6
Environmental Stress Physiology with Reference to Mass Cultures

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Abstract
When dealing with a photoautotrophic growth system, the biological process that drives growth and productivity is the ability of the cells via their photosynthetic apparatus to capture light and convert it efficiently to chemical energy. This chapter examines the basic principles of algal photosynthesis and growth physiology in order to provide the reader with the necessary background for a more comprehensive understanding of the effect of stress on algal photosynthesis and growth. Particular emphasis has been put on the light acclimation process of outdoor microalgae cultures and on the synergistic effect of light and other environmental stress such as low temperature, salinity, and high oxygen concentrations on algal photosynthesis and productivity. Chlorophyll fluorescence measurement has been suggested as a powerful tool to rapidly monitor the effect of stress on algal photosynthesis and growth. We strongly believe that the future of algal biotechnology highly depends on the ability to better understand its biological limitations and what needs to be overcome in order to significantly improve productivity.

Keywords photosynthesis; growth physiology; light acclimation; chlorophyll fluorescence; environmental stress; productivity

6.1 INTRODUCTION

Algal ecology, physiology, and biochemistry have been reviewed extensively over the decades. Contributions by Lewin (1962), Carr & Whitton (1973), Fogg (1975), and Falkowski & Raven (2007) are just a few examples of textbooks that cover a wide range of topics related to the subject of this work, which is mainly devoted to the physiological response of algae to environmental stress.

Response to stimuli or change in its environment is an inherent characteristic of any living organism. Changes in environmental conditions may thus be defined on the basis of the response that the cell undergoes as a result of the sensed change, either a limiting factor or a stress factor. For the sake of simplicity, we define a limiting factor as one that determines the rate of growth or biochemical reaction, and that a change in its level will result in a change in the rate without any requirement for an acclimation process. Stress will thus be defined as an environmental condition that results in a metabolic imbalance that requires biochemical and metabolic adjustments before a new steady state of growth can be established.

Handbook of Microalgal Culture: Applied Phycology and Biotechnology, Second Edition. Edited by Amos Richmond and Qiang Hu.

Schematically, the sequence of events associated with the response of living cells to an environmental change may be described as follows:

Steady state → Environmental change → Sensing mechanism → Response mechanism adaptation → New steady state

Outdoor algal cultures are exposed to a variety of changes in environmental conditions. These changes take place in two different timescales. One is the diurnal cycle that includes variation in light and temperature in a 24-h cycle. The other is a seasonal cycle that varies according to the climatic and geographical location of the particular habitat in which the algae are growing. In dense algal cultures used in algal biotechnology, a third cycle is imposed by mixing and culture depth (optical path length of reactor), which mainly results in a light–dark cycle which fluctuates in terms of fraction of seconds or minutes as compared to the hours or months in the other two cycles.

Microalgae have indeed developed diverse mechanisms for sensing and acclimating to changes in their environment (for reviews see Pfannschimdt et al., 2001; Li et al., 2009). Acclimation responses observed include the alteration of light-harvesting complex synthesis and degradation in response to changes in light quality and intensity. Such alterations are aimed to help balance efficiently the absorption of excitation energy and the production of reducing power (NADPH) and chemical energy (ATP) with their utilization for growth and reproduction. Inability to maintain this balance due to excess excitation of the photosynthetic reaction centers may result in the production of toxic reactive oxygen species (ROS) that may lead to photooxidative death. As implied, many of the stress responses and adaptive processes are associated with the photosynthetic apparatus. In recent years, it has become evident that photosynthesis itself contributes important signals to this light control of gene expression by means of changes in the reduction/oxidation state of signaling molecules which are induced by changes in quality and quantity of incident light. This provides a feedback-response loop in which the expression of photosynthesis genes is coupled to the function of the photosynthetic process and highlight its dual role in energy fixation and the reception of environmental information (Pfannschimdt, 2003). The great variety of these signaling mechanisms is summarized under the term "redox control." The concept of photosynthesis as a sensor for environmental information was originally introduced as the "grand design of photosynthesis" by Arnon (1982) and further extended by Anderson et al. (1995) and Huner et al. (1998).

6.2 LIGHT AND PHOTOSYNTHESIS RATE

6.2.1 *P* versus *I* curve

The light response curve (*P*/*I*) of microalgae has been used as a tool in analyzing the response of photosynthetically grown cells to the light environment and at the same time to analyze the response of the photosynthetic apparatus to environmental conditions. The *P*/*I* curve can be divided into three distinct regions: a light-limited region, in which photosynthesis increases with increasing irradiance; a light-saturated region, in which photosynthesis is independent of irradiance; and a photoinhibited region, in which photosynthesis decreases with further increase in irradiance. In addition, an intermediate region where there is either a gradual or abrupt transition from light-limited to light-saturated photosynthesis has been identified (Prioul & Chartier, 1977; Leverenz, 1987). A typical response of photosynthesis (CO_2 assimilation or O_2 evolution) to increasing irradiance is shown in Figure 6.1. At low irradiance, photosynthesis rates are linearly proportional to irradiance. In this region of the *P*/*I* curve, the rate of photon absorption

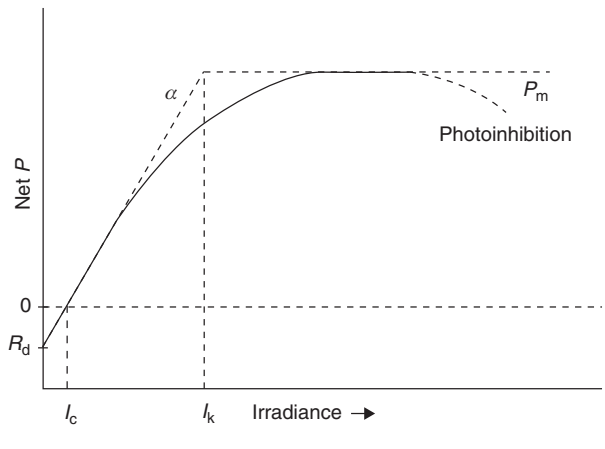

Figure 6.1. A schematic diagram of photosynthesis (*P*) versus irradiance (*I*) curve, showing the typical photosynthetic parameters. The light-saturated rate is denoted P_{max}. At low irradiance, photosynthesis rate is approximately a linear function of irradiance, and the ratio between photosynthesis and irradiance is often denoted by the symbol α. The saturation irradiance, I_k, is given as intercept between α and P_{max}. At irradiance above the optimum, photosynthesis rates usually shows a decline from the light-saturate value. Dark respiration is denoted by R_d. The compensation irradiance I_c, where no net oxygen evolution is observed.

determines the rate of steady-state electron transport from water to CO_2, and thus it is called the light-limited region. The initial slope of the *P*/*I* curve is usually denoted by the symbol α (Jassby & Platt, 1976). The slope can be normalized to chlorophyll (Chl) biomass α^B and the units are (O_2 evolved or CO_2 fixed per unit chlorophyll)/(quanta per unit area). When the initial slope is measured on a plant leaf or in a dense microalgal culture in which all the photosynthetically active radiation is absorbed, the slope can be taken as a direct measure of the maximum quantum yield of photosynthesis. However, in natural phytoplankton communities or optically thin cultures of microalgae, light absorption is a small fraction of the incident light and the initial slope may not be used as a direct measure of the quantum yield. Yet, the initial slope is proportional to the quantum yield and can be used to compare cultures in which the light absorption has not changed. One means of deriving the maximum quantum yield from α^B is to measure the spectral irradiance and the spectral-averaged optical absorption cross section normalized to Chl *a*, a^* (with units $m^2\ mg^{-1}$ Chl *a*). This normalization is convenient as it allows the calculation of the absorbed light from measurements of Chl *a* and incident spectral irradiance. From knowledge of $a*$ and α^B, the maximum quantum yield can thus be calculated:

$$\Phi_m = \alpha^B / a^* \tag{6.1}$$

For exhaustive information on the definition and calculation of the optical cross section in microalgae, see Dubinsky (1992), Kromkamp & Limbeek (1993), and Falkowski & Raven (2007).

At higher irradiances, the relationship between absorbed light and rate of photosynthesis is not linear anymore; consequently, the quantum efficiency decreases. Eventually, photosynthesis becomes light saturated and the photosynthesis rate reaches the maximum (P_{max}) and remains constant with increasing irradiance. By definition, the rate of photon absorption at light saturation exceeds the rate of steady-state electron transport from water to CO_2. In addition to these fundamental parameters, some other derived parameters are widely used. The compensation irradiance for photosynthesis, $I_c = R_d/\alpha$, that is, the light irradiance where oxygen evolved by photosynthesis is completely consumed by respiration, and hence no net oxygen evolution is observed. The saturation irradiance I_k, that is, the point on the *P*/*I* curve indicating the irradiance at which control of photosynthesis passes from light absorption and photochemical energy conversion to reductant utilization; it is a convenient indicator of photoacclimational status. I_k can be defined as $I_k = P_{max}/\alpha$. At irradiance less than I_k, electron transport capacity exceeds the rate at which photons are absorbed and delivered to PS II, and at irradiance values greater than I_k, the converse is true. In addition, the predominant fluorescence-quenching mechanism (see Chapter 2) at PFDs (photon flux densities) less than I_k is photochemical, that is, photosynthetic, whereas above I_k it is non-photochemical, involving thermal dissipation. I_k has the advantage of being independent of the units used for expressing photosynthesis (O_2 or CO_2, weight, area, cell, carbon, or Chl *a* basis). As a result, it is tempting to use I_k as a parameter for comparison of photoacclimation status both within and between the species and to assume that its inverse relation to α implies a similar relation to maximum photosynthesis efficiency, that is, ignoring the dependence of I_k on P_{max}. As pointed out by Henley (1993), I_k is often a better indicator of P_{max} than of α. In other words, a lower value of I_k does not imply necessarily a higher value of α (or quantum yield), but it could be simply due to a lower value of P_{max}. For example, low temperature typically affects P_{max} more than α; consequently, a low value of I_k indicates an inefficient use of high light intensities rather than an efficient use of low ones (Fig. 6.2).

The quantitative description of the light dependence of photosynthesis dates from Blackman's studies (1905) of limiting factors in plant productivity. Blackman considered the rate of plant production to be linearly dependent on the availability of a single limiting factor at low resource supply and independent of the availability of this factor above some threshold value. It was soon recognized that the transition between limiting and saturating resource availability may not be as abrupt as postulated by Blackman kinetics, and a number of formulations of the *P*/*I* curve were proposed by plant physiologists, oceanographers, and limnologists (Jassby & Platt, 1976). It has been frequently observed that some *P*/*I* data exhibit variability in the bending part of the curve (convexity), that is, the sharpness of the transition from PFD limitation to saturation. Leverenz et al. (1990) demonstrated in *Chlamydomonas reinhardtii* that the shape of the *P*/*I* curve depended on the degree of photoinhibition; the transition from light-limitation to light-saturation became less abrupt with increased photoinhibition. The convexity index θ (Leverenz et al., 1990), originally termed *M* by Prioul & Chartier (1977), ranges from 1.0, when the curve is of the Blackman type, to 0.0 when the response is a rectangular hyperbola (Fig. 6.3). The latter situation may occur with stressed microalgae (Leverenz et al., 1990). It is beyond the scope of this chapter to go into the pros and cons of different models for fitting *P*/*I* curves, which have already been discussed in detail elsewhere (Jassby & Platt, 1976; Eilers & Peeters, 1988). However, the general consensus is that the choice

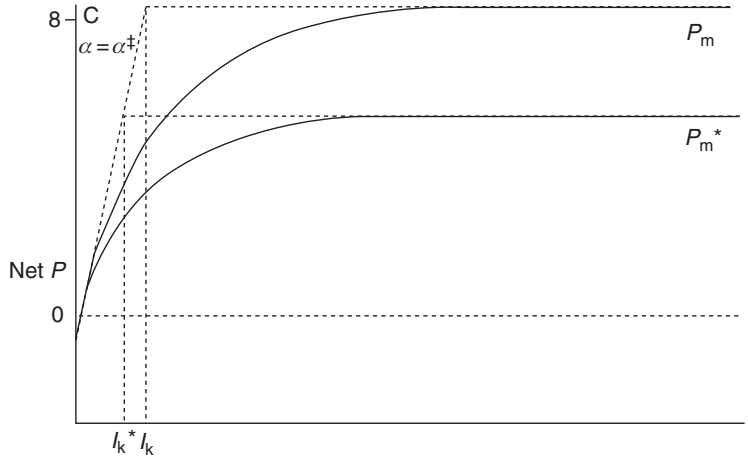

Figure 6.2. Light response curves having same α but different P_{max} and the resultant differences in I_k. This pattern may result from temperature changes.

of model has a profound effect on the estimate of the light-limited rate of photosynthesis, α, but most of those models return similar estimates of the light-saturated rate P_m. Therefore, where photoacclimation of the *P*/*I* response has been reported after growing cultures under a variety of conditions, an internal consistency in the parameters can still be expected (assuming that they where estimated using the same model), but this does not necessarily hold for comparisons based on different models and fitting protocols (see MacIntyre et al., 2002, for review). However, despite the uncertainties introduced by differences in methodology, the *P*/*I* curve is widely accepted as a useful relationship for studying the physiology of microalgae and cyanobacteria (Henley, 1993).

6.2.1.1 PS II quantum yield as a proxy measure of photosynthesis

Chlorophyll fluorescence measurement has become one of the most powerful and widely used techniques available to plant physiologists and ecophysiologists (Chapter 2), and in recent years its utilization has been extended to

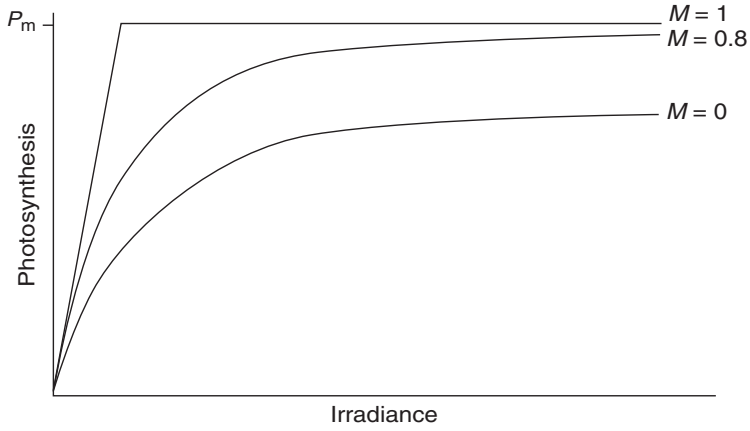

Figure 6.3. Graphical display of *P*/*I* curves with different rates of convexity (*M*). Blackman curve ($M = 1$); non-rectangular hyperbola ($M = 0.8$); rectangular hyperbola ($M = 0$). *P*, photosynthesis rate; *I*, incident irradiance.

mass culture as a tool for evaluating their physiological status (Torzillo et al., 1996, 1998; Kromkamp et al., 2009; Sukenik et al., 2009). One of the main attractions of chlorophyll fluorescence is that it can give a measure of photosynthesis (Maxwell & Johnson 2000; Baker, 2008). Indeed, the introduction of a simple-to-use fluorescence parameter for measuring the effective quantum yield of PS II ($F'_m - F_s/F'_m = \Delta F/F'_m$, Chapter 2) in the light by Genty et al. (1989), and subsequent observations that this parameter correlated well with quantum yields of other photosynthetic processes, for example, oxygen evolution and CO_2 fixation (Masojidek et al., 2001), have led to the widespread use of protocols for converting the quantum yield of PS II into actual rates of electron flow (Kromkamp & Forster, 2003). Electron transport rate (ETR) can be calculated using the following equation:

$$\mathrm{ETR} = \mathrm{PFD} \times a^* \times 0.5 \times \Delta F/F'_m \times (\mu\mathrm{mol\ e^-mg\ Chl^{-1}\ s^{-1}})$$

where PFD is the photon flux density (μmol photons m^{-2} s^{-1}), a^* is the optical cross section (m^2 g^{-1} Chl, see Section 6.2.1), $\Delta F/F'_m$ is the effective quantum yields of PS II (dimensionless), 0.5 as it is assumed that 50% of the absorbed light is delivered to PS II and 50% to PS I (i.e., two photons are required to move one electron through the photosynthetic chain) in order to achieve a balanced excitation pressure on both photosystems (Gilbert et al., 2000), and that no cyclic electron transport by PS I is occurring. However, in higher plants, and presumably in green algae, the ratio between PS I and PS II cross sections may be close to one (Boichenko, 1998); the ratios in other algal groups may deviate significantly. Moreover, optical cross section a^* can change during the day with irradiance (Dubinsky et al., 1995). Therefore, measure of optical cross section is suggested for correct comparison of light response curve parameters gathered through fluorescence measurements in cultures grown under different environmental conditions (Kromkamp et al., 2009).

6.2.2 Photoacclimation

As mentioned previously, the rapid changes in ambient light quantity and quality to which microalgae are exposed resulted in the development of a remarkable capacity for photoacclimation. The timescale according to which the cells have to respond to those changes varies from seconds to days. As a result, algal cells have developed multiple response mechanisms enabling them to deal with excess of energy. The state transition and non-photochemical mechanisms (see Chapter 2) operate to adjust the amount of light energy delivered to PS II on a timescale of seconds to minutes. Other processes reflecting changes in light quality or intensity may take longer and require structural and biochemical modifications. The long-term acclimation to irradiance is referred to as photoacclimation. This process involves changes at optical, biophysical, biochemical, ultrastructural, physiological, and molecular levels (Falkowski, 1980; Falkowski & La Roche, 1991; Fisher et al., 1998; MacIntyre et al., 2002). A common trend characterizes the mechanism of photoacclimation, that is, an increase in Chl *a*, and in other light-harvesting pigments, as growth irradiance decreases. The increase in pigment content during acclimation to low light results in a decrease in the optical cross section a^* (m^2 mg^{-1} Chl *a*), thus reducing the gain in light harvesting. Indeed, in a microalgal culture, a doubling of cellular chlorophyll does not bring about a doubling in the rate of light absorption (Dubinsky et al., 1995). Conversely, cells acclimated to high irradiance generally show relatively high carotenoid content relative to Chl *a*. Under stress conditions, some carotenoids such as β-carotene in *Dunaliella salina* and astaxanthin in *Haematococcus pluvialis* accumulate in globules outside of chloroplast do not transfer excitation energy to reaction center and act as a screen to protect photosynthetic reaction center from excessive excitation, while xanthophyll cycle pigments, in particular, zeaxanthin, under high light can reduce excitation of PS II reaction centers by dissipating excess of excitation energy via non-photochemical quenching (NPQ) (see Chapter 2). Because these carotenoids absorb light without a concomitant increase in the energy transfer to reaction center, organisms acclimated to high light often show lower maximum quantum yields.

The length of time required to accomplish the changes in pigmentation varies from species to species, from hours to several days. The rapid decrease in cellular chlorophyll after transition from low light (LL) to high light (HL) is assisted by the dilution of the pigment in the course of cell division and does not result from active pigment destruction (Berner et al., 1989). In contrast, the slower photoacclimation process during the transition from HL to LL is because dilution of pigments as a result of cell division acts in the opposite sense to the direction of photoacclimation, which is directed to increase cellular pigmentation (Fisher et al., 1996). Therefore, cells that are transferred from HL to LL reach the steady state in cellular chlorophyll considerably later than those photoacclimating in the opposite sense.

Prezelin & Sweeney (1979), Falkowski & Owens (1980), Perry et al. (1981), and Ley & Mauzerall (1982) have suggested that phytoplankton responds to decreased light intensities by increasing either the size or the number of

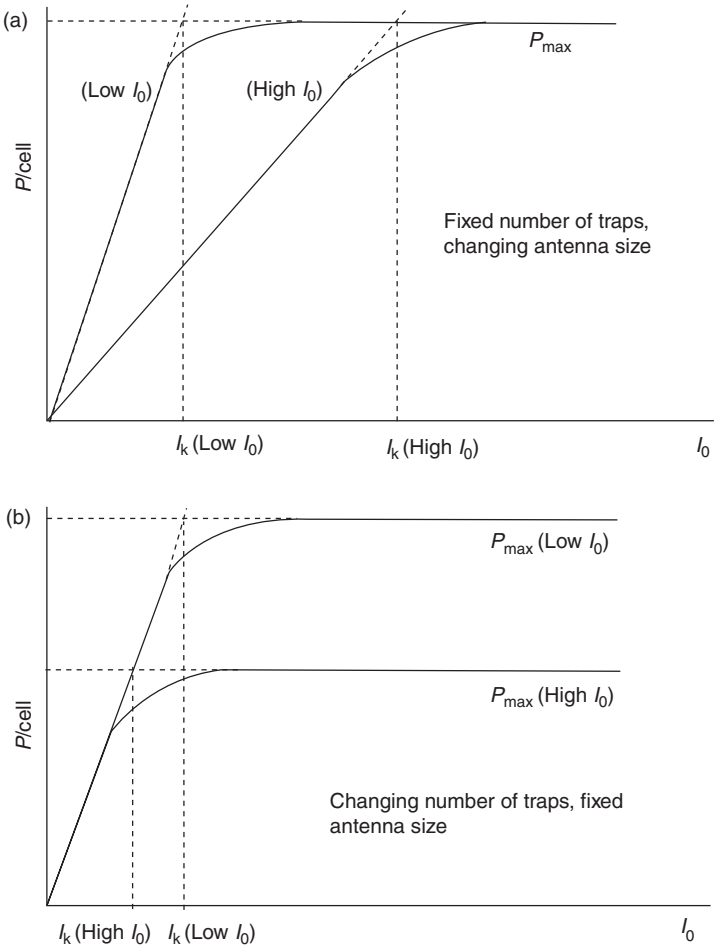

Figure 6.4. Model of saturation curves. (a) Model of adjustment to low and high light irradiances by changing the size of a fixed number of PSUs. (b) Model of adjustment of the photosynthesis unit to low and high light by changing the number of PSUs and not their size. From Ramus (1981), with permission from John Wiley & Sons.

photosynthetic units (PSU)[1] within a cell. Prezelin & Sweeney (1979) have suggested that the two strategies of light–shade adaptation could be distinguished on the basis of the characteristics of photosynthesis–irradiance curves. A similar proposal has been also made for macroalgae (Ramus, 1981). The rationale for this proposal is that an increase in the size of PSUs should result in compensation for low light by providing a constant amount of photons to reaction centers. In such a case, the maximum rate of cellular photosynthesis would remain constant under decreased light (Fig. 6.4a); less light will be required to saturate photosynthesis and PSUs will become more efficient. While an increase in the numbers of PSUs per cell will result in an increased maximum photosynthesis rate, more light will be required to saturate photosynthesis and hence a higher

[1]The concept of photosynthetic units, originally proposed by Emerson and Arnold in the 1930s, was based on kinetic measurements of oxygen production. A photosynthetic unit has been operationally defined as the number of pigment molecules involved in the evolution of one molecule of O_2, or the reduction of one molecule of CO_2, when the chloroplast pigments are excited by one flash of light so short that the components involved in the process will not function twice during their lifetime and so strong that a further increase in flash intensity does not lead to an increase in the measured value (Falkowski, 1980). Schmid & Gaffron (1968) estimated from oxygen flash yield studies that the number of chlorophyll molecules per PSU ranges between 1800 and 2500.

value of I_k (Fig. 6.4b). Both the photoadaptation strategies have been observed in all algal classes studied so far. For example, it was found that *Dunaliella tertiolecta* (Chlorophyte) and *Nannochloropsis* (Eustigmatophyte) adapt to low light by increasing the number of PSUs (Falkowski & Owens, 1980; Fisher et al., 1996, 1998), while *Chlorella pyrenoidosa*, *C. reinhardtii* (Chlorophyte), and *Glenodimium* (Dinoflagellate) adapt to low light by increasing the PSU size (Meyers & Graham, 1971; Prezelin, 1976; Neale & Melis, 1986).

In cyanobacteria, the antenna system for PS I is totally different from that for PS II (see Chapter 2). The light-harvesting antenna serving PS I is exclusively constituted of Chl *a* while the one serving PS II consists mainly of phycobilisome. PS II, which is excited by phycobilisome, has a relatively larger optical absorption cross section compared to PS I which is excited by Chl *a*. To balance the electron flow between PS II and PS I, cyanobacteria generally have more PS I reaction centers relative to PS II. This ratio can be altered both by light intensity and by spectral distribution of irradiance. Cyanobacterial cells grown under high irradiance have a lower PS I/PS II ratio and lower phycobilisome content compared to cells grown under low light (Kawamura et al., 1979; Murakami & Fujita, 1991; Hihara et al., 1998; Sonoike et al., 2001). This change is considered as a compensation for PS II antenna size because the size of the phycobilisome is preferentially reduced under high light conditions. Campbell & Öquist (1996), using chlorophyll fluorescence, found that the light intensity to which the cells are acclimated could be predicted by the light response curve of the NPQ parameter. In a wide range of cyanobacteria with different pigment contents, morphologies, and light histories, NPQ resulted minimally near the PFD in which the cells were grown. In cyanobacteria, like in green plants, the intersystem redox state appears to direct the transcription of PS II and PS I reaction centers (Fujita et al., 1988). The state transitions can thus be viewed as a short-term response to redox state of the plastoquinol pool, and photoacclimation can be viewed as a long-term response to the same control mechanism. The redox state of plastoquinone pool acts as a biological light meter that can signal the status of intersystem electron traffic and affect feedback responses in the light-harvesting systems (Escoubas et al., 1995; Huner et al., 1998).

In microalgae, the xanthophyll cycle-related dissipation of excessively absorbed energy is of major importance for the prevention of photoinhibitory damage to PS II. De-epoxidation of violaxanthin (Vx) to zeaxanthin (Zx) leads to enhanced dissipation of excess excitation energy in the PS II antenna system, thereby preventing inactivation and damage to the photosynthetic apparatus. Excess excitation energy is dissipated as heat, a process that can be registered as NPQ of Chl *a* fluorescence (see Chapter 2). In vascular plants and green algae, enhanced thermal dissipation requires the presence of both a trans-thylakoidal proton gradient and high concentrations of Zx (Horton & Ruban, 1992). Recent studies have shown that the PS II PsbS protein is an essential prerequisite of NPQ in higher plants (Li et al., 2000). It is beyond the scope of this chapter to delve into the different models proposed to explain the mechanism of antenna energy dissipation; however, there seems to be a general consensus that NPQ is connected to a structural change of the PS II light-harvesting complex (LHC II) (Horton et al., 2005, 2008).

Unlike in higher plants, the role of the xanthophyll cycle at least in some microalgal cultures (*Chlorella*, *Scenedesmus*, *Haematococcus*, *Chlorococcum*, *Spongiochloris*) resulted ambiguous, and its contribution to energy dissipation significantly varied among species (Masojidek et al., 1999, 2004).

The acclimation to high light of *Phaeodactylum tricornutum* cultures grown in outdoors in both open ponds and tubular photobioreactors (PBRs) was studied by means of chlorophyll fluorescence, pigment analysis, and growth (Torzillo et al., 2012). Diluted cultures grown in PBRs experienced the highest light stress which resulted in dramatic changes in both chlorophyll fluorescence and photosynthesis parameters. These changes were accompanied by a higher induction of the diadinoxanthin cycle pigments evidenced by increasing ratio between diatoxanthin and diadinoxanthin during the day, and a higher value of NPQ.

In contrast to NPQ in higher plants, microalgae, and diatoms (Niyogi, 1999; Ruban et al., 2004; Goss & Jakob, 2010), in the case of cyanobacteria, it has always been assumed to not use an antenna-related quenching mechanism to decrease the amount of energy funneled to RCII (Campbell et al., 1998). Recently, however, evidence has been presented for the existence of at least three distinct mechanisms for dissipating excess energy in cyanobacteria. One of these photoprotective mechanisms is related to the phycobilisomes (PBS), the extramembranal antenna of cyanobacteria PS II. In this photoprotective mechanism, the soluble orange carotenoid-binding protein (OCP) in *Synechocystis* sp. PCC 6803 plays an essential role (Wilson et al., 2006; Kirilovsky, 2007). However, mechanism of this novel NPQ photoprotective process in cyanobacteria awaits elucidation.

Understanding the mechanism of how excess solar energy is dissipated by the photosynthetic apparatus under high-irradiance stress is a major problem, not only for

researchers studying fundamental aspects of photosynthesis but also for applied photosynthesis research. Indeed, the optimization of growth conditions in outdoor cultures for mass production necessarily requires knowledge of the fate of light energy within the photosynthetic apparatus. For example, diluted outdoor cultures of microalgae may utilize less than 40% of the light they absorb at peak irradiance in summer, the rest must be dissipated as heat (Chapter 2; Cuaresma et al., 2009, 2011). As a guideline for algal biotechnologists, the optical path length of bioreactor, algal concentration, and mixing rate, particularly under outdoor conditions, should be optimized in order to avoid acclimation of the cells to low light on one hand, and the induction of photoprotective mechanisms during the exposure of the cells to potential harmful light intensities on the other. According to Masojidek et al. (2010), a midday depression of maximum PS II photochemical yield (F_v/F_m) of 20–25% compared with morning values in microalgae cultures is still compatible with well-performing cultures. Lower or higher depression of F_v/F_m can be taken as an indication of low-light acclimated or photoinhibited cultures, respectively.

6.2.3 Photoinhibition

6.2.3.1 Basic principles

Photoinhibition of photosynthesis is defined as a light-induced depression of photosynthesis that is manifested as a decrease in the maximum quantum yield of photosynthesis, a decrease in the convexity of the photosynthetic light response curve and, in the case of prolonged exposure to excessive light, a decrease in the rate of light-saturated photosynthesis (Leverenz et al., 1990; Long et al., 1994). The term photoinhibition has also been used to mean damage to PS II and the term photoprotection to mean changes assumed to protect PS II against damage (Demmig-Adams & Adams, 1992). Photoprotection probably reflects a way of adjusting the rate of dissipation of absorbed radiation energy so that the excitation energy density in the PS II antenna is sufficient to drive photosynthesis at a rate that meets the demands of assimilatory reactions. This would reduce the possibility of overexcitation of the PS II reaction center. However, a clear distinction between damage and photoprotection is not always possible (Demmig-Adams & Adams, 1992; Ögren & Evans, 1992). The possibility of overexcitation of PS II increases when photosynthetic organisms are grown under suboptimal conditions; this would reduce the photosynthetic rate and lead to an increase in the dissipation of absorbed energy through non-radiative processes. Since the capacity for photoprotection is limited, certain conditions can lead to damage and loss of active PS II reaction centers. However, recent studies have shown that neither interruption of the photosynthetic fixation of CO_2 nor a variety of environmental stress accelerates photodamage to PS II, which are thought to inhibit the repair of photodamaged PS II (Nishiyama et al., 2006; Murata et al., 2007; Takahashi & Murata, 2008). Their findings suggest that an excess of light energy absorbed by antenna pigments accelerates photoinhibition through suppression of the repair cycle of PS II.

It is thought that the light-dependent damage (photodamage) to PS II occurs under light of any intensity and can be considered unavoidable in photosynthetic organisms (Anderson & Chow, 2002). Photosynthetic organisms are able, however, to overcome photodamage by the rapid and efficient repair of PS II under physiological conditions. The repair process requires protein synthesis. In the "classical" interpretation scheme of the molecular mechanism of photoinhibition, photodamage was interpreted as the result of a direct attack of ROS to the photochemical reaction center of PS II. Production of ROS is the result of either reduction of Q_A, the primary electron acceptor of PS II (Vass et al., 1992), or by charge recombination between the acceptor side and the donor side of PS II (Keren et al., 1997). The resultant ROS then attack the photochemical reaction center of PS II directly. However, recent studies in higher plants and in the cyanobacterium *Synechocystis* have indicated that photodamage is initiated by the direct effect of light on the oxygen-evolving complex and that ROS inhibit the repair of photodamaged PS II by primarily suppressing the synthesis of proteins *de novo* (Murata et al., 2007).

Environmental conditions that reduce carbon metabolism, such as chilling and freezing temperatures (Long et al., 1983; Torzillo et al., 1996; Vonshak et al., 2001), high temperature (Bongi & Long, 1987), and nitrogen deficiency (Herzig & Falkowski, 1989), strongly increase the susceptibility to photoinhibition. Recent studies have attributed such enhanced photoinhibition to the synergistic effects of the light-dependent damage and the inhibition of repair by environmental factors (e.g., salt or low-temperature stress), and they have demonstrated that the synthesis of protein *de novo* was markedly affected under stress conditions (Nishiyama et al., 2005). Inhibition of CO_2 fixation in *C. reinhardtii* failed to accelerate photodamage to PS II but did inhibit the repair of damaged PS II (Takahashi & Murata, 2005). The increased level of PS II photoinhibition was attributed to inhibition of the synthesis of PS II proteins, in particular, the D1 protein at the translation step (Takahashi & Murata, 2008). It is believed that limitation in the CO_2 fixation lead to a decrease in

the utilization of NADPH, and a consequent decline of its oxided form $NADP^+$, which is a major acceptor of electrons in PSI, and its depletion can increase the electron transport from PSI to molecular oxygen with the generation of H_2O_2, when water–water cycle cannot cope with the increased amount of H_2O_2 to be scavenged (Takahashi & Murata, 2008). Therefore, consistent with this hypothesis, factors that limit fixation of CO_2 in the Calvin cycle, such as high-temperature stress, low-temperature stress, and salt stress, all accelerate photoinhibition by inhibiting the repair of photodamaged PS II.

Studies of photoinhibition on aquatic plants have been reviewed by Neale (1987) and Long et al. (1994). Hereafter, we will analyze how and to what extent the *P*/*I* curve can be modified by photoinhibition and the consequences of photoinhibition to microalgal productivity outdoors. Initially, photosynthesis increases linearly with irradiance and the maximum quantum yield is determined from the initial slope of the curve. The shaded area in Figure 6.5a above the light response curve indicates the amount of absorbed light that is not used for photosynthesis; while the dashed line in Figure 6.5b represents a theoretical Blackman response curve (see previous section), where photosynthesis operates at the maximum quantum yield efficiency until light saturation occurs and photosynthesis is limited by the dark reactions. The shaded area enclosed by the Blackman curve and the effective light response curve of photosynthesis (solid line) indicates the absorbed light energy that could potentially be used for photosynthesis, but is lost due to intrinsic characteristics and regulatory processes of the photosynthetic apparatus (Baker, 1996). In this region of the *P*/*I* curve, changes occur within the thylakoids that result in a smaller proportion of absorbed light being used for photochemistry due to an increase in the rate at which energy absorbed by the antennae of PS II is dissipated as heat. This light-induced quenching of excitation energy is thought to occur in the light-harvesting antennae of PS II and is associated with the decrease in the pH of thylakoids lumen during the formation of the thylakoid ΔpH and the consequent conversion of the carotenoid violaxanthin to zeaxanthin via the xanthophyll cycle (Demmig-Adams & Adams, 1992). In Figure 6.5c, the effect of severe photoinhibition on the light response curve (lower solid line) is shown. The shaded area between the normal and photoinhibited curves represents the absorbed light energy lost to photosynthesis due to photoinhibition of the culture. In this case, decreases in the maximum quantum yield, in the convexity, and in the maximum photosynthesis rate (P_{max}) are accentuated. This situation can occur when microalgal cultures are exposed to strong light (some ten times more than the growth irradiance) or to a combination of high light and other stress (e.g., low temperature). This behavior has been found, for example, in *Arthrospira platensis* cultures following exposure to high irradiance (Torzillo & Vonshak, 1994). However, in some cases, the situation is not so clear and the term photoinhibition has been frequently interpreted in different ways. Confusion has been generated by the means used to estimate photoinhibition. Decrease in the maximum

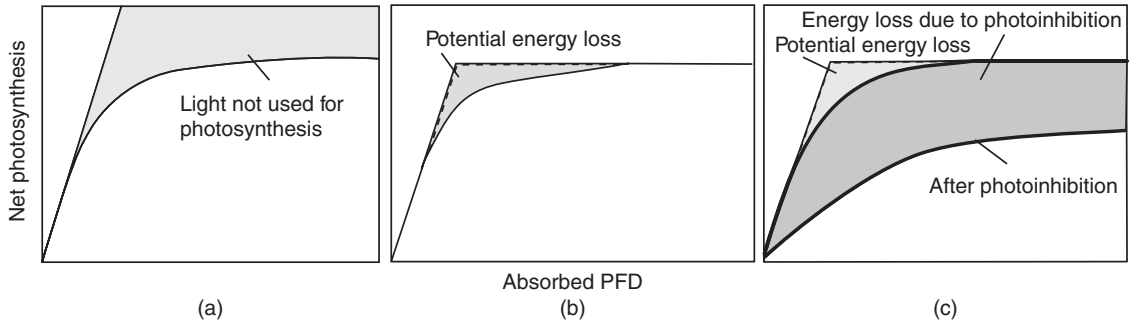

Figure 6.5. (a) Typical photosynthesis response curve to increasing irradiance. The shaded area above the curve and the extrapolated initial slope show the amount of absorbed light that is not used for photosynthesis; (b) the shaded area enclosed between the Blackman curve and the effective light response curve indicates the absorbed light energy that could potentially be used for photosynthesis, but is dissipated through non-photochemical quenching (heat) as a result of regulatory processes which are intrinsic of the photosynthetic apparatus; (c) the effect on *P*/*I* curve of severe photoinhibition which results in a decrease in both the quantum yield and P_{max}. The shaded area under the normal *P*/*I* and the photoinhibited curves indicate the absorbed light energy lost to photosynthesis due to photoinhibition. Modified from Baker (1996), with permission of Springer.

quantum yield and in the convexity of the *P*/*I* curve has been observed to precede decrease in P_{max}, and a significant decrease in quantum yield may occur without any decrease in P_{max}. Probably, the original definition of photoinhibition given by Kok (1956), that is, a light-dependent reduction in photosynthetic efficiency, may still provide the most useful working definition of photoinhibition of photosynthesis for leaves and whole organisms.

For both microalgae and terrestrial plants, photodamage to PS II reaction centers can be detected with high sensitivity from changes in variable chlorophyll fluorescence (Björkman, 1987; Neale, 1987; Long et al., 1994; Maxwell & Johnson, 2000; Baker, 2008). The F_v/F_m ratio (variable to maximum fluorescence) is a convenient measure of the potential maximal quantum yield of PS II, and it has been assumed as an index of photoinhibition (Björkman & Demmig, 1987). Decrease in the F_v/F_m ratio has been found highly correlated to reduction in the quantum yield of oxygen evolution or CO_2 uptake (Genty et al., 1989). In the field, F_v/F_m frequently exhibits diurnal depression that are roughly symmetric to light intensity and are mirrored by corresponding changes in the quantum yield of photosynthesis (Neale, 1987; Long et al., 1994; Torzillo et al., 1996). However, in aquatic systems as well as in some microalgal cultures, short-term photosynthesis measurements may often indicate maximum light-saturated photosynthesis rates (P_{max}) at noontime, that is, in correspondence to the lowest value of F_v/F_m and quantum yield. This paradoxical co-occurrence of midday maxima in both photoinhibition and photosynthesis appears inconsistent with the debilitating effect of PS II damage as evidenced by the decrease in F_v/F_m and quantum yield of photosynthesis. An explanation of this phenomenon has been furnished by Behrenfeld et al. (1998). The effect of photoinhibition depends upon which step in the photosynthetic electron transport chain is rate limiting at a given incident irradiance. Photosynthetic activity at sub-saturating light irradiance is rate limited by light absorption, and excitation energy transfer to PS II reaction centers is a near-linear function of irradiance. Conversely, at saturating light intensities, photosynthesis is limited on the acceptor side of PS II, generally by the capacity of enzymatic processes in the Calvin cycle (Sukenik et al., 1987), which in effect restricts electron turnover through PS II. Behrenfeld et al. (1998) have demonstrated in *Thalassiosira weissflogii*, adapted to low light intensity, that changes in carbon fixation are not observed until rate limitation is shifted from the Calvin cycle reactions to electron transport through PS II. In *T. weissflogii*, changes in P_{max} were not observed until the reduction of active reaction centers had reached 50% of initial. These findings demonstrate that photoinhibition leads indeed, first of all, to a reduction in the quantum yield of photosynthesis which is mirrored in a decline of the F_v/F_m ratio, nevertheless, the overall electron transport (P_{max}) can remain virtually unaltered despite substantial PS II photodamage (Kok, 1956; Leverenz et al., 1990; Behrenfeld et al., 1998; Vonshak et al., 2001). What then could be the expected consequences of these findings to microalgal biomass yield?

It must be pointed out that algal productivity depends primarily on light energy conversion efficiency, that is, the absorption and utilization of light by the photosynthetic apparatus to assimilate CO_2 into dry matter. Dense algal cultures are therefore predominantly grown at light limitation and, consequently, their photosynthetic performance would be more dependent on the initial slope rather than on the light-saturated portion of the *P*/*I* curve. Hence, a reduction in the F_v/F_m ratio due to excessive light absorption in the top layers of an outdoor algal culture will result in a decrease in the biomass yield (Masojidek et al., 2010).

6.2.4 Photoinhibition in outdoor cultures

Traditionally, light has been referred to as the main limiting factor in mass culturing of microalgae (Burlew, 1953; Richmond & Vonshak, 1978). This assumption was based on the observation that outdoor algal cultures are kept in a dense suspension where light penetrates only through a small fraction of the culture. Also, for practical reasons, it was obvious that none of the mixing devices used in such systems could be able to induce a light–dark cycle to overlap with the timescale of the flashing light effect demonstrated in the laboratory. The first to demonstrate that dense *A. platensis* cultures grown outdoors are undergoing a photoinhibitory process were Vonshak & Guy (1992). They clearly demonstrated that by shading the cultures and preventing them from full exposure to solar radiation, a higher productivity could be maintained.

Later on, by the use of *in situ* chlorophyll fluorescence technique it was demonstrated that in dense *Arthrospira* cultures grown under optimal conditions, exposure to full sunlight results in a midday decrease in F_v/F_m (i.e., the maximum quantum yield achievable) of approximately 10% of the early morning value; the depressing effect of high light increases to 20% when the $\Delta F/F'_m$ (i.e., the effective quantum yield of PS II) is measured (Torzillo et al., 1996). These and other results indicate that a reduction in the efficiency of photosynthesis in the middle of the day cannot be avoided even with ultra dense cultures grown at the optimum biomass concentration (Hu et al., 1996).

This kind of photoinhibition has been called diurnal photoinhibition (Ögren & Evans, 1992) in which recovery of F_v/F_m is usually completed by dusk. In a later study, Lu & Vonshak (1999) studied the response of outdoor cultures of *A. platensis* using the polyphasic rise of chlorophyll fluorescence transients, which provide information on the primary photochemistry of PS II. The maximum efficiency of PS II photochemistry (F_v/F_m) declined in response to daily increasing irradiance and recovered as daily irradiance decreased. The greatest inhibition (15%) in F_v/F_m was observed at 12:00 which responded to the highest irradiance. The daily change in the concentration of PS II reaction centers followed the same pattern as F_v/F_m. However, no significant changes in the probability of electron transport beyond Q_A (Ψ_o) were observed during the day. The results suggest that the decrease in F_v/F_m induced by photoinhibition in outdoor *Arthrospira* cultures was a result of an inactivation of PS II reaction centers.

6.2.4.1 Effect of suboptimal temperature on photoinhibition and culture productivity

Even photon fluxes equivalent to about half of full sunlight frequently produce a persistent photoinhibition when applied in controlled environments in combination with other environmental stress limiting the C-metabolism, that is, low temperature and/or high oxygen concentration in the cultures.

Algal cultures grown outdoors are usually exposed to a combination of environmental stress. The most common combination is high light and low temperature. It happens while fluctuations in light intensity occur in a range of 1–2 h, the increase of temperature is a much slower process and takes about 4–5 h. This kind of de-synchronization between the two most important environmental factors, which affect photosynthesis and growth of outdoor algal cultures, results in a unique stress condition under which photoinhibition may indeed be induced at relatively low light intensity due to the suboptimal temperature conditions (Vonshak et al., 2001).

Acclimation to low temperature of two *A. platensis* strains, M2 and Kenya, was studied by Vonshak & Novoplansky (2008). Both strains showed similar growth rates when grown at 30°C, which can be considered optimal for this cyanobacterium, but once acclimated to low temperature, the strain M2 showed greater decline in growth, suggesting that the Kenya strain acclimated better to low temperature by downregulating its photosynthetic activity through (i) decreasing antenna size; (ii) decreasing the reaction center density; and (iii) increasing the energy dissipation. In this study, it was pointed out the importance of screening and selecting strains for mass culture on the basis of their ability to withstand and acclimate to low temperature. Indeed, this parameter can fluctuate in open ponds from 15°C in the morning hours to 35°C in the middle of day, causing a significant reduction in productivity and changes of the biomass composition (Torzillo & Vonshak, 1994; Vonshak et al., 2001).

The effect of low temperature on photosynthesis and growth of outdoor cultures of *Arthrospira* in tubular reactors has been investigated using saturating pulse fluorescence (Torzillo et al., 1996). Diurnal changes in maximum photochemical quantum yield of PS II of dark-adapted cultures, F_v/F_m ratio, or in the effective photochemical quantum yield of PS II, $\Delta F/F'_m$ ratio, were measured under steady-state photosynthesis in cultures grown at 25°C (i.e., 10°C below the optimum). A reduction of 30% in the F_v/F_m ratio was found in the middle of the day in the culture grown at 25°C (Fig. 6.6); at the same time of the day $\Delta F/F'_m$ decreased up to 52% in the culture grown at 25°C. In the evening, recovery of the morning values of F_v/F_m and $\Delta F/F'_m$ ratios was incomplete. Photoinhibition reduced the daily productivity of the culture by 33% with respect to another culture grown at 35°C. These results strongly support the hypothesis that photoinhibition can take place even in relatively dense outdoor cultures of *Arthrospira* when they are exposed to the combination of high light and suboptimal temperature (Torzillo et al., 1996).

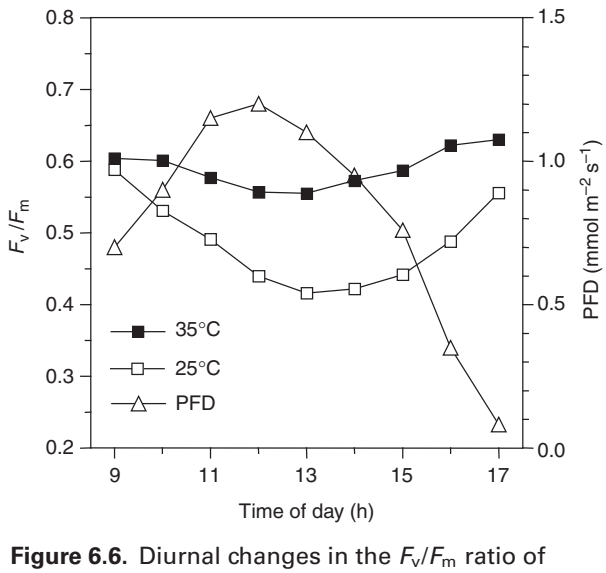

Figure 6.6. Diurnal changes in the F_v/F_m ratio of *Arthrospira platensis* cultures grown outdoors in tubular photobioreactors at two temperatures.

The effect of low temperature on photoinhibition was also investigated in outdoor cultures of *Monodus subterraneus* (Eustigmatophyta) by Vonshak et al. (2001) by measuring the diel changes in photosynthetic oxygen evolution and several photochemical parameters. Cultures were maintained at two temperature regimes. In one, the rise in temperature was initiated in the morning as a result of the increase in solar radiation up to the optimal temperature of 28°C, while in the other culture a heating device was used to increase the rate of warming up in the early morning. It was found that, although the two cultures were maintained most of the day at the same temperature and light intensity, cultures exposed to suboptimal morning temperature, for only a short time, showed a larger decrease in almost all the photosynthetic parameters. By comparing the diel changes in maximal photochemistry efficiency of PS II, the relative ETR, photochemical, and non-photochemical chlorophyll fluorescence quenching of the cultures, they concluded that even a relatively short exposure to suboptimal morning temperatures induced a photoinhibitory damage. The higher photochemical activity of the heated culture was also reflected in a 60% increase in productivity compared to the non-heated one.

Increased susceptibility to photoinhibition can be caused by several mechanisms: (i) low temperature slows the rate of CO_2 fixation thus causing the overreduction of electron transport compounds at a given photon fluence rate; (ii) low temperature inhibits the scavenging of active oxygen species, known to protect PS II against photoinhibition; (iii) low temperature inhibits the PS II repair cycle, that is, the interplay between degradation and *de novo* synthesis of the D1 protein degraded during photoinhibition (Murata et al., 2007).

Lee-Feng & Vonshak (2011) used two *A. platensis* (Kenya and M2) strains to compare the changes in antioxidant enzyme activities in response to low temperature-induced photoinhibition. When transferred to 15°C from 33°C, cells exhibited an immediate cessation of growth followed by a new acclimated growth rate. Although both strains had similar growth rates at 33°C, once transferred to a lower temperature, Kenya had a faster growth rate than M2. The activity of superoxide dismutase from Kenya was higher than from M2 and increased remarkably with acclimation time. Ascorbate-dependent peroxidase activity of the Kenya strain declined when transferred to the low temperature environment while peroxidase activity of M2 decreased in the beginning and then increased with time. The finding may support the hypothesis that strains capable of employing higher antioxidant enzyme activities through a response to stress represent a better ability to apply a more efficient regulatory strategy of response to low temperature-induced photoinhibition. It may be suggested that such strains may also perform better in large-scale production facilities.

6.2.4.2 Effect of high oxygen concentration and low temperature on photoinhibition and culture productivity

A number of studies have suggested that reaction of dioxygen (O_2) resulting in active oxygen species initiate early destructive processes of photoinhibition (see Krause, 1994, for review). Reduction of oxygen leads to the formation of the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$), while electronic excitation leads to the formation of singlet state oxygen ($^1O_2$). These ROS can damage cellular components, that is, proteins, lipids, and nucleic acids. The photosynthetic electron transport system represents the major source of ROS having the potential to generate singlet oxygen, hydrogen peroxide, and the superoxide radical. The generation of ROS is enhanced when the photosynthetic apparatus absorbs excess light or high light in conjunction with other stressors such as nutrient limitation or depletion (Asada, 1994). According to Nishiyama et al. (2004), ROS primarily affect the synthesis of the D1 protein *de novo* at the translation elongation step and thus inhibit the repair of PS II. Indeed, elimination of molecular oxygen, a precursor of $^1O_2$ from cells had no effect on the rate of photodamage (Nishiyama et al., 2004). Moreover, disruption of a gene for biosynthesis of α-tocopherol, an efficient scavenger of singlet oxygen decelerated the repair of PS II without affecting photodamage to PS II (Inoue et al., 2011). These findings have forced researchers to reevaluate the validity of proposed ROS-dependent phenomena, such as "acceptor-side" and "charge-recombination" mechanisms (Nishiyama et al., 2011). A reevaluation of the role of thermal dissipation of excitation energy (NPQ) in the protection of PS II against photoinhibition has been also proposed (Nishiyama et al., 2004). The protection of PS II by thermal dissipation seems to play a role in preventing the generation of ROS by reducing the PS II-mediated transport of electrons rather than in protecting PS II from photodamage. The effect of ROS on the PS II photodamage and repair were studied separately in cells of the cyanobacterium *Synechocystis* sp., PCC 6803 (Nishiyama et al., 2004). Increases in the intracellular levels of H_2O_2 in the culture medium or inactivation of genes for H_2O_2-scavenging enzymes, stimulated the apparent photoinhibition of PS II by inhibiting the repair cycle of PS II but not by accelerating photodamage to PS II (Nyshiyama et al., 2001). Moreover, increased cellular

levels of $^1O_2$ also stimulated the apparent photoinhibition of PS II by inhibiting the repair of PS II, but not by accelerating photodamage to PS II (Nishiyama et al., 2004). These observations indicated that ROS act primarily by inhibiting the repair of PS II under oxidative conditions (Nishiyama et al., 2006).

However, the role of O_2 during high light stress is dualistic. High dissolved oxygen concentration is a sign of a healthy photoautotrophic culture with high photosynthesis. Oxygen can protect the photosynthetic apparatus from photoinhibition by dissipation of excessive energy through photorespiration or via the Mehler reaction (Krause & Cornic, 1987; Wu et al., 1991). On the other hand, persisted high concentration of oxygen will lead to formation of excess amounts of harmful oxygen radicals through multiple mechanisms that can have a potentially deleterious effect on photosynthetic structures (Asada & Takahashi, 1987).

Conditions of high O_2 and low CO_2 concentrations are known to stimulate photorespiration in terrestrial plants, but there is no evidence that this process takes place in cyanobacteria (Colman, 1989). The active intracellular accumulation of inorganic carbon developed by cyanobacteria may be sufficient to inhibit ribulose-1,5-bisphosphate oxygenase activity and therefore to suppress photorespiration (Chapter 2).

The first evidence of oxygen inhibition on *Arthrospira maxima* growth was reported by Torzillo et al. (1984). They showed that laboratory cultures grown under an oxygen partial pressure of 0.7 atm caused a growth decrease of about 40% compared to a culture grown at 0.2 atm of O_2. Similar results have been also reported by Marquez et al. (1995). They clearly showed that the inhibition caused by O_2 on the growth was dependent on the oxygen concentration tested. Since both photosynthesis and growth of *Arthrospira* cultures were inhibited to a comparable extent, the authors suggested that inhibition of oxygen may cause some damage to the photosynthetic apparatus. The effect of high oxygen concentration on the photosynthesis and growth of *Arthrospira* was further studied using chlorophyll fluorescence on laboratory cultures bubbled with pure oxygen so that the O_2 concentration reached 36 mg L^{-1} (Vonshak et al., 1996). It was found that the effective photochemical efficiency of PS II ($\Delta F/F'_m$) was much more depressed than maximum photochemical quantum yield of PS II (F_v/F_m), and that the decline of these two parameters occurred according to two very different kinetics. These facts might indicate that photoinhibition and photooxidation in *Arthrospira* could occur as parallel processes and not be necessarily sequential.

Attempts to investigate the mode and type of oxygen species involved in the oxidative damage of *Arthrospira* cells have also been performed (Singh et al., 1995). The results showed that the singlet oxygen was the most predominant oxygen species generated during high light stress, while superoxide and hydroxyl radicals played a minor role in the photodynamic damage of *Arthrospira* cells.

Chlorophyll fluorescence was also used to investigate the effect of oxygen on the productivity of *Chlorella sorokiniana* (Ugwu et al., 2007). The F_v/F_m ratio declined as the dissolved oxygen concentration increased from 120% to 320% of air saturation, and this change was paralleled by a reduction in the culture productivity.

The response of the photosynthetic apparatus of *A. maxima* cells to increased concentrations of ROS was studied by Ganesh et al. (2007). It was found that the growth rate diminished as the cells were exposed to increased amount of H_2O_2. With the dose of 1 mmol H_2O_2/(g cell), the growth rate was diminished by 27%. ROS led to a decrease in the amount of phycocyanin and carotenoids. The authors concluded that the decrease in photosynthetic efficiency was mainly due to damage to PBS and PS II, while PS I was unaffected.

The combination of high oxygen concentration and high light intensity is very frequent in outdoor cultures of *Arthrospira*, particularly when grown in closed systems. For example, in PBRs made with tubes of about 5 cm internal diameter, in well-growing cultures, the oxygen concentration can increase at a rate of 2–3 mg L^{-1} min^{-1}. This results in an oxygen concentration of up to 70–80 mg L^{-1} even with a gas exchange with air every 50 s and with a relatively high turbulence rate (Vonshak et al., 1996; Torzillo et al., 1998).

The combination of high oxygen concentration and low temperature in outdoor cultures can occur at the beginning of the cooler season, when the culture temperature drops much below the optimum, but irradiance is still enough to drive photosynthesis at an appreciable rate. Such conditions can be very common in desert areas where the morning temperature of the culture is far below the optimum while light intensity is high enough to induce photoinhibition. The synergistic effect of high oxygen concentration and low temperature was studied in outdoor cultures of *Arthrospira* grown in tubular PBRs by using an online chlorophyll fluorescence technique (Torzillo et al., 1998). The results have shown that the combination of low temperature and high oxygen concentration had a considerable impact on PS II photoinhibition measured as changes in the F_v/F_m ratio, resulting in a strong reduction of the growth of the culture where a mere reduction of 10°C below the optimum was

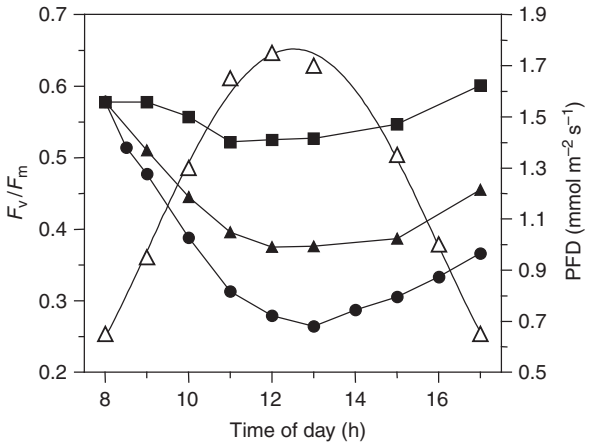

Figure 6.7. Effect of oxygen concentration and temperature on the F_v/F_m ratio of *Arthrospira platensis* cultures grown outdoors in photobioreactors. (■) Low oxygen–optimal temperature; (▲) high oxygen–optimal temperature; (●) high oxygen–low temperature; (△) photon flux density.

imposed and the oxygen concentration was allowed to rise to 70–80 mg L^{-1} (Fig. 6.7). A great part of the radiation absorbed by the photosynthetic apparatus was dissipated since a significant reduction in the effective photochemical quantum yield of PS II ($\Delta F/F'_m$) was observed during the day. However, while in the low oxygen culture, recovery was complete by dusk, in the high oxygen culture, persistent photoinhibition took place since the recovery of both F_v/F_m and $\Delta F/F'_m$ was incomplete at the end of the day. Photoinhibition reduced the daily productivity of the culture grown under high oxygen stress by about 33%, and that of the culture grown under high oxygen–low temperature stress by 60% (Table 6.1). Photoinhibition stress induced by high oxygen stress and low temperature in outdoor cultures of *A. platensis* acclimated to high or low light conditions was investigated by Torzillo et al. (2003). Productivity of low light-acclimated cultures was lower than that measured in high light ones whether grown under stress or optimal conditions. In low light-adapted cultures, the F_v/F_m ratio declined from 0.6 in the morning to 0.15 in the middle of the day, and the corresponding values of photosynthetic activity (oxygen evolution) declined to almost zero. D1 protein content at the end of the day in the low light-acclimated cultures grown under high oxygen and suboptimal temperature of 25°C, was found about 50% lower than in the morning (Torzillo et al., 2003).

There is little doubt that oxygen accumulation in the culture represents the main obstacle to the development of closed PBRs in industrial scale unit. Indeed, in this system the advantage to grow algal cultures at a very high biomass concentration is greatly reduced by oxygen accumulation. On the other hand, the fragility of the cells usually dictates strict limitation of the culture circulation speed, which aims at increasing gas exchange with air and increasing the turbulence of the culture. Therefore, the application of an efficient degassing system to prevent high oxygen accumulation is a prerequisite for successful design of industrial scale PBRs.

6.2.5 Some practical considerations

Maximum quantum yield of PS II (F_v/F_m) in healthy microalgal cultures ranges from 0.7 to 0.8. Once exposed to full solar radiation at midday, a decline of up to 90% of the initial value may be observed. This means that photon use efficiency drops to about 10% of that observed in the

Table 6.1. Effect of oxygen concentration and temperature on biomass productivity and chlorophyll synthesis in *Arthrospira platensis* cultures grown outdoors in photobioreactors

Culture conditions	Oxygen concentration (mg L^{-1})	Temperature (°C)	Chlorophyll synthesis (mg L^{-1} d^{-1})	Biomass synthesis (mg L^{-1} d^{-1})
LO–OT	22 ± 2	35	6.02 ± 0.18	570 ± 28
HO–OT	60 ± 19	35	2.57 ± 0.05	380 ± 18
HO–LT	58 ± 16	25	0.22 ± 0.01	230 ± 10

Data are the mean ± SD of triplicate experiments.
SD, standard deviation of triplicate experiments; LO–OT, low oxygen–optimal temperature; HO–OT, high oxygen–optimal temperature; HO–LT, high oxygen–low temperature.

early morning. The reason for this decline in efficiency is explained that even under intensive mixing the first layers of cells absorb light in excess to what they can use in the photosynthetic process, resulting in a need for dissipation of energy through NPQ. The problem has been figured out already, 50 years ago, and basically three types of approaches have been proposed:

1. Increase of cell density and the mixing rate of the cultures in order to prevent the saturation effect;
2. Use of special designs of PBRs in which it is possible to improve light distribution in the culture;
3. Search for strains having small antenna size and thus higher photosynthesis saturation levels.

The first strategy has been pursued by Richmond and coworkers (Chapter 11) since the beginning of algal biotechnology outdoors, and particularly in the recent years with the use of ultra high cell densities of *Arthrospira* and other microalgae in flat PBRs. However, even when *Arthrospira* cultures were grown at the optimal cell density, and with very high mixing rates, it was not possible to prevent a reduction in the F_v/F_m ratio in midday (Hu et al., 1996).

Special designs of PBRs have been studied quite intensively in Florence (Italy). Carlozzi & Torzillo (1996) attacked the problem by devising and constructing a strongly curved tubular photobioreactor for mass culture of *Arthrospira*. This tubular photobioreactor was studied to create convective mixing in the tube lumen so as the cells of the core region are carried toward the tube wall to receive illumination. Considering the higher power required to support such a convective mixing, however (about 40% higher than conventional reactors), the small increase in biomass yield obtained (17%) did not justify this device. Other special designs have been tested by Laws et al. (1987). Finally, photobioreactor designs devised to promote light dilution have been proposed by Torzillo et al. (1993), Tredici & Zittelli (1997), Carlozzi (2003), and more recently by Wijffels & Barbosa (2010). High photosynthetic efficiency was achieved by Cuaresma et al. (2011) with vertical laboratory photobioreactor designs, for example, 1.3 g of biomass produced per mol of PAR photons supplied, which compares favorably to the theoretical maximum yield, 1.8 g mol^{-1}. However, there is still no clear evidence whether photobioreactor designs utilizing the light dilution effect can effectively improve the economic feasibility of the process at an industrial scale level.

The third approach in search for algal strains with small antenna is based on the following rationale. Strains having a small antenna size will minimize absorbance of light by the outer layers of cells, thereby reducing the dissipation of light through NPQ and the risk of photoinhibition. This should result in a higher overall photosynthetic productivity in outdoor cultures. Indeed, small antenna-size cells are characterized by higher photosynthesis rates (Nakajima & Ueda, 1997, 2000; Neidhardt et al., 1998; Melis et al., 1999). In this fashion, small antenna size will permit the increase of culture density and/or of the culture depth, and consequently an increase in biomass output rate (Melis, 2009; Formighieri et al., 2012).

Small antenna size may be obtained through acclimation of cells to strong light in the laboratory; however, once cells are transferred outdoors the antenna size would readily revert to that of normally pigmented cells upon lowering of light intensity during the day and as a result of the increased cell density due to daily growth. Since no small antenna-size species have been found in nature, it seems necessary to develop mutants with stable characteristics, that is, cell type with an antenna size that is similar to that observed in high light-acclimated cells. These findings may have profound and immediate consequences on the industrial scale biomass production of microalgae where an increase in population density and productivity would allow a reduction of costs.

6.3 SALINITY STRESS

The response of algae and cyanobacteria to changes in the osmotic environment have attracted considerable attention since they are inhabitants of many of the biotopes characterized by big variations in salinities and may thus serve as model organisms for the study of the response of photosynthetic organisms to osmotic stress. The fundamental aspects of salt adaptation were intensively reviewed: Kirst (1989) dealing with tolerance of marine macroalgae and phytoplankton species to salinity; and Oren (1999) reviewed the energetic costs of salt adaptation. Salt acclimation of cyanobacteria was reviewed by Reed & Stewart (1988), and by Erdmann & Hageman (2001). This section of the chapter will mainly deal with the interaction of the photosynthetic apparatus of microalgae and salinity stress.

Photosynthesis of algae is inhibited by osmotic stress (Vonshak & Richmond, 1981; Gilmour et al., 1984; Kirst, 1989; Endo et al., 1995). Such a decrease in photosynthesis may be associated with the inhibition of PS II activity (Gilmour et al., 1984, 1985; Endo et al., 1995). In *D. tertiolecta*, the osmotic stress inhibits the noncyclic electron transport and stimulates the cyclic electron transport and the fluorescence emission arising from PS I at 77 K, suggesting that the inhibition of PS II activity results from

the ΔpH-dependent downregulation and the state II transition (Gilmour et al., 1984, 1985). Endo et al. (1995) have recently shown that in *C. reinhardtii*, the inhibition of quantum yield of PS II photochemistry by osmotic stress is due to an increase in NPQ, which is attributable to a state II transition. In the red algae *Porphyra perforata*, Satoh et al. (1983) demonstrated that the decrease in excitation energy reaching PS II reaction centers and the inhibition of the oxidizing side of PS II by salt stress resulted in a decrease in PS II activity. It seems that many cyanobacteria are capable of compensating the reduction of energy supply from the photosynthetic pathway by significantly increasing their respiratory activity (Vonshak et al., 1988; Gabbay-Azaria et al., 1992; Pescheck et al., 1994; Zeng & Vonshak, 1998). Studies on the kinetics of the response of *A. platensis* to salinity stress (Lu et al., 1999) revealed that the responses of the maximal photochemical efficiency of PS II to high salinity were composed of two phases. The first phase took place in the first 4 h characterized by an immediate decrease in F_v/F_m, in the first 15 min after exposure followed by a recovery to around 90% of initial value in about 2–4 h. This phase was independent of light. The second phase proceeded after 4 h, in which F_v/F_m declined to 70% of initial value at 12 h in the light, but no further decrease in F_v/F_m was observed in the dark, indicating that photoinhibition was induced by salinity stress. While the ability to respond immediately to a change in the osmotic environment is a prerequisite to enable the algal cells to survive the change, the next step is the ability of the cells to adapt and establish a new steady state of growth. In many of the algal systems studied, a decline in productivity is observed once adapted to excessive salinity and clearly associated with a decrease in their photosynthetic capacity. It was reported that one of the primary sites of damage to the photosynthetic apparatus by environmental stress is located in PS II (Baker, 1991). In cyanobacteria, the effect of salt stress on PS II has not been studied as intensively as in higher plants. Jeanjean et al. (1993) reported that no significant changes were noticed in the activity of PS II electron transport in *Synechocystis* sp. PCC 6803 adapted to 0.55 M NaCl. However, in cells adapted to a higher level of salinity (0.684 M), PS II activity was decreased (Schubert & Hagemann, 1990). Using transients in Chl *a* fluorescence analysis, Lu & Vonshak (2002) and Lu et al. (1998) analyzed the modifications that took place in *A. platensis* cells adapted to elevated salinity.

The findings pointed out that after the initial decline in all the photosynthetic activities some of them were restored. No modification was induced by salt stress at the donor side of PS II. This finding also suggests that the decrease in PS II activity, seen during adaptation to salt stress, may be due to damage at the acceptor side of PS II and/or in the PS II reaction centers. Since no significant decrease in the probability of electron transport beyond Q_A (Ψ_o) was observed, it may as well indicate that the acceptor side also was not the main site of damage induced by salt stress. It therefore appears most likely that salt stress causes damage to the PS II reaction center itself. The decrease in the rate constant of energy trapping by PS II reaction centers in salt-adapted cells, derived from the fluorescence parameters $(1/F_o)-(1/F_m)$, also suggests that the main effect induced by salt stress lies in the PS II reaction centers. An increase in the complementary area above the fluorescence induction curve in salt-adapted cells exposed to 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) clearly demonstrated that salt stress resulted in an increase in the proportion of closed PS II reaction centers.

However, recently the separate effects of salt stress on damage and repair have been examined in *Synechocystis* (Allakhverdiev et al., 2002; Allakhverdiev & Murata, 2004). Salt stress inhibited the repair of photodamaged PS II but did not accelerate damage to PS II. Therefore, it appears that the enhanced photoinhibition that was observed in earlier studies might have been due to the synergistic effects of light and salt stress, that is, damage by light and inhibition of repair PS II cycle by salt stress. The labeling of proteins in *Synechocystis in vivo* showed that the synthesis of the D1 protein *de novo* was markedly suppressed by salt stress due to 0.5 M NaCl (Allakhverdiev et al., 2002; Allakhverdiev & Murata, 2004). It was also noted that salt stress suppressed not only the synthesis of D1 protein but also the synthesis of almost all other proteins. Hence, it is conceivable that inhibition by salt stress of the repair of PS II is attributable to suppression of the synthesis of proteins *de novo*. It appeared that a major target for inhibition by salt stress may be at the transcriptional step (Allakhverdiev et al., 2002; Allakhverdiev & Murata, 2004).

Since salt stress limits the fixation of CO_2 by inactivating Rubisco, it is then expected that when such conditions are combined with strong light, the generation of ROS is accelerated, which in turn inhibit protein synthesis (Murata et al., 2007).

Another modification observed in the salt-adapted cells was an increase in the respiratory rate thereby affecting PS II, since the respiratory electron transport chain is often coupled with the photosynthetic electron transport chain in cyanobacteria. However, the higher excitation pressure that this would place on PS II could be overcome by a decrease in the absorption cross section of PS II (as reflected by a decrease in the ratio of phycocyanin/chlorophyll), a

decrease in the rate constant of excitation energy trapping by PS II reaction centers and by increased PS I activity. This would result in a decrease in energy transfer between PBS and PS II, and shifts the distribution of excitation energy more in favor of PS I. Enhancement in PS I activity should increase cyclic electron transport. Several reports have shown that cyclic electron flow increases under salinity stress (Jeanjean et al., 1993; Hibino et al., 1996). Thus, it seems that an increase in PS I activity in salt-adapted cells may protect PS II from excessive excitation energy under salt stress. On the other hand, the increases in PS I activity and in the respiratory rate of salt-adapted cells may provide more energy for the synthesis of organic osmolytes and for the extrusion of Na^+ in cells to maintain osmotic balance.

It was thus suggested that adaptation of the PS II apparatus to salt stress in *Arthrospira* cells appears to involve a decrease in the absorption cross section (decreased ratio of phycocyanin/chlorophyll), and in modifications to PS II photochemistry. An increase in PS I activity parallels the decrease in the maximum quantum efficiency of PS II photochemistry and may regulate excitation energy equilibration to maintain balanced electron transport in salt-adapted *Arthrospira* cells. Through an increase in the proportion of closed PS II reaction centers, the PS II apparatus was thus protected from further excess excitation energy.

The interplay between light intensity and salinity on growth and lipid production by laboratory batch cultures of *Nannochloropsis* sp. grown on nitrogen-replete and nitrogen-depleted media was investigated by Pal et al. (2011). It was found that the cells grown on nitrogen-replete medium, the increase in light intensity and salinity, increased the dry weight and total lipid content due to enhanced formation of triacyglycerols (TAG). Cells grown under stressful conditions (e.g., 700 μmol photon m^{-2} s^{-1} and 40 mg L^{-1} NaCl), content of the major long-chain polyunsaturated fatty acid (LC-PUFA), eicosapentaenoic acid (EPA), was significantly reduced while TAG reached 25% of the dry weight. In contrast, combined high salinity and high light conditions were detrimental to lipid productivity (Pal et al., 2011).

6.4 CONCLUDING REMARKS

The recent increase in energy prices led to the rediscovery of the potential of algal biomass as a source of renewable energy. Unfortunately, the research initiated in the early 1980s was terminated some years later and not much work was carried out on this specific issue. There is no doubt that algal biomass represent a potential source of renewable energy. Yet, we should realize that the gap in knowledge cannot be overcome in a short time and all the claims of high productivity achieved are as of today based on either extrapolation from small-scale, short-term experiments or the use of measuring units that are not reflecting a real increase in productivity and are rather the results of mathematical calculations.

In this chapter, we have tried to point out what we believe are the real major obstacles for improved algal productivity in large-scale production systems. Outdoor algal cultures are exposed to diurnal and seasonal changes significantly affecting the photosynthetic activity that is reflected in the overall productivity of the cultures. Even when the final product of an algal production facility is not the total biomass produced, but rather a specific product like pigments or lipids, an important part of the overall process requires the production of biomass in order to be able to induce the cells to produce and accumulate the desired product.

Many attempts were made to outline a set of criteria that will help in screening and isolating of algal strains that will perform better in outdoor systems. We would like to propose that the selection of strains differing in their ability to respond and adapt their photosynthetic apparatus to variation in light and temperature might serve as a reliable and fast tool for such a screening process. Using the ability to induce mutations that modify PS II or selecting strains from a variety of habitats may help in such a screening process. Our work with *Arthrospira* (Lee-Feng & Vonshak, 2011) and *Porphyridium* (Kalpana et al., 2009) may serve as an example to such an approach.

The lack of a better understanding of the mechanism of how excess solar energy is dissipated by the photosynthetic apparatus under high-irradiance stress is a major problem, not only for researchers studying fundamental aspects of photosynthesis but also for applied photosynthesis research. Indeed, the optimization of growth conditions in outdoor cultures for mass production necessarily requires knowledge of the fate of light energy within the photosynthetic apparatus. On the other hand, in algal biotechnology the warning signals must be recognized as soon as possible in order to prevent a significant reduction in daily productivity or situations that, in few days, may culminate in the loss of the culture. Since environmental stress affects the function of PS II, directly or indirectly, Chl *a* fluorescence technique represents a useful tool to get rapid evidence of stress conditions affecting the photosynthetic activity of the culture and to get a quantification of the effect of stress on biomass yield.

Outdoor dense microalgae cultures may experience large variations in light intensity due to the changes in daily

irradiance and mixing. Although turbulent mixing is aimed to expose the cells to average uniform irradiance, relatively long exposure of cells to excess light cannot be avoided. On the other hand, a strategy striving to counteract photoinhibition at midday, through an increase in cell concentration, would lead to acclimation of the cells to low irradiance, which may result in an increase in the PS II antenna size and thus to an increased risk of overexcitation (Neidhardt et al., 1998; Falkowski & Raven, 2007). Moreover, the increased fraction of cells deprived of light in the deeper layers may bring about an increase in the energy dissipated through respiration.

The effect of photoinhibition on the productivity of microalgal cultures substantially increases if additional stress, for example, suboptimal temperatures or high oxygen concentration are superimposed. Thus, a larger proportion of the radiation absorbed by the photosynthetic apparatus is dissipated through non-photochemical pathways resulting in a reduced biomass yield. Production of genetically modified strains with small antennae size seems to be a promising way to circumvent the problem of light penetration into the culture and at the same time to reduce the impact of photoinhibition on biomass productivity of outdoor microalgal cultures.

6.5 SUMMARY

Microalgae represent a unique experimental system to study stress responses of photosynthetic organisms. In higher plants, response and adaptation to stress takes place in two levels: the metabolic level and the morphological/structural level. In many cases, it is difficult, if not impossible, to determine which is the initial response and which one is just a result of the initial modification. Since microalgae lack the morphological structure that characterizes higher plants, they may be used as a unique experimental system to study metabolic and molecular processes associated with the response and adaptation of photosynthetic organisms to stress. The study of stress physiology and acclimation of microalgae also has an important application in further development of the biotechnology for mass culturing of microalgae.

- When culturing algal cells under outdoor conditions, cells are exposed to severe changes in light and temperature much faster than the timescale required for the cells to be able to acclimate. A better understanding of those parameters and the ability to monitor those conditions will provide the growers with a better knowledge on how to optimize growth and productivity.
- Induction of accumulation of high value products is often associated with stress conditions. A better understanding of the physiological response may help provide a better production system and process for the desired product, and at a later stage, give an insight of the potential for genetic modification of desired strains.
- The potential use of microalgae as part of a biological system for bioremediation/detoxification and waste treatment is also associated with growing the cells under stress conditions.
- Microalgae represent a promising alternative to convert CO2 into high-added value products and biofuels. Algae biorefineries may thus alleviate food versus fuel conflicts and may become particularly advantageous for regions with limited biomass availability and land unusable for agriculture.

Understanding the process associated with these unique environmental conditions may help in choosing the right culture conditions as well as selecting strains in order to improve the efficiency of the biological process.

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