultures are grown at low densities, the low signal/noise ratio can be improved by increasing the effective pathlength by collecting the culture on a filter and scanning the filter (Yentsch 1962). The absorption enhancement on the filter caused by scattering must be corrected, either by using an empirical comparison with a suspension in a cuvette (Mitchell and Kiefer 1984, Bricaud and Stramski 1990, Cleveland and Weidmann 1993) or by modeling (Roesler 1998). Published corrections for absorption enhancement vary by almost an order of magnitude (Roesler 1998).
2. The error caused by directional scattering losses in suspensions can be reduced by placing an opal-glass diffuser between the sample and the detector in a spectrophotometer (Shibata 1958), placing the sample immediately adjacent to the detector (Bricaud et al. 1983), or using either an integrating sphere (Nelson and Prézelin 1993) or a reflecting tube instrument (Kirk 1992, Zaneveld et al. 1994). Even so, scattering errors in suspensions may persist, as indicated by the non-zero values of apparent absorption at 750 nm, where pigments do not absorb (Roesler 1998).

Interspecific and intraspecific differences in the absorption characteristics of microalgae are a consequence of their size and pigment complement:

1. The absolute amount of light absorbed by a cell depends on its size and the amount of pigment in it (Agustí 1991, Finkel 2001).
2. The chlorophyll-specific absorption depends on how densely the photosynthetic pigments are packed within the cell. As the internal concentration of pigments rises, Chl*a*-specific absorption decreases because of internal self-shading, particularly in large cells (Morel and Bricaud 1981, Berner et al. 1989, Dubinsky 1992). This can be seen as the absolute magnitude of Chl*a*-specific absorption, a^{Chl}, in Figure 19.11. The effect of pigment packaging on absorption is most easily discerned at the Chl*a* red peak (674 nm), where absorption by other pigments is negligible; specific absorption is much higher in the similarly sized small species, *Aureococcus anophagefferens*, *Pycnococcus provasolii*, and *Synechococcus bacillaris*, which have Chl*a* quotas of less than 0.1 pg · Cell^{-1}, than in the larger species, which have Chl*a* quotas of 2 to 3 pg · Cell^{-1} (Table 19.2).
3. The primary regulation of the pigment complement is under genotypic control. Although all microalgae contain Chl*a*, the distribution of accessory pigments (Chl*b* vs. one or more types of Chl*c*, specific carotenoids, xanthophylls, and/or phycobilins) is taxonomically determined. Jeffrey et

TABLE 19.2 Pigment data for the cells shown in Fig. 19.11: *Aureococcus anophagefferens* (pelagophyte); *Dunaliella tertiolecta* (chlorophyte); *Pycnococcus provasolii* (prasinophyte); *Storeatula major* (cryptophyte); *Synechococcus bacillaris* (cyanobacterium) and *Thalassiosira weissflogii* (diatom). Size is approximate diameter (μm). Chl*a* · cell^{-1} in (pg). Pigment:Chl*a* ratios in (g · g^{-1}).

Species	Size	Chla	Chlb	Chlc	MG-DVP	[a]PSC	[b]PPC	[c]Xanth
		Cell	Chla	Chla	Chla	Chla	Chla	Chla
A. anophagefferens	2	0.09		0.378		0.693	0.016	0.100
D. tertiolecta	8	2.00	0.256			0.159	0.253	0.071
P. provasolii	2	0.07	0.793		0.054	0.449	0.510	0.072
S. major	5	1.70		0.118			0.338	
S. bacillaris	1	0.03					0.075	0.280
T. weissflogii	16	2.78		0.093		0.340	0.021	0.063

Chlc, the sum of all *c* chlorophylls; MG-DVP, Mg-2,4-divinyl pheoporphyrin a_5 monomethyl ester; PSC, photosynthetic carotenoids; PPC, photoprotective carotenoids; Xanth, xanthophylls.

[a]The distribution by taxon is: 19′-butanoyloxyfucoxanthin, fucoxanthin (*A. anophagefferens*); neoxanthin (*D. tertiolecta*); neoxanthin, prasinoxanthin (*P. provasolii*); fucoxanthin (*T. weissflogii*). *S. major* and *S. bacillaris* also contain phycobilins, which were not quantified.

[b]The distribution by taxon is β-carotene (*A. anophagefferens*); lutein, β-carotene (*D. tertiolecta*); lutein, β-carotene, astaxanthin (*P. provasolii*); β-carotene, alloxanthin, monadoxanthin, crocoxanthin (*S. major*); β-carotene (*S. bacillaris*); β-carotene (*T. weissflogii*).

[c]The distribution by taxon is diadinoxanthin, diatoxanthin (*A. anophagefferens* and *T. weissflogii*); violaxanthin, antheraxanthin, zeaxanthin (*D. tertiolecta* and *P. provasolii*); zeaxanthin (*S. bacillaris*).

al. (1997) provide a comprehensive review of the structures and distributions of the pigments. The increase in Chl*a*-specific absorption between 425 and 525 nm in *A. anophagefferens* relative to the similarly sized *P. provasolii* (see Fig. 19.11a) is due to its higher quota of photosynthetic carotenoids and the presence of Chl*c* rather than Chl*b* (see Fig. 19.11b, Table 19.2). The high absorption between 540 and 570 nm in *Storeatula major* and between 550 and 650 nm in *S. bacillaris* relative to the other species is a consequence of their being the only species shown that have phycobilins (see Fig. 19.11a,b).

4. Secondary regulation of the pigment complement is under phenotypic control, because microalgae respond to changes in their environment. Within a species, the relative abundance of both photosynthetic and photoprotective pigments depends on growth irradiance (reviewed by MacIntyre et al. 2002), on nutrient status (Sosik and Mitchell 1991, Wilhelm and Manns 1991, Latasa and Berdalet 1994, Goericke and Montoya 1998), and in the short-term, on recent exposure to bright light (Demmig-Adams 1990, Arsalane et al. 1994, Olaizola et al. 1994, Casper-Lindley and Björkman 1998).

The consequence of the size- and pigment-determined efficiency of absorption is variability in the efficiency with which the cells utilize light of different spectral quality. The relative quantum output of sunlight and several common light sources is shown in Figure 19.11c. The spectra have been weighted so that they have the same integrated value between 400 and 700 nm (i.e., they have the same value of PAR). The efficiency with which each light source is absorbed by the cells is not equivalent. It is intuitively obvious from comparison of the output spectra and absorption spectra that all of the species will absorb the light from the blue LED efficiently because its output is concentrated in the part of the spectrum where absorption by chlorophylls and carotenoids (which all the species have) is highest. Conversely, none will absorb light from the quartz halogen lamp as efficiently; its output increases with wavelength and only *S. bacillaris* has comparable efficiencies of absorption at the red and blue ends of the spectrum. The blue LED will, therefore, have a higher value of PUR than the quartz halogen lamps, although both have the same value of PAR.

Quantitative treatment of PUR relies on convolution of the output and absorption spectra. There is no meter that measures PUR, although the spectral weighting that is involved in calculating it is not complex. There are two ways in which this can be done. The first, which we designate as a "biological" description because it is expressed explicitly in terms of the cell's pigment content, is by simple convolution of the output and absorption spectra (Gallegos et al. 1990).

$$PUR^{Chl} = E_a = \sum_{\lambda=400}^{\lambda=700} E(\lambda) a^{Chl}(\lambda), \qquad (7)$$

where E_a is the light absorbed by the cells per unit Chl*a*, $E(\lambda)$ is the quantum output at wavelength λ and $a^{Chl}(\lambda)$ is the corresponding Chl*a*-specific absorption coefficient. As E has units of μ mol photons $m^{-2} \cdot s^{-1}$, and a^{Chl} has units of $m^2 \cdot (mg\ Chl)^{-1}$, PUR^{Chl} has units of mol photons $\cdot$ $(g\ Chl)^{-1} \cdot s^{-1}$ after reconciliation of units of quantity. Although the units may seem peculiar initially, they represent the efficiency with which photons are absorbed by the pigments present in the cells.

An alternative expression of PUR is in "physical" terms, without explicit reference to the absorption characteristics of the cell, as a flux of photons per unit area. We designate this simply PUR. It has the same units as PAR and is obtained by applying a dimensionless weighting function, A_e, to it (Markager and Vincent 2001):

$$PUR = A_e \cdot PAR \qquad (8)$$

The numerical value of the weighting function is the ratio of the light absorbed under the spectral regimen of interest (E_a, Equation 7) to E'_a, the light absorbed under a theoretical, spectrally flat regimen of equal photon flux over PAR, E' (see Fig. 19.11c). Because the output of the theoretical regimen is wavelength independent, E'_a is equal to the product of E' and the mean value of $a^{Chl}(\lambda)$ between 400 and 700 nm, a^{Chl}:

$$A_e = \frac{E_a}{E'_a} = \frac{\sum_{\lambda=400}^{\lambda=700} E(\lambda) \cdot a^{Chl}(\lambda)}{E' \cdot a^{Chl}} \qquad (9)$$

Although PUR^{Chl} and PUR differ in presentation and units, there is no significant difference in their computation; the same convolution is involved in both. PUR^{Chl} preserves interspecific and intraspecific differences in the efficiency of Chl*a*-specific absorption: Note that PUR^{Chl} (Table 19.3) is substantially higher under each light regimen for the three small cells that have high a^{Chl} in Figure 19.11a than for their larger counterparts. In contrast, removal of pigment density and pigment package effects from PUR (Table 19.3) makes it easier to compare the relative absorption characteristics of cells with different pigment complements. For both measures, matching the light output of two different sources is a simple matter of taking the ratio of either PUR^{Chl} or PUR. For example, the culture of *A. anophagefferens*

TABLE 19.3 Light absorption and PUR. Data in the upper panel are calculated values of PUR^{Chl} (mol photons · [g Chl*a*]$^{-1}$ · s^{-1}) for the cells in Fig. 19.11a and the sources in Fig. 19.11c, all with an intensity of 2000 µmol photons · m^{-2} · s^{-1}, measured as PAR (400–700 nm). Data in the lower panel are the dimensionless spectral weighting coefficients, A_r, used to convert PAR to PUR (Equation 8) by reference to the flat spectrum E' in Fig. 19.11C.

PUR^{chl} (E_o and E_o') by source

Species	*Sunlight*	*Fluorescent*	*Quartz Halogen*	*White LED*	*Blue LED*	E_o'
A. anophagefferens	58.3	37.7	23.9	46.1	66.4	38.9
D. tertiolecta	21.0	14.0	12.4	16.5	23.4	15.6
P. provasolii	47.0	31.4	22.6	39.2	60.7	33.2
S. major	24.6	18.5	14.2	20.6	24.6	18.8
S. bacillaris	50.2	39.8	33.0	41.0	44.1	39.6
T. weissflogii	20.1	14.0	11.7	15.9	19.5	15.0

$A_r(E_o/E_o')$ by source

Species	*Sunlight*	*Fluorescent*	*Quartz Halogen*	*White LED*	*Blue LED*	E'
A. anophagefferens	1.50	0.97	0.61	1.18	1.71	1.00
D. tertiolecta	1.35	0.90	0.80	1.06	1.50	1.00
P. provasolii	1.42	0.95	0.68	1.18	1.83	1.00
S. major	1.31	0.98	0.76	1.10	1.31	1.00
S. bacillaris	1.27	1.01	0.83	1.04	1.12	1.00
T. weissflogii	1.34	0.93	0.78	1.06	1.30	1.00

shown in Figure 19.11a was grown under a fluorescent lamp at an irradiance of 80 µmol photons m^{-2} · s^{-1} PAR. If its photosynthetic rate were to be measured under a quartz halogen lamp, the equivalent flux of absorbed photons would be achieved at 127 µmol photons · m^{-2} · s^{-1} PAR (= 80 · [Fluorescent PUR^{Chl}/Quartz Halogen PUR^{Chl}] = 80 · [Fluorescent A_r/Quartz Halogen A_r]; see Table 19.3 for coefficients). For the culture of *S. bacillaris*, which was also grown at 80 µmol photons · m^{-2} · s^{-1} PAR under the fluorescent lamps, the equivalent flux of absorbed photons under the quartz halogen lights would be at 97 µmol photons · m^{-2} · s^{-1} PAR (Table 19.3).

Although this equivalence holds for total absorption, it does not hold strictly for absorption of light that is photosynthetically active. This is given by the photosynthetic cross-section, which describes the absorption by photosynthetic pigments alone, and which is equivalent to total absorption less the contributions of photoprotective pigments and pigmented non-photosynthetic material such as cell walls and other absorbing materials such as flavins and haemes (Sosik and Mitchell 1995, Suggett et al. 2003, 2004). In those taxa that have an approximately equal distribution of pigments between PSII and PSI, the efficiency of utilization for incident irradiance follows the absorption spectrum, which differs little in shape from the excitation spectrum for Chl*a* fluorescence. Those photons that are absorbed contribute approximately equally to photosynthesis and growth so the quantum yield of photosynthesis (the amount of carbon fixed per photon absorbed) is either independent of or only weakly dependent on wavelength. This relationship has been reported for representatives of several chromophytic groups, the dinoflagellates, diatoms, and prymnesiophytes (Schofield et al. 1990, 1996, Nielsen and Sakshaug 1993). Consequently when these cells are acclimated to different spectral regimens, the growth rates show a strong dependence on both irradiance and spectral quality when irradiance is expressed as PAR. However, there is only a very weak dependence of the quantum yield of growth (the achieved growth rate per photon absorbed) on spectral quality.

In contrast, the photosynthetic action spectrum cannot be approximated from the absorption spectrum

in those taxa that have phycobilins as accessory pigments, the cyanobacteria, cryptophytes, and rhodophytes (Haxo and Blinks 1960, Neori et al. 1986). In these taxa, the phycobilins are associated with PSII, whereas Chl*a* is predominantly associated with PSI (Myers et al. 1980, Bidigare et al. 1989). Consequently, light of a spectral quality that is absorbed by phycobilins but not Chl*a* or by Chl*a* but not phycobilins cannot stimulate both photosystems, so the quantum yield is dependent on wavelength. Comparison of both absorption spectra, which include the contributions of both PSII and PSI and fluorescence excitation spectra, which are specific to PSII, may allow irradiances of different spectral quality to be reconciled for the phycobilin-containing taxa (Lutz et al. 2001).

5.2. Photosynthesis-Irradiance Curves

Photosynthesis expressed as a function of irradiance (PE) is highly responsive to environmental conditions (see Fig. 19.3). Understanding this variability and the kinetics of its change in response to environmental perturbation (Post et al. 1985, Prézelin et al. 1986b, Cullen and Lewis 1988, MacIntyre and Geider 1996, Anning et al. 2000, 2001) is central both to interpretation of comparable measurements made in the field and to building and testing models of PE response and growth. Research extends from descriptions of regulation under nutrient-replete (Sukenik et al. 1990) or nutrient-limited growth (Laws and Bannister 1980, Herzig and Falkowski 1989, Greene et al. 1991) to descriptions of regulation under conditions designed to simulate natural variability in irradiance and nutrient availability during mixing (Kroon et al. 1992a, Smith et al. 1992, Kromkamp and Limbeek 1993, Ibelings et al. 1994). In all cases, an important objective is to develop an insight into regulation of photosynthesis and productivity in nature, based on an improved understanding of microalgal physiology.

Photosynthesis can be measured as rates of carbon accumulation or oxygen evolution. The two can be reconciled by applying the photosynthetic quotient, which is the molar ratio of O_2 evolved per CO_2 assimilated. Falkowski and Raven (1997) review variability in the stoichiometry of photosynthetic and respiratory quotients. The high sensitivity of the ^{14}C method compared to oxygen exchange has led to its widespread adoption in limnology and oceanography since its introduction by Steemann-Nielsen (1952) because microalgal biomass is typically low in most waters. The technique has been widely used in both the laboratory and the field and interpreting the acreage of data collected to date has been a driving force in the development of models of photosynthesis and growth (Bannister and Laws 1980, Geider et al. 1996, 1998, Flynn and Hipkin 1999). Numerous factors should be considered when analyzing PE data, whether comparing within or across studies.

1. When comparing photosynthesis and growth, unless the PE incubator and the growth chamber use the same kind of lights, light data collected with a broad-band light sensor (PAR) must be reconciled to account for the proportion that can be absorbed by the algae (PUR, see earlier discussion).
2. The apparent dynamics in PE response across growth conditions are driven in part by the way in which they are normalized to biomass (Anning et al. 2000, MacIntyre et al. 2002). Cell-, Chl*a*-, and carbon-specific responses behave in different ways because of systematic variability in Chl*a*:C (see Fig. 19.2) and the cellular quotas of Chl*a* and carbon (Thompson et al. 1991).
3. The carbon-uptake rate in a PE curve is time dependent (MacIntyre et al. 2000, 2002) so that the values of the fitted parameters depend on the duration of the incubation. The ^{14}C method usually estimates production as the difference between labeling at the beginning and end of an incubation. Because it integrates changing rates over its duration, it is relatively insensitive to fluctuations in the rate when compared with a method that monitors changes continuously (MacIntyre et al. 2002). Cumulative uptake can also yield misleading results when the objective is to describe inhibition of photosynthesis as a function of exposure (Neale 2000): uptake during the initial part of an incubation will be measured even if the cells are dead by the end of it.
4. If the duration of the incubation is short enough that labeled carbon is not lost by respiration, ^{14}C uptake should approximate gross photosynthesis (Williams 1993). The ^{14}C method itself cannot measure the respiration rate. There have been relatively few comparisons of ^{14}C uptake with net and gross photosynthesis, as measured by oxygen exchange (Williams et al. 1979, Grande et al. 1989b), but ^{14}C uptake approximates gross photosynthesis when incubations are short. It is closer to net photosynthesis in longer (24-hour) incubations (Eppley and Sharp 1975), because the labeled carbon is cycled internally and some is lost

by respiration. Short-term ^{14}C uptake normalized to cellular carbon is, therefore, likely to overestimate growth rate unless corrected for an estimate of respiratory loss. In practice, this is not as easy as it might appear. The respiration rate increases with irradiance (Fig. 19.12) as other anabolic pathways such as nitrogen assimilation increase in activity to balance carbon fixation (Turpin et al. 1988) and, at high irradiance, as oxygen-consuming energy-dissipating pathways such as the Mehler cycle are induced (Grande et al. 1989a, Kana 1993, Lewitus and Kana 1995). Correcting short-term ^{14}C uptake by an estimate of respiration in the dark, measured as O_2 consumption by an oxygen electrode, will tend to overestimate net productivity, and it still requires an estimate of the photosynthetic quotient to reconcile the rates.

5. The ^{14}C method either does or does not measure loss of dissolved organic carbon (DOC), depending on the protocol. Where uptake is measured by acidifying a whole water sample and adding scintillation cocktail directly to it, the method measures the sum of POC synthesis and any DOC loss, subject to the labeling pattern (Smith and Horner 1981). Where uptake is measured by filtering the sample after the incubation and counting ^{14}C retention on the filter, the method

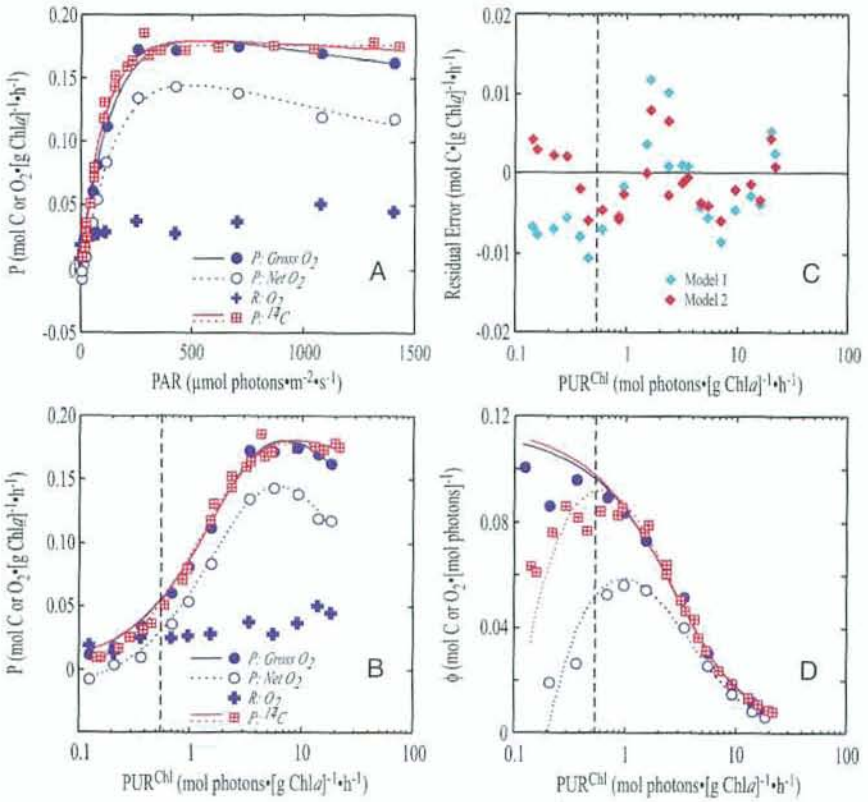

FIGURE 19.12. **(a)** Variation of gross and net photosynthesis and respiration as a function of irradiance (Todd Kana and MacIntyre, unpublished data). Rates were measured using an $^{18}O/^{16}O$ technique that measures both gross photosynthesis and respiration simultaneously with a membrane-inlet mass spectrometer (Kana 1990), subject to the limitations of the isotope dilution equations. Superimposed are data on ^{14}C uptake, measured in a photosynthetron (Lewis and Smith 1983). The fits are either a fit with two exponents (Platt et al. 1980) (Model 1, *solid lines*) or the same fit modified with an intercept term (Model 2, *dashed lines*). The light sources for both techniques differed. Irradiance is expressed as PAR. **(b)** The same data in which incident irradiance has been corrected for the spectral quality of the two light sources and recast as PUR^{Chl} (see text for details). Note the log scale to emphasize differences between the data and fits at low irradiance. The vertical dashed line is growth irradiance. **(c)** Irradiance-dependence of residual errors (observed—expected values) for the ^{14}C data, as fit by Models 1 and 2. Note the magnitude and divergence at and below growth irradiance (*the dashed line*). **(d)** Variation in quantum yields of photosynthesis with irradiance. Note the divergence between the prediction of Model 1 and the ^{14}C data.

measures only POC synthesis, although there is some retention of DOC on the filter (Maske and Garcia-Mendoza 1994). This will cause an underestimate of productivity if there is uniform labeling of pools (Smith and Horner 1981). Although DOC production is likely low under conditions of nutrient-replete growth (Sharp 1977), it may rise under nitrogen starvation (Otero and Vincenzini 2004). Excretion of DOC also increases at high irradiance (Mague et al. 1980, Joint and Pomroy 1981), where it appears to be an energy-dissipating mechanism (Wood and Van Valen 1990, Gordillo et al. 2001), particularly under low nutrient conditions, as cells dump carbon fixed in excess of their ability to synthesis proteins. In benthic diatoms and in many cyanobacteria, almost 50% of carbon fixed may be excreted as extracellular polymeric substance (EPS) (Alcoverro et al. 2000, Smith and Underwood 2000, Staats et al. 2000). Failing to account for this will bias the estimated relative assimilation rates of carbon and other elements.

6. The influence of the model (hyperbolic tangent, exponential, quadratic, etc.) used to fit the PE curve on the value of the parameters has been reviewed extensively (Jassby and Platt 1976, Platt et al. 1980, Zimmerman et al. 1987, Eilers and Peeters 1988, Frenette et al. 1993, MacIntyre et al. 2002). The consensus is that all models give similar estimates of P_m but differ in their estimates of α because of differences in their architecture. It is important, therefore, to be consistent in using a single model when comparing estimates of α or the saturating irradiance E_k ($= P_m/\alpha$) in cultures grown under different conditions. However, most models give accurate estimates of the rates (as indicated by the distribution of residual errors) over most of the irradiance range.
7. Because the cellular organic carbon being respired during short-term incubations is not labeled with isotope, the ^{14}C method tends to overestimate net photosynthesis at low irradiance (Steemann Nielsen 1953). This is illustrated in Figure 19.12, which shows gross photosynthesis, determined by the $^{18}O/^{16}O$ method (Kana 1990) and ^{14}C incorporation. The fits given by Model 1 (Platt et al. 1980) are almost identical for the O_2 and ^{14}C data (Table 19.4), although there is a pronounced divergence between the fits and the ^{14}C data at low irradiance, as the fit is forced through zero. As a result, the residual errors in low light are high (see Fig. 19.12c), and the rate of carbon fixation at

TABLE 19.4 Curve fit parameters for the PE data in Fig. 19.12.

$$\text{Model 1:} = P_s\left[1-\exp\left(\frac{-\alpha E}{P_s}\right)\right]\exp\left(\frac{-\beta E}{P_s}\right); \quad P_m = P_s\left(\frac{\alpha}{\alpha+\beta}\right)\left(\frac{\beta}{\alpha+\beta}\right)^{\beta/\alpha}$$

$$\text{Model 2:} = P_s\left[1-\exp\left(\frac{-\alpha E}{P_s}\right)\right]\exp\left(\frac{-\beta E}{P_s}\right)+P_0; \quad P_m = P_s\left(\frac{\alpha}{\alpha+\beta}\right)\left(\frac{\beta}{\alpha+\beta}\right)^{\beta/\alpha}-P_0$$

Data	P_m	P_s	α	β	P_0
Model 1					
Gross O_2	0.180	0.197 ± 0.008	0.113 ± 0.005	0.0021 ± 0.0007	—
^{14}C	0.180	0.186 ± 0.007	0.115 ± 0.006	0.0006 ± 0.0005	—
Model 2					
Net O_2	0.144	0.194 ± 0.008	0.104 ± 0.008	0.0039 ± 0.0009	0.021 ± 0.003
^{14}C	0.177	0.194 ± 0.005	0.144 ± 0.010	0.0003 ± 0.0003	0.015 ± 0.004

Data were fit to Model 1 (Platt et al. 1980) or Model 2. P_m is the light-saturated rated of photosynthesis; P_s is the light-saturated rate of photosynthesis that would be achieved if β were 0; P_0 is an intercept; α is the light-limited rate of photosynthesis; and β is a term describing the decrease in photosynthesis. P_m, P_s, and P_0 are in (mol O_2 or C · [g Chla] $^{-1}$ · hr^{-1}). α and β are in (mol O_2 or C · [g Chla]$^{-1}$ · hr $^{-1}$) (mol photons · [g Chla]$^{-1}$ · hr^{-1})$^{-1}$ = (mol O_2 or C · [mol photons]$^{-1}$). Note that no errors are given for P_m, as the fitting protocol that was used did not return the covariation matrix for the output parameters, information that is needed to calculate the error correctly (Davis 1986, Zimmerman et al. 1987).

growth irradiance is overestimated relative to the observed data. This might reflect an increase in the respiration rate (which cannot be measured by the ^{14}C technique) relative to gross photosynthesis at low irradiance or might simply be an artifact of the ^{14}C method. When the ^{14}C data are fitted with a model modified by inclusion of a non-zero intercept (Table 19.4), the residual error is decreased at all irradiances but particularly at low ones (including in this case the growth irradiance) (see Fig. 19.12c). This provides a better description of the observed ^{14}C uptake at low irradiance. Model 2 (Table 19.4) is clearly the appropriate one for fitting net photosynthesis, as measured by oxygen exchange (see Fig. 19.12), in which case the intercept term is the dark respiration rate. A conservative approach for short-term ^{14}C-uptake data is to force the fit through the origin (Sakshaug et al. 1997); it may be prudent to avoid making many measurements at low irradiance, where the ^{14}C method is prone to systematic error.

An alternative treatment of the PE relationship is to recast it in terms of quantum yield, ϕ (see Fig. 19.12d), which is the efficiency with which absorbed light is converted to carbon (mol C · [mol photons]$^{-1}$):

$$\phi = \frac{P_E}{E_a} = \frac{P_E}{PUR^{Chl}} = \frac{P_E}{E \cdot A_c \cdot a^{Chl}} \qquad (10)$$

Part of the impetus for doing so is the potential for using active fluorescence methods to predict productivity: F_v/F_m and F_v'/F_m' are the maximum and achieved quantum yields of Photosystem II activity (Genty et al. 1989, 1990). The relationship between ϕ and irradiance can be described in terms of the maximum value, ϕ_m, a theoretical limit reached where irradiance tends to zero, and the saturation irradiance, E_k. Where the PE curve is expressed in terms of PAR, ϕ_m is equivalent to the quantum yield in the region of the initial slope, α, when it is expressed in chlorophyll-specific units:

$$\phi_m = \frac{\alpha^{Chl}}{A_c \cdot a^{Chl}}, \qquad (11)$$

where a PE curve is expressed in terms of PUR^{Chl}, the ratio of P to PUR^{Chl} at any point is ϕ (compare Fig. 19.12b and 19.12d) and the initial slope of the curve fit is ϕ_m. Note that if the curve is fitted with P_0, ϕ is calculated as $[P\text{-}P_0]/PUR^{Chl}$. Because the photosynthetic rate at low irradiance is $[\alpha E\text{-}P_0]$, rather than $[\alpha E]$, ϕ_m cannot be calculated with Equation 11 and doing so can overestimate it by a large margin (see Table 19.4).

Attempting to model growth rate from PE data is also subject to uncertainties because of the temporal variability of the PE relationship. Both Chl*a*- and cell-specific P_m and α vary through the light period when cells are grown on a light–dark cycle (McCaull and Platt 1977, Prézelin and Sweeney 1977, Harding et al. 1982, Prézelin et al. 1986a). Any attempt to model growth of cells on a light–dark cycle must account for the periodicity in PE response and attempt to integrate it over the same period that growth measurements are made.

5.3. Ultraviolet Radiation

Much research has been conducted to assess the effects of UV radiation (UVR) on microalgae. Although there have been descriptions of important mechanisms (Vincent and Neale 2000), interactions (Neale et al. 1998b), and adaptations to minimize damage (Roy 2000), and although many different sensitivities of microalgae to UV have been demonstrated (Vernet 2000), many experimental studies of microalgae cultures using UVR are difficult or impossible to relate to each other or to nature. Vernet (2000) concluded that "in spite of a large body of studies available, we are far from understanding how microalgae respond to environmental UVR." Reasons for this included the following:

1. The use of unrealistic irradiances in laboratory studies (sometimes vastly exceeding natural exposures).
2. A lack of relevant hypotheses about the influence of UVR in natural environments, where exposures vary with time and where the competing processes of damage and repair to the essential (but not immutably linked) processes of photosynthesis, nutrient assimilation, and cell division must be characterized very carefully to make any sense of what occurs in the real world.

Many of the problems with intercomparison between UV experiments can be avoided through careful selection and measurement of irradiance sources (lamps) (Cullen and Neale 1994). The objective should be to ensure that the experimental exposure can be related *quantitatively* to conditions in nature. Further, the spectrum should not unnaturally target one physiological process over another (e.g., DNA damage vs. photosynthesis) unless that is an intentional part of the experimental design.

The concept of spectral weighting functions (biological weighting functions and action spectra) is centrally

important to UV research (Coohill 1989, Neale 2000). Simply put, radiant exposures cannot be compared in a biologically relevant way unless the relative effectiveness of different wavelengths is taken into account. This is also the case with PUR versus PAR (see previous discussion), but with UV the errors associated with using unweighted or incorrectly weighted irradiance can be much larger. The requirements for equipment are daunting, as is the complexity of quantitative analysis. Accurate measurement of UVR is extremely demanding and expensive; cheap spectrometers are incapable of excluding stray photons from their detectors when measuring rare but destructive UVB in the presence of natural visible radiation and so are inaccurate. (Manufacturers of optical instruments, such as scanning spectrophotometers, provide materials to explain the challenges of accurate radiometry.)

A comparison of different measures of UVR illustrates the complexity of matching experimental spectral irradiance with natural exposures (Table 19.5) (Cullen and Neale 1997). The table does not lend itself to rapid comprehension; neither does the quantitative comparison of experimental exposures to natural regimens of radiation. For any of the four measures in Table 19.5, values within a row represent relative exposure from the different sources: the UVB ($W \cdot m^{-2}$) from a bare sunlamp is nearly the same as surface conditions at 45° latitude; the solar simulator emits nearly twice the natural UVB. Comparison of the patterns between rows shows how important weightings are. When the spectra are weighted for DNA damage, the bare sunlamp is nearly threefold more damaging than surface irradiance at 45°, but when weighted for inhibition of photosynthesis, it is closer to the natural exposure rate. The implication is that a bare sunlamp would induce disproportionately high DNA damage as compared to nature, even though unweighted UVB ($W \cdot m^{-2}$) is about the same. In turn, the filtered sunlamp (a common treat-

TABLE 19.5 Different measures of UV radiation for three sources of irradiance, compared to three natural situations.

	Artificial Sources			*Natural Spectra*		
	Bare lamp	*Filtered lamp*	*Solar Simulator*	*45° Surface*	*60° S Ozone Hole*	*60° S Ozone Hole 5 m*
Measure of Damaging Irradiance						
UVB ($W \cdot m^{-2}$)	1.61	0.31	2.80	1.62	1.47	0.26
DNA_{300}	1.28	0.15	0.76	0.45	0.42	0.08
P. tricornutum	0.96	0.12	1.18	0.89	0.67	0.18
A. sanguinea (low light)	0.34	0.04	0.61	0.50	0.35	0.10
Effectiveness Ratio						
$(DNA/PHS)_{diatom}$	1.33	1.28	0.65	0.50	0.63	0.47
$(DNA/PHS)_{dinoflagellate(LL)}$	3.79	3.82	1.25	0.89	1.23	0.82

Sources: Spectra from two sunlamps (FS40 T12-UVB, Light Sources Inc., illuminated 200 hours before use), measured at 60 cm, are typical for laboratory studies on cultures. Data are from Cullen and Lesser (1991). Cellulose acetate film (0.13 mm thickness, aged 100 hours at 3 cm from the lamps) was used to attenuate shorter wavelengths that are not encountered in nature. The spectrum for a solar simulator (filtered through a Schott WG-295 long-pass filter) represents a more realistic simulation of solar radiation (Browman et al. 2003). The midday spectrum at 45° N is from a model; those at 60° S as influenced by the ozone hole (140 DU) are from measurements and a model for the Weddell Sea on a typically cloudy day (Cullen and Neale 1997).

Measures of damaging irradiance: Unweighted UV-B radiation (280–320 nm) is reported in $W \cdot m^{-2}$. DNA_{300} is weighted for the action spectrum of DNA damage in alfalfa seedlings (Quaite et al. 1992) and normalized to $1.0\,nm^{-1}$ at 300 nm. Photosynthesis-inhibiting radiation, E^* (Cullen et al. 1992a, Neale 2000) is calculated from biological weighting functions (BWFs) for the diatom, *Phaeodactylum tricornutum* (Cullen et al. 1992a) and the dinoflagellate, *Akashiwo sanguinea* (Hirasaka) Hansen et Moestrup (formerly *Gymnodinium sanguineum Hirasaka*), grown in low light (Neale et al. 1998a).

Effectiveness ratio: For each source of irradiance, the ratio of effectiveness for DNA damage (likely to affect growth rate), as compared to inhibition of photosynthesis, is calculated for both the diatom and the dinoflagellate. All effectiveness ratios are relative but comparable. For example, when compared to any of the natural spectra, each artificial source is more damaging to DNA then to photosynthesis, but the difference depends on the BWF for photosynthesis. Extrapolation of experimental results to nature requires explicit consideration of biologically weighted irradiance in both the laboratory and the field (after Cullen and Neale 1997).

ment) can induce only a fraction of near-surface inhibition of photosynthesis, closer to what would be encountered at a depth of several meters. If time is not invested in getting it right, results will not be comparable to nature. More fundamentally, if spectral irradiance is not measured, biologically weighted, and reported, results will not be comparable between studies or with nature.

Relative ratios of DNA damaging radiation to photosynthesis inhibiting radiation are reported for each spectrum in Table 19.5. The absolute values have no meaning because we have no basis for comparing DNA damage directly to inhibition of photosynthesis (but see Neale 2000), although a comparison of ratios within rows shows how unnatural some lamps can be. Generally, sunlamps are much more damaging to DNA than to photosynthesis, as compared to nature. The bias depends on the sensitivity of algal photosynthesis to UV, which depends in turn on species and preconditioning (Neale 2000). These calculations do not even consider the unnatural consequences of high ratios of UV : PAR common in many experiments (Vernet 2000) and the kinetics of photoinhibition versus DNA damage. Photoinhibition may rapidly reach a balance between damage and repair, so the reduction in photosynthesis is a function of irradiance (Cullen et al. 1992a), or it may not, so inhibition is a function of cumulative exposure (Neale et al. 1998c). In contrast, DNA damage is well described as a function of cumulative exposure on short time scales (Huot et al. 2000). Clearly, when embarking on studies of the physiological effects of UV, careful preparation is warranted.

6.0. FUTURE RESEARCH

Much research in microalgal ecology has been driven by the development of more rapid and more sensitive techniques for detecting biomass or some characteristic of the physiological responses of the organisms. Whether there is a corresponding advance in our understanding of the organisms' ecological role in their natural habitat is debatable. We argue that advances are most likely to come from hypothesis-driven research (Platt 1964) and that framing a suitable question is more important than collecting huge quantities of data whose interpretation is largely conjectural. That said, the development of new technologies does permit observation on temporal and spatial scales that were unachievable two decades ago. For instance, it is now possible to monitor a culture continuously using automated samplers and analyze nutrient concentrations (in filtered samples), cell number and particle size distributions, and spectral absorption in UV and visible by the cells (Bernard et al. 1996, Le Floc'h et al. 2002). Application of modeled relationships to these data then allows absorption at 678 nm to be corrected for pigment packaging, based on particle size distributions, to yield Chl*a* concentration (Le Floc'h et al. 2002), and for particle number and differential nutrient concentrations to be converted to cell nutrient quotas (Bernard et al. 2001), both with impressive accuracy. Techniques such as these are immensely powerful in determining physiological responses to the stochastic changes in nutrients or irradiance that are more representative of the natural environment than the stable conditions typically used in physiological studies. Such observations can and should be used to test and either refine or refute the predictions of first-principles models of physiological response to environmental change (Geider et al. 1998). The rapid development of autonomous moorings in oceanic, coastal, and estuarine waters provides a natural control for observations of natural populations themselves while describing the rate of change of the environmental characteristics to which the organisms respond (Sosik et al. 2003). A clearer understanding of the mechanisms that drive natural cycles of microalgal productivity will come largely from quantitative comparisons between natural assemblages and cultures. Although regulation of nutrient uptake, pigments, PE response, and growth appears to have common patterns across the taxa that have been studied, the coefficients that describe species-specific characteristics are few in number and span ranges of up to an order of magnitude (Raven and Johnston 1991, Smayda 1997, MacIntyre et al. 2002). Much basic descriptive work remains to be done.

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