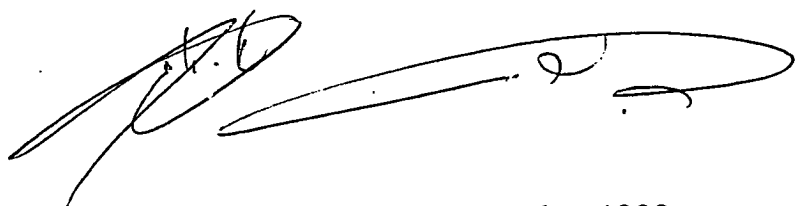# The Response of *Dunaliella bardawil* to the Natural Changes in the Sunlight Spectrum and Intensity

A dissertation submitted for the degree of Doctor for Philosophy

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Presented to

The Scientific Council of

The Weizmann Institute of Science

September 1999

UMI Number: DP16888

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This work has been carried out under the supervision of

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Acknowledgments

This work is in the memory of my late father, Ami Turnheim, who would have been proud to see this project accomplished.

The Response of *Dunaliella bardawil* to the Natural Changes in the Sunlight Spectrum and Intensity

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Abbreviations

ß-car = ß - Carotene

Blue Z. = Blue zelofan paper

Cbr = Carotene Biosynthesis Related protein

Chl. = Chlorophyll

Chl a/Chl b ratio = Chlorophyll a to Chlorophyll b ratio

$CuSO_4$ = Cuprum Sulfate

Dich = Potassium Dichromate (KH_2Cr_2)

DLB = Double Lens Bioreactor

ELIP = Early light-inducible protein

FR = Far red (710 -740 nm)

LHCII = Light Harvesting complex of Photosystem II

MB = Methylene Blue

PAR = Photosynthetic Active Radiation range (400-700 nm)

PSI = Photosystem I

PSII = Photosystem II

PSU = photosystem unit size

RCII = Reaction Center of photosystem II

RuBP = ribulose biphosphate

UV = Ultra Vaiolet light

UV-A = 315-400 nm

UV-B = 280-315 nm

Vio = Violaxanthin

Zea = Zeaxanthin

1. Introduction

The conversion of light energy into chemical energy through the process of photosynthesis is essential for every living organ on earth. The efficiency of photosynthesis depends on numerous limiting factors such as the availability of water and nutrients, ambient temperature, concentration of CO_2 and O_2 in the ambient air, pH and the quality and quantity of the light source.

1.1 Consider the light source

All energy for life, originates in the form of electromagnetic radiation coming from the sun. Light serves both as the energy source for photosynthesis and it also provides the information regulating various physiological processes, therefore it is an important environmental factor for plant growth and development.

The sunlight spectrum and intensity, and the interactions between them are affected by sun's inclination in the sky, different weather conditions, day time and seasons of the year.

The original spectrum of the sun is very close to that of an ideal black body of 5700K (Kirk, 1983) which correlates by Plank's spectral distribution law to have a peak maximum at about 500 nm. Both the intensity and the spectrum of the sunlight are changed after passing through the earth's atmosphere due to scattering of the light by dust particles and aerosols and it's absorption by water vapor, Oxygen, Ozone and Carbon Dioxide present in the atmosphere. The proportion of the incident solar flux removed by the atmosphere increases as the solar elevation (the angle of the sun's disc to the horizontal) decreases, in accordance with the increase in path length of the solar beam through the atmosphere.

The scattering and absorption processes which take place within the atmosphere not only reduce the intensity but also change the spectral distribution of the direct solar beam. The pattern of the sunlight spectrum after passing through the atmosphere is defined by Rayleigh's Law which states that the efficiency of scattering by air molecules (which are much smaller than the wavelengths of solar radiation, λ) is proportional to $1/\lambda^4$. Scattering of solar radiation is therefore much more intense at the short-wavelength end of the spectrum so that most of the radiation scattered by the atmosphere is in the visible and UV range. In the case of scattering by dust particles (which are generally not sufficiently small relative to the wavelength of most of the solar radiation), different type of scattering known as Mie scattering is exhibited. The Mie scattering takes place mainly in a forward direction and is also characterized by a much weaker dependence on wavelength compared to Rayleigh's Law. Considering these processes, it is postulated that the photosynthetic active radiation range (PAR) constitutes about 50% of the total irradiance at the earth's surface (Kirk, 1983). Clearly, as the solar elevation diminishes, the atmospheric path becomes longer and the ratio of short- (blue) to long- (red) wavelength

light in the direct solar beam decreases because of the intensified removal of the more easily scattered short - wavelength light. On the other hand, as solar elevation diminishes, the relative contribution of skylight (i.e. the scattered light, which is particularly rich in the shorter wavelengths) to total irradiance increases. There is therefore no simple relation between solar elevation and the spectral distribution of total irradiance.

In their natural habitat, plants are exposed to sunlight, therefore - light quality, intensity, direction and temporal variation may have tremendous effects on plant growth. Their pigments and receptors have, thus adapted to the unique structure of the sun's spectral output.

For these reasons, the response of photosynthesis and primary productivity to quantum flux density and to the spectral conditions has been the subject of intensive investigation for over a century (Bjorkman, 1981; Falkowski, 1984; Anderson and Osmond, 1987; Senger and Bauer, 1987; Dring, 1988; Demmig-Adams, Adams III et al., 1989; Smith, Samson et al., 1993; Chamovits and Deng, 1996; Thiel, Döhring et al., 1996) and others.

1.2 The photosynthetic Response to the variations in the light environment

Higher plants and algae adapt to their light environment by regulating the composition, structure and function of thylakoid membranes, pigments composition and the overall rates of photosynthesis in order to effectively utilize the amount of available light.

1.2.1 Acclimation to changes in the light intensity -

The sun/shade acclimation of the photosynthetic machinery involves a series of responses, which allow plants to adapt to the prevailing light conditions in order to optimize growth. Optimization of growth depends on effective utilization of the light quantity and quality and on the short and long term changes in these features.

Among the dynamic properties of the photosynthetic apparatus, which change in response to the light environment is the total chlorophyll concentration. Total chlorophyll increases under low light conditions (Bjorkman, 1981; Anderson and Osmond, 1987) in order to increase the capacity of light capturing. Under high light conditions, the opposite process occurs. Mostly, the increase in total chlorophyll is due to *de novo* synthesis of chlorophyll b (Chl b) (Leong, 1984). Since it is known that Chl b is present only in the auxiliary Chl a/b of LHCI and LHCII, while the core complexes of PSI and PSII contain only chl a (Smith, Morrissey et al., 1990), variations in the Chl a/Chl b ratio imply a variable PSI and PSII size in the thylakoid membrane (Lichtenthaler, Kuhn et al., 1982; Malkin and Fork, 1981; Webb and Melis, 1995). These changes in total chlorophyll and in Chl a/Chl b ratio, alter both the size and composition of the Chl antennae of PSI and PSII, and the PSII/PSI stochiometry in the thylakoid membrane

accordingly to the changes in the light intensity (Webb and Melis, 1995; Falkowski, 1984; Pick, Gounaris et al., 1987).

In addition, changes in the number and density of thylakoid membranes and the amount of electron carriers (such as plastoquinone, cytochromes f, b_6 and b-559), changes in RuBP carboxylase concentrations and in the relative amounts of chlorophyll-protein complexes in the thylakoid membranes are observed upon differing light environment (Anderson and Osmond, 1987; Leong, 1984; Post, Dubinsky et al., 1984; Smith, Morrissey et al., 1990).

The phenomenon described above appears to be highly conserved in all photosynthetic organisms examined (Webb and Melis, 1995). However, the amplitude of the response differs significantly among different species. Green algae display a significantly greater amplitude of the response to differing light environment than higher plants, making them a better model organism in such studies. Moreover, the adaptation processes reflected by the changes in Chla/Chlb ratio in the photosynthetic system of green algae were proved to be fast enough to enable to respond to daily changes in sunlight in contrast to higher plants (Pick, Gounaris et al., 1987).

1.2.2 The response to changes in the light quality -

The existence of mechanisms of acclimation to changes in the light quality is well established. The light is perceived by a complex system of different photoreceptors, which detect different light spectra. Among the most widespread photoreceptors which are known today are the Phytochromes, blue light/UV-A photoreceptors (which are known as the Cryptochrome family of photoreceptors) and UV-B receptors (Dring, 1988; Smith, Samson et al., 1993; Chamovits and Deng, 1996; Hellingwerf, Hoff et al., 1996; Cashmore, 1997; Jenkins, 1997; Batschauer, 1998) and references therein.

The photosynthetic pigments are suggested as well, to comprise an additional group of photoreceptors which include the various chlorophylls and carotenoids that are involved primarily in the transfer of light energy to the electron transport chain of photosynthesis (Chamovits and Deng, 1996).

The phytochrome family of photoreceptors which is the most-characterized and is responsible for the detection of far red (FR) and red (R) light is known to exist in higher plants and in green algae (Dring, 1988) and has been speculated to exist as well in the halotolerant green algae *Dunaliella bardawil* (Ruyters, 1988; Sánchez-Saavedra, Jiménez et al., 1996).

The reduction in the red to far-red light ratio (R/FR) under natural shade has profound effects on plant growth and development such as stem elongation, seed germination and etiolation in higher plants (Morgan and Smith, 1976; Smith, Samson et al., 1993; Batschauer, 1998). In addition, it affects chloroplast movement in the filamentous green algae *Mougeotia*, photoperiodic responses in green, red and brown algae and germination in blue-green algae (Dring, 1988). Furthermore, the involvement of phytochrome in various blue-light mediated

responses has previously been observed by photobiologists, leading to the proposal of some kind of co-action between the photoreceptors (Ahmad and Cashmore, 1996).

Nevertheless, while in higher plants most effects are mediated by phytochrome, in lower plants - particularly in green algae, the blue light photoreceptors (Cryptochrome) has been found to be dominant (Ahmad and Casmore, 1996).

Eventhough blue light responses in higher plants, algal, bacterial or fungal systems are known and documented for over a century, (Humbeck and Senger, 1984; Rau and Schrott, 1987; Ahmad and Cashmore, 1996), knowledge about the structure of photoreceptors responsible for several physiologically well-characterized responses to UV and blue light was still lacking. The reason for it, was partly due to the difficulty in obtaining a good *in vitro* assay system for flavin-type blue light regulated phenomena (since the action spectra of the familiar blue light responses were consistent with a flavin chromophore), and in part due to the group of non-photoreceptor pigments absorbing in the blue light range (cytochromes, chlorophylls, carotenoids and flavins bound to metabolic enzymes). Among the familiar responses to blue light is - phototropism, anthocyanin production, inhibition of hypocotyl elongation and stomatal opening in many plants. In algae and *Neurospora* , carotenoid synthesis and control of cell division were observed to be affected by blue light as well as chloroplast movement in ferns (Rau, 1980) .

The recent first isolation of a blue light photoreceptor, CR1 (for cryptochrome 1), from *Arabidopsis thaliana* by molecular genetic techniques was a major progress in this field (Ahmad and Cashmore, 1993; Ahmad and Cashmore, 1996; Cashmore, 1997) and there is evidence about the existence of more than one cryptochrome which unlike the phytochromes, would likely be quite diverse in molecular nature and chromophore content. Ironically, the first identified cryptochrome appeared to rely for a significant component of its action spectrum in blue light on a non-flavin chromophore (pterin).

The possibility that blue-light signal transduction pathways in algal and plants systems are similar, was raised, based on the recent cloning of a *chlamidomonas* gene that encoded a protein similar to CR1 (Chamovits and Deng, 1996).

Under certain conditions, phytochrome responses may be dependent on a functional cryptochrome so that blue and red light responses may interact with each other (Dring, 1988; Chamovits and Deng, 1996; Cashmore, 1997).

1.2.3 Interaction between light intensity and light quality in the photosynthetic responses-

In addition to the strategies of light acclimation presented above, which are known to be either influenced by changes in the light intensity (1.2.1) or by changes in the light quality (1.2.2), it was indicated in several cases (Lichtenthaler, Buschmann et al., 1980; Humbeck and Senger, 1984; López-Juez and Hughes, 1995) that some of the responses which are typical to changes in the light intensity, are stimulated by changes in the light spectrum. For example, it has been shown that chloroplast response to high light (i.e. higher chl a/chl b ratio, lower granal stacking

etc.) can be simulated in barley and radish seedlings with blue light. Under red light, a shade-type chloroplast was obtained (Buschmann, Meier et al., 1978). Moreover, López-Juez and Hughes, (1995) showed that there is a role for a blue photoreceptor in detecting low *versus* high fluence rate of light, which subsequently controls the light acclimation responses. However, he suggested that photosynthesis or other mechanisms of fluence rate photoperception must also be involved.

In general, it was concluded that for higher plants, blue light was more effective in producing high light responses. Lichtenthaler, Buschmann et al., (1980) suggested that since sun leaves receive more blue light than shade leaves, blue light seems to be responsible for the formation of sun-type chloroplasts in nature.

In concern with algae, the situation is not clear. Golden, (1995) showed in Cyanobacteria (*Synechococcus* sp. strain PCC 7942) that the characteristic low to high light shift responses are induced by low-fluence blue light with red light canceling the inductive blue signal. Therefore, it is speculated that subtle shifts in the blue/red light ratio may provide information about changes in the light environment that are linked to the light intensity. While the Cyanobacteria resemble higher plants in this aspect, Senger, Humbeck et al., (1992) reported that blue light mimicked low light environment for *Scenedesmus obliquus* (green algae), and it was concluded that according to the different biotops (of higher plants and algae) blue light mimics the shade conditions for green algae (Humbeck and Senger, 1984; Thielmann, Galland et al., 1991).

1.2.4 Protection strategies against excessive light conditions - Coping with conditions of light stress.

Under various environmental conditions, plants are exposed to levels of sunlight in excess of those required for photosynthesis. When the rate of photons absorption exceeds the rate of utilization of excitation energy, it can accumulate within the photochemical system and potentially lead to destructive reactions. Such a situation is defined as photoinhibition (Foyer, Lelandais et al., 1994 and others). Photoinhibition results under very high light intensities or under other conditions in which light intensity exceeds the capacity of the cell to transport electrons (such as limitations in CO_2 or other nutrients). The internal damages (within the photosynthetic membranes) are manifested externally by a lower rate of growth, and a lower light-saturation rate and quantum yield of photosynthesis.

In addition to the internal damages which may induce a decrease in the photosynthetic efficiency, there are processes of photoacclimation which induce changes in the capacity of energy perception by the photochemical apparatus. It is not clear how the relative contribution of these two contrasting possibilities of photoinhibition and photoacclimation to a decrease in photosynthesis efficiency can be distinguished (Demmig-Adams, 1990).

In addition to the ability of adapting to changes in the light intensity (as described above), photosynthetic organisms have developed pathways to safely dissipate excessive energy as

heat, thus decreasing the energy arriving at the reaction centers and preventing the damage caused by intense light.

The halotolerant unicellular green algae *Dunaliella bardawil* , has the ability to accumulate large amounts of ß-carotene (reaching more than 10% of the algal dry weight) which is concentrated in oily globules located in the interthylakoid space of the chloroplast (Ben Amotz, Katz et al., 1982). This massive ß-carotene accumulation occurs under defined growth conditions such as high light intensity, high salt concentration, extreme temperatures and sulfate or nitrate deficiency (Ben-Amotz and Avron, 1983).

In addition, *D. bardawil* demonstrates a remarkable degree of environmental adaptation to salt due to its ability of synthesizing massive amounts of intracellular glycerol which serves to balance osmotically the extra cellular salt concentration, therefore it is widely distributed in natural habitats.

The unique adaptability of *Dunaliella* to grow under a wide range of environmental conditions as well as under high light intensity has made it a favorable model system for detailed physiological and biochemical investigations in our study.

Three of the mechanisms of photoprotection which are possessed by the *Dunaliella*, are discussed in turn below.

The first mechanism is the response to light stress by the de-epoxidation of the carotenoid Violaxanthin via Antheraxanthin to form Zeaxanthin (by the "Xanthophyll cycle" - in general (Demmig-Adams and Adams, 1992) and in *Dunaliella* (Levy, Tal et al., 1993; Cowan, Logie et al., 1995)). Zeaxanthin, which is accumulated mostly in the antennae of both photosystems (Lee and Thornber, 1995), is proposed to participate in protection of the photosynthetic apparatus against photodestructive damages (Pfündel and Bilger, 1994). It is known to promote the dissipation of excessive light energy absorbed by LHCII into heat - either by direct quenching of chlorophyll excited state (since the energy level of singlet excited carotenoid like zeaxanthin, theoretically allows a direct energy transfer from the singlet excited state chlorophyll a to zeaxanthin), or by indirect mode of promoting the LHCII aggregation, giving rise to a quenching structure of LHCII (Ruban, Young et al., 1994). When highly illuminated cells are returned to normal light, reepoxidation of zeaxanthin to violaxanthin occurs. The Xanthophyll cycle is widely conserved among all photosynthetic organisms (except a few algal classes; Demmig-Adams and Adams, 1992). However, in general low-light grown organisms have a smaller pool of xanthophyll-cycle pigments per chlorophyll compared to high light grown organisms (Casper-Lindley and Björkman, 1998; Demmig-Adams, 1990; Demmig-Adams and Adams III, 1994).

Measurements of the action spectrum for the xanthophyll cycle indicated that it is induced by spectral range of 450-550 nm and 600-700 nm, while the 550-600 nm range has no inducing effect (Lee and Yamamoto, 1968).

The second mechanism is the light stress induced accumulation of secondary carotenoids such as ß-carotene in the case of *D. bardawil* (Ben-Amotz and Avron, 1981; Ben-Amotz and Avron,

1983; Ben-Amotz, Lers et al., 1988; Lers, Biener et al., 1989). In addition, the accumulation of Astaxanthin in *Haematococcus pluvialis* was also reported (Chaumont and Thepenier, 1995).

In addition to their function as accessory pigments in photosynthesis, carotenoids normally protect photosynthetic pigment-protein complexes from the oxidative damaging effect by inactivating reactive oxygen (Britton, 1988). Experiments with isolated PSII reaction centers have shown that ß-carotene quenches singlet state oxygen ($^1O_2$, which is formed by chlorophyll triplets within the photosynthetic membranes under conditions of excess light) directly, affording considerable protection against photodamage (Telfer and Barber, 1995). The phenomena of ß-carotene accumulation in response to high light intensity or nutrients limitation, in a large number of lipoidal globules located in the interthylakoid space by the *D. bardawil* has been extensively studied (Ben-Amotz and Avron, 1983; Ben-Amotz, Lers et al., 1988; Ben-Amotz and Avron, 1989a and 1989b; Lers, Biener et al., 1989; Jiménez and Pick, 1993; Shaish, Avron et al., 1993) and others. High ß-carotene containing *D. bardawil* was shown to be strongly protected against damage induced by excessive irradiation (Ben-Amotz, Gressel et al., 1987). In this case, due to the pattern of the ß-carotene absorption spectrum, and since protection against photoinhibition showed to be most dominant when blue light was used as the photoinhibitory agent (compared with other light qualities) it was suggested that its main role is in screening through absorption of the blue region of the spectrum (Ben-Amotz, Shaish et al., 1989).

The light quality that stimulates carotenogenesis in *Dunaliella* is ill-defined. Wallen and Geen, (1971) found that for *D.tertiolecta*, blue and green light increased carotenoid synthesis, whereas Ben-Amotz and Avron, (1989b) indicated that under lab conditions the extent of accumulation of ß-carotene in *D. bardawil* is a direct function of the integral amount of light to which the algae was exposed to during a division cycle, independently of light quality. However, they tested the influence of white light (400 - 730 nm), green light (combination of 546 + 578 nm) and red light (645 - 730 nm), in the range of intensity between 25 to 500 w/m^2 (which is lower significantly from the values in nature).

Sánchez-Saavedra, Jiménez et al., (1996) tested the dependence of accumulation of ß-carotene by *D. bardawil* and its photosynthetic response on light quality under different R/FR ratios. It has been demonstrated that the addition of Far-red light (of $\lambda > 700$ nm) to white light (400-700 nm) has a significant influence on growth and photosynthesis, inducing an increase in growth rate, cell volume and carotenoid content. They argued that the increase in FR light could simulate the light field in dense culture, and suggested the involvement of phytochrome in these processes. It should be noticed that again this study was carried out in the lab under continuous illumination of light intensity much lower than the natural sunlight. However, it was suggested that besides the effects of light and nutrients deficiency, photosynthetically produced oxygen radicals are involved in triggering massive ß-carotene accumulation in *D. bardawil*. (Shaish, Avron et al., 1993). However, although photoregulation of carotenoid synthesis is not fully

resolved, it has so far been reported for algae, fungi and bacteria that there exists a blue light control of carotenoid biosynthesis (Rau, 1980; Rau and Schrott, 1987).

The third mechanism is the light stress induced response of Cbr synthesis (Cbr, Carotene Biosynthesis related protein) a pathway of photoprotection which was uniquely identified in *D. bardawil* (Lers, Levy et al., 1991; Levy, Gokhman et al., 1992; Levy, Tal et al., 1993). Cbr accumulates within hours in the thylakoid membranes of light-stressed *Dunaliella* cells and is present as long as light stress persists. The Cbr protein showed to be closely related to early light-induced proteins (Elips) of higher plants which are known to be controlled by blue light and light stress conditions (Adamska, Ohad et al., 1992). Lately, it was proposed that Cbr forms a photoprotective complex together with zeaxanthin within the light harvesting antennae under the conditions of light stress or sulfate starvation (Levy, Tal et al., 1993; Braun, Banet et al., 1996). This proposal was based on the evidence observed for the physical association between Cbr and zeaxanthin and on the parallel relationship detected between Cbr induction and Violaxanthin conversion to Zeaxanthin under light stress conditions. These processes decayed both upon return to normal light.

The exact pathway of Cbr induction in *D. bardawil* is not yet fully elucidate. Banet (1997), tested the action spectra of Cbr accumulation in *D. bardawil* under lab conditions. He showed that UV-A (300-410 nm), blue light (400-480 nm), red light (600-700 nm) and to a lesser extent green light (500-600 nm) induced Cbr accumulation. However, blue light showed to be the most efficient. Antennae chlorophyll was suggested by Banet (1997), as the sensing chromophore for both Cbr accumulation and violaxanthin de-epoxidation. His data supported as well the suggestion that Cbr might facilitate the organization of the xanthophyll zeaxanthin within the LHCII complex.

Nevertheless, the light intensity in those experiments was in the range of 15 to 120 μE which is significantly lower than in nature.

1.3 Field studies of photosynthesis and Biotechnology aspects

Utilization of the photosynthetic machinery for the production of energy, chemicals and food by mass culturing microalgae started in the 1950's (Berlew, 1953). Since then, many other applications of large scale reactors have been studied (Goldman, 1979; Pirt, Lee et al., 1983; Richmond, 1986; Matsunaga, Takayama et al., 1991; Qiang, Guterman et al., 1996) and references therein.

The different methods consisted of open pools, laboratory growth chambers and outdoor growth chambers.

When temperature and nutrients do not limit growth, productivity depends on the irradiance impinging on the culture surface and on the efficiency of radiation conversion into chemical energy. One of the essential requirements for massive growth of algae is to optimize the light

distribution by increasing the surface (which receives the light) to volume (of the photosynthetic culture) ratio, in the growth system (Tredici and Materassi, 1992). In order to improve production by using increased surface to volume ratio, enclosed, covered tubular photobioreactor systems were introduced (Benemann, Tillet et al., 1987; Tekoah, 1994; Tredici and Chini Zittelli, 1997 and others).

On the other hand, outdoor growth systems have been constructed as inexpensive open ponds utilizing large areas that are otherwise wasted (see for example, outdoor growing of *Dunaliella* in Ben-Amotz, (1995) and Grobbelaar, (1995)), or as more sophisticated chambers combining closed reactors with the ability of maintaining continuous growth methods (Gitelson, Qiuang et al., 1996; Qiang, Guterman et al., 1996; Tredici and Chini Zittelli, 1997).

The outdoor bioreactors retain the advantage of unlimited natural sunlight as the energy source, which provides the conditions for high cell production and up scaling growth-systems. Another advantage is the ability of investigating photosynthetic performance and acclimation processes under natural light habitat. This aspect of *in situ* investigations was carried out mostly by analyzing aquatic plants without removing them from their natural environment (mainly in concern with light stress responses see Häder, Herrmann et al., 1996; Porst, Herrmann et al., 1997 and others). Surely, under field conditions, the light environment for ecophysiological plant research is not reproducible and difficulties in controlling and monitoring other factors such as - temperature, pH, nutrients etc. can be raised. Hence, much effort has centered in the recent years on acquiring irradiation systems with an emission spectrum simulating solar radiation in the field of environmental photobiology (Hermann, Häder et al., 1996; Thiel, Döhring et al., 1996). However, such a sunlight simulator should be able to simulate the diurnal changes in the sunlight spectrum (and intensity) and demands therefore complex electronic control of a series of individual lighting circuits which feed various lamp types with different spectra of emission (Thiel, Döhring et al., 1996).

The novel photobioreactor (DLB) which was used in this study (2.1.3.1) enabled the monitor and manipulation of the natural sunlight spectrum and intensity under field conditions, together with the control of nutrients, temperature and pH. In addition, it enabled us to assess the responses of the *Dunaliella* photosynthetic machinery to the temporal changes in the sunlight characteristics.

1.4 Objectives of Research

We hypothesized that part of the sensing mechanism for light intensity in *D. bardawil*, is sensitive to spectral changes.

The objective was to investigate the role of the natural daily and seasonal changes in the sunlight spectrum in the adaptation strategies to different light intensities in the green algae *D. bardawil*.

We also hypothesized that accomplishment of the main objective would enable to enhance photosynthetic productivity of *D. bardawil* and its by-products, by optimizing light conditions in the filed.

Achieving the objectives of this research relies on combining a field experiment under natural light, together with tightly controlled conditions approaching to those used in the lab.

2. Materials and Methods

2.1 Experiments

2.1.1 The model organism - *Dunaliella bardawil*
Wild-type *Dunaliella bardawil* Ben-Amotz and Avron which is a locally isolated species (American Type Culture Collection, Rockville, MD, No 30861) was used.

2.1.2 Routine initial growth conditions -
The algae were cultivated within a Pyrex cylinder (40 cm long, 5 cm diameter) in a growth medium as described in Ben-Amotz and Avron (1989a) containing 2M NaCl. The light source was a cool white fluorescent lamp (200 $\mu E/m^2sec$ measured at the cylinder interface) and the culture was continuously mixed by air bubbling inside the cylinder.

2.1.3 The experimental set up -
2.1.3.1 The photobioreactor - All experiments were carried out within tubular bioreactors (Fig 2.1a, the double lens bioreactor, DLB) consisting two concentric Pyrex glass tubes of 100 cm long with diameters ratio of 1.5 (7.5/5 cm). The *Dunaliella* culture was introduced into the inner tube, while the space in-between the tubes was filled with precooled water (25-28⁰c) in circulation (Fig 2.1). Sunlight was the light source in all experiments.
The DLB was designed as an optical system that concentrates light arriving at the surface of the external tube onto the smaller surface of the internal tube (the double wall lens concentrator principle). In order to improve light spatial and temporal distribution and to maximize the actual intensity of radiation reaching the organism, the following optical requirements have been considered:

* In an ideal concentrating tubes system, the ratio between radius of outer/inner tubes should be equal to the value of the refractive index of the tube and the solution inside the tubes. This insures that all the incident light at the outer surface will reach the inner tube's surface, and a concentration power equals to R/r (with R equals to the radius of the outer tube) is approached (see the theory in appendix 1).

* In order to achieve the state of minimal energy loss, the refractive index of the solution in the cooling jacket and the refractive index of the medium inside the inner tube should be equal.

* In order to maximize the amount of light and the duration time of the light reaching any single cell in the inner tube, the ratio between the area which is exposed to the light and the volume in

which the organism (which "consumes" the light) grows, should be maximized (Tredici, Carlozzi et al., 1991) . The mathematical description of the surface to volume (S/V) ratio is :

$$(1) \qquad S/V = 2\pi rl/\pi r^2 l = 2/r$$

(with r equals the radius of inner tube and l equals the length of the tube)

Since S/V is inversely correlated with r, small r values will induce large s/v ratio. However too small r, considering the bioreactor's structure and the light source, will decrease the amount of incident light reaching the inner tube and the size of illuminated area over its surface (see fig 2.2). The right balance between the two considerations above determines the size of r.

* The water layer in the space in-between the tubes has to be thick enough to absorb the undesired Infrared range of the sunlight spectrum.

* In order to get maximal light intensity integrated over the daytime, the incident angle of the plane of sunlight radiation reaching the tube's surface should be 90^0 during the apparent daily movement of the sun.
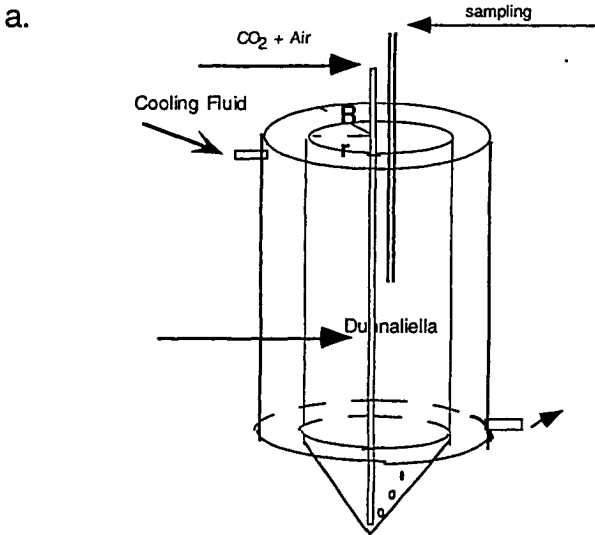
Figure 2.1: The experimental system. (a) A cross section of the bioreactor.

b.
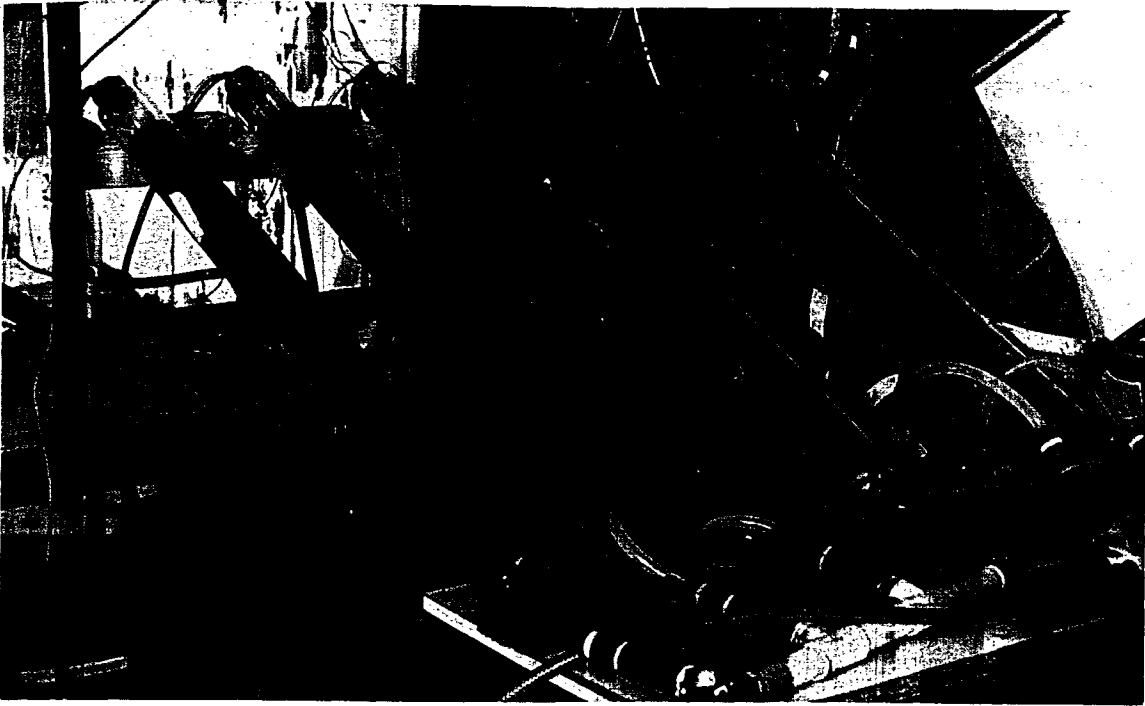
Figure 2.1: The experimental system. (b) View of the experimental system.
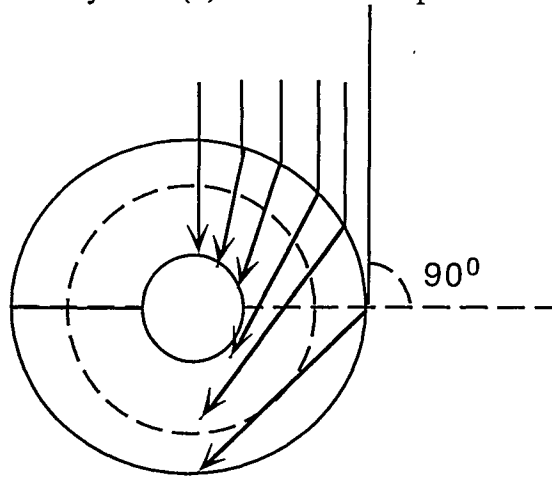
Figure 2.2: A cross section of the double wall lens bioreactor (DLB) demonstrating the considerations involved in choosing the optimal size of r (inner tube's radius), the large circle represents the outer tube's surface. The inner circle represents the situation where the radius is too small, therefore results in a large S/V ratio on one hand but in some radiation lost on the other. The dashed circle represents the case where r is too large therefore all the light reaches the inner tube but only a small S/V ratio is achieved.

After conducting a preliminary experiment with 2 different tubes dimensions and considering all the above demands, the final dimensions of bioreactor that were used are - 7.5 cm and 5 cm for outer and inner diameters respectively.

The optical properties of this system were calculated[1] to be - diameters ratio of 1.5, surface to volume ratio of 0.51 and an average concentration factor of 1.2 the ordinary insolation reaching

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[1] Diameters ratio was calculated by simple division of R/r. The Surface to Volume ratio and the concentration factor of the bioreactor were calculated after considering the area of the inner tube's circumference which receives

the inner tube's surface. Both refractive indexes of algae's growth medium and of the cooling water were measured, indicating values of 1.352 and 1.333 at 25^0 respectively. Since the measured values are similar, no adjustment was needed.

The DLB was situated in a south - north position, tilted in a complementary 90^0 angle to the plane of sunlight radiation (depending on the time of the year). The daily integrated intensity reaching the bioreactor was estimated to be 2.5 (during summer time) to 4 (in winter) times the integrated sunlight intensity reaching a flat pool. The DLB was located at the Solar Tower at the Weizmann Institute of Science, Rehovot, in the open air. In order to minimize experimental artifacts and variability, treatment and control were always running simultaneously in triplicates for each experiment (using a total number of 6 bioreactors).

2.1.3.2 Optimization of growth conditions and light treatments - In order to mix and to aerate the culture, and also to flush the system of excess O_2 evolved in photosynthesis - mixture of air and CO_2 was bubbled into the tube. The air flux (~200 ml/min.) was achieved by using an aquarium air pump, the additional CO_2 evolved from a CO_2 gas balloon. Beside the gas mixture bubbling pipe (of 95 cm long), a shorter pipe (45 cm) was also inserted to the inner tube connected to a 10 ml syringe in order to sample the culture (fig 2.1a).

* pH of the culture was set to be at maximum 8.2 (as recommended in the literature, (Ben-Amotz and Avron, 1989a)) by using a pH electrode connected to a pH controller (El-Hamma, Aqualab APC-6200). An electric valve was controlled by the pH controller and was set to open and let CO_2 be injected in the air flow inlet each time when the pH was above 8.2.

* Temperature - computer controlled feedback system capable of maintaining a desired constant temperature within the bioreactor was used. This system allowed temperature fluctuations during the winter and summer daily cycles to be limited between 25^0-28^0C at the inner tube. The water used for temperature compensation was circulating from a water reservoir (of a suitable temperature) in to the space between the inner and outer tube and back to the reservoir.

* Growth medium- The medium used in the experiments was the same as used during the routine growth (Ben-Amotz and Avron, 1989a). In order to identify any limitation of nutrients, the possible effects of changes in nutrients concentration during the course of a 1 week of experiment on growth and pigments characteristics was tested. First, the nutrients concentrations of the macro elements Nitrogen, Phosphate and Potassium were monitored, and second, the effects of re-establishing initial concentrations of NO_3, KH_2PO_4 and $MgSO_4$ after 3

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the light reaching from the outer tube. The size of this area was calculated by using the critical angle ß, (=62.7^0 see appendix 1), then by calculating the central angle which is exposed to the light (=228^0, equals to 63% of the circumference of the tube) and by assuming that the sunlight covers 50% of the outer tube's area.

days was tested by comparing control treatments with nutrients replenished treatments. It was shown that NO_3 was completely consumed, PO_4 was consumed to ~10% of its initial value, and K^+ concentration decreased by only 10% after 3 days of growth. The deficiency in nitrate and phosphate in the control after 3 days, induced an increased ß-carotene synthesis and a decrease in growth rate respectively compared to nutrients replenished cultures. $MgSO_4$ seemed to be a non limiting factor in the growth culture. Therefore, in order to isolate the influence of any limiting factors on the experiments beside sunlight intensity it was decided to add KNO_3 and KH_2PO_4 re-establishing the initial concentration value on the third day of each experiment.

* Control of sunlight - The control over the sunlight spectrum in the experiments testing the impact of spectrum on photosynthetic acclimation processes was achieved by either using filters solutions in the space between the tubes or by covering the tubes with colored zelofan paper. The absorption spectra of the filters are shown in fig 2.3. The control group of bioreactors were covered by polyethylene which functioned as a neutral density filter in order to compare the light intensity between treatment and control.

The control over the daily distribution of the sunlight intensity was achieved by changing the inclination angle of the bioreactor to the ground (which is complementary to the angle between the bioreactor and the sunlight plane of radiation). In most cases, the bioreactors were situated to be 90^0 to the sunlight plane of radiation, so that the intensity impinging on the bioreactors' surface was maximal and the daily intensity distribution over the surface was parabolic (reaching to maximum intensity at noon time and decreasing again during the afternoon).

In a set of experiments (3.1.7), which tested the response of the algae to different daily sunlight distribution, 3 bioreactors exposed to a constant light dose throughout the day (i.e. treatment) were compared to bioreactors which were exposed to the natural cycle of the daily change in intensity (control). The control group of bioreactors was situated in 90^0 to the sunlight plane of radiation, while the treatment group was situated in an angle $< 90^0$ (= α_2) such that light intensity at mid-day still was similar to the intensity at 10:00 AM (time of optimum photosynthesis). An example of the calculation for the treatments inclination angle in June is brought below :

Parameters of sunlight intensity which were measured - in June at mid-day (I_{high}) =900w/m^2

at 10:00 AM (I_{low}) = 760w/m^2

In order to calculate α_2, so that light intensity over the day will remain ~760w/m^2, the parameters below were substituted in the following equation:

$$(2) \qquad I_{low} / I_{high} = \cos \alpha_2 / \cos \alpha_1$$

$\alpha_1 = 8.5^0$, the inclination angle between the ground and the control bioreactor which accepts maximum radiation (of 900w/m^2) in June at noontime. This angle is complementary to give 90^0 to the sunlight plane of radiation in June.

$\alpha_2 =$ the inclination angle of the bioreactor to the ground which will result in 760w/m^0 impinging on the tubes surface through most of the day time in June.

$I_{low} = 760$ w/m^2, the sunlight intensity at 10:00 AM, this intensity will be accepted under α_2 also at noontime.

$I_{high} = 900$w/m^2, the sunlight intensity at noon time measured at angle α_1.

The resulting value for α_2 is 31^0 between the bioreactor and the ground, and 59^0 between the tube and the sunlight plane of radiation.
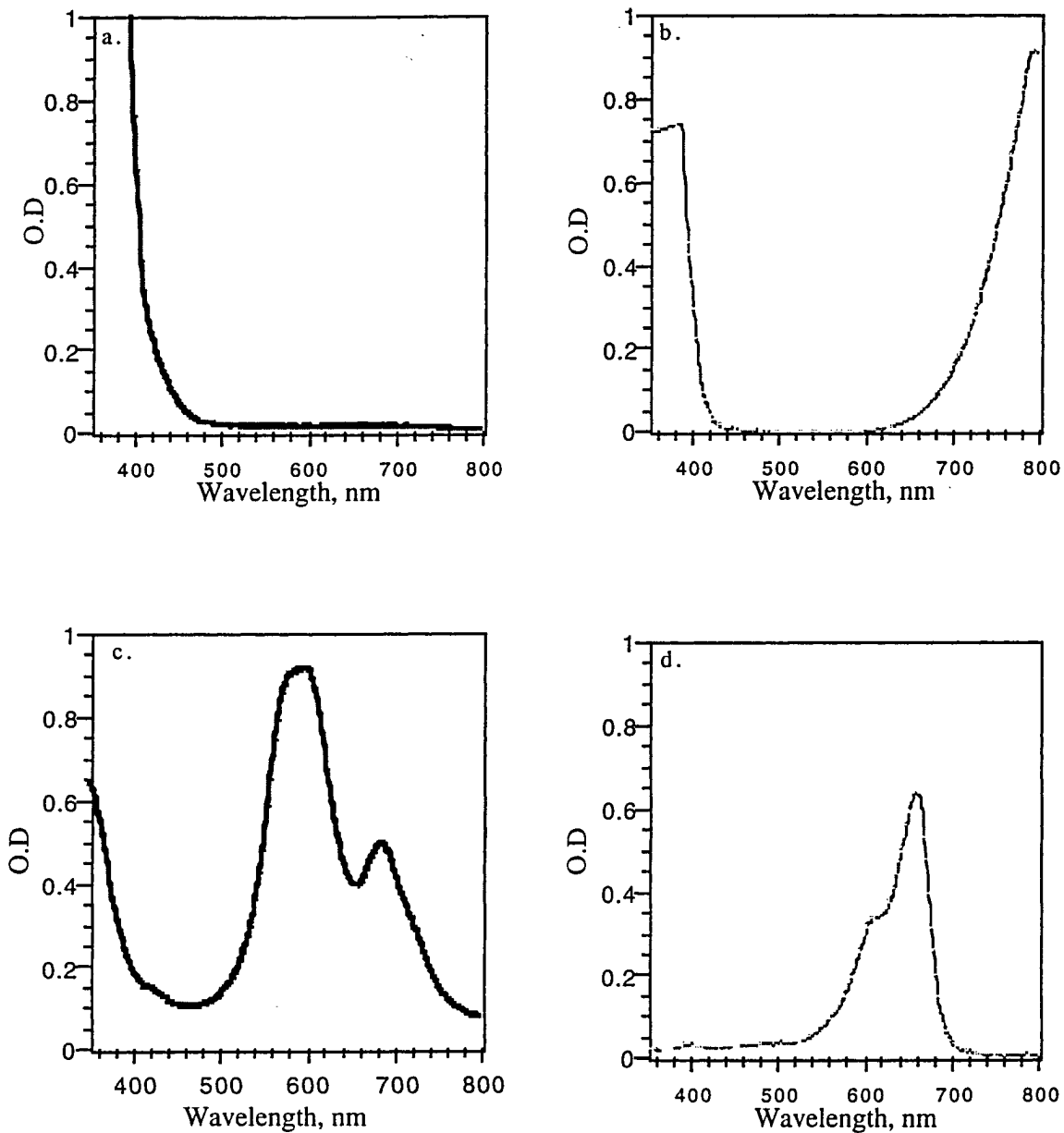
Figure 2.3: Absorption spectra of the filters. (a) Potassium Dichromate ($6.20*10^{-2}$ M in water) (b) Copper Sulfate ($3.60*10^{-4}$ M in water). (c) Blue Zelofan paper. (d) Methylene Blue ($1.06*10^{-5}$ M). See also Fig. 3.23 for the overlapping image of the spectra.

2.1.4 Experimental procedure - Two types of experiments monitoring different time scales were carried out -

2.1.4.1 Monitoring the short term (hours) photosynthetic acclimation - Duration of experiment was 3 days, a procedure of sampling once to twice a day in the first two days and then every 3-5 hr's on the third day of experiment was carried out.

2.1.4.2 Monitoring the photosynthetic acclimation in the long term (annual) - Results of the third mid-day of the "short term" experiments were compared all over the year during 1995-97.

In the beginning of every experiment, cells in initial concentration of $3.5*10^5$ cells/ml which were previously grown in the lab under continuous illumination (as described in 2.1.2) were transferred to the bioreactors located outdoors.

In each experiment, all samples went through cells counting, pigments determination and one of the following analysis: Quantum yield of photosynthesis determined by the rate of O_2 evolution, the estimation of photosynthesis efficiency of PSII and the photosynthetic antennae size by fluorescence induction technique (Malkin and Kok, 1966), Cbr analysis by SDS-Polyacrylamide Gel Electrophoresis and Western Blotting, or Xanthophyll epoxidation state determination by HPLC.

Sunlight spectrum and intensity were measured simultaneously with sampling the algae during the day, in order to look for a correlation between the change in light regime and physiological state of the algae as well as its photosynthetic production.

Experiments were carried out throughout the year in order to detect the seasonal effect as well.

2.2 Measurements principles

2.2.1 Sunlight measurements -

Sunlight intensity measurements of global and direct radiation were obtained from the control room of the Solar Tower at the Weizmann Institute by light intensity meter (Eppely radiometer), that integrated intensity over all wavelengths throughout the day.

Sunlight spectrum was measured by a computer operated spectrometer (resolution of ±10 nm) built of an OCLI circular variable filter for visible and near infrared light (catalogue number cv 400-1200). The filtered light was received by a photo-diode, the signal from the diode was amplified using a HAMMATSU C2719 amplifier, and converted using a National Instruments Lab PC card to a digital file. These files were further processed using wavelength and amplitude calibration files. The sunlight passed through a 2 mm hole at the top of an aluminum tube. The whole device was mounted onto a dish, that was adjusted by a computerized tracking system, to be at 0^0 angle to the sun throughout the day (Fig. 2.4).
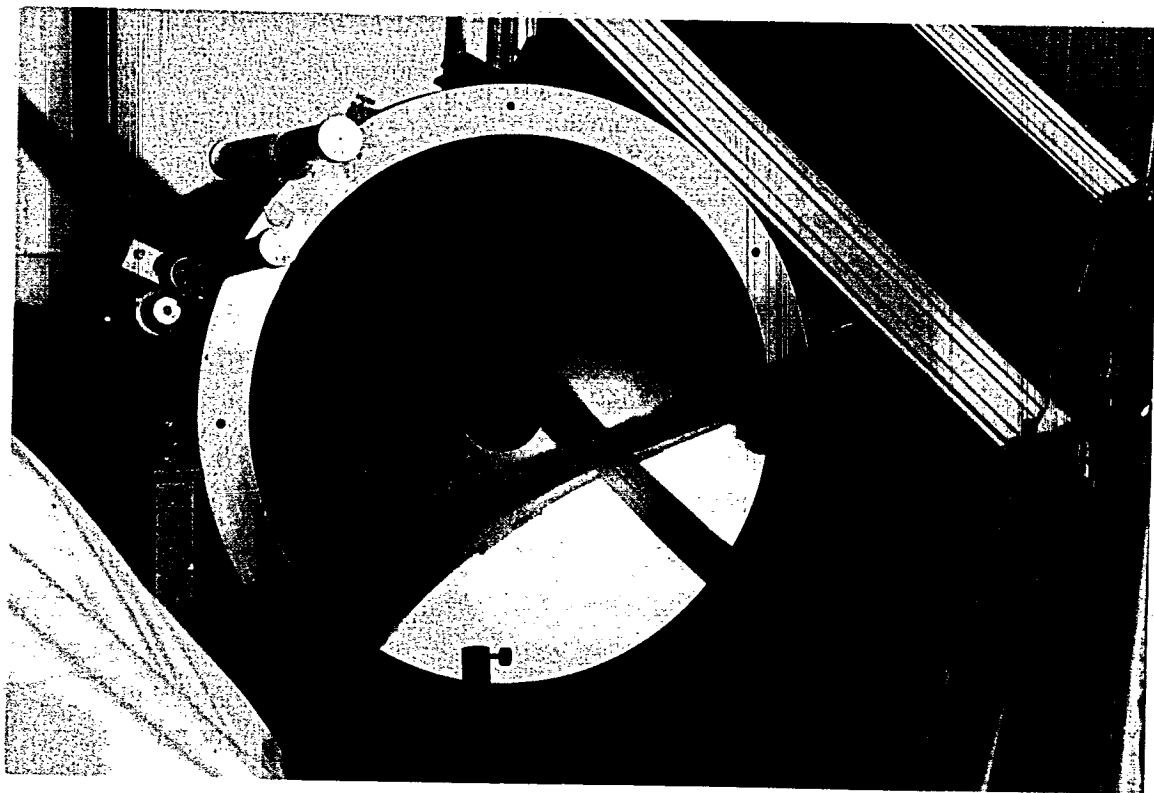
Figure 2.4: The spectrometer mounted on the tracking dish.

2.2.2 Photosynthetic performance -

    2.2.2.1 Oxygen evolution measurement was carried out for determination of photosynthetic quantum yield - By using a Clark-type DW2/2 oxygen electrode unit designed by Delieu and Walker, (1981) connected to a CB1-D O_2 control box, Hansatech Instruments. The output signal was recorded by a dual channel model BD112 flatbed recorder (Kipp and Zonen). As the light source, a slide projector (having maximal light intensity of 2900 $\mu E/m^2sec$) connected to an optical fiber was used. Different combinations of neutral density filters (Balzers AG) were used in order to achieve the different light intensities. For each response curve, initially acclimation time of 3 minutes under maximal light intensity was performed, optimal time of exposure to each light intensity was determined to be 2 min. Photosynthetic quantum yield was determined by the slope of the linear part of the light response curve (i. e. the range measured under low light intensities), maximal rate of photosynthesis was extracted from the light saturated part of the curve.

2.2.2.2 Fluorescence Induction measurement - was carried out for determination of photosynthetic efficiency and the size of RCII. The measurements were carried out in vivo, on 3 ml samples taken from the Bioreactor at different times during the experiments (see 2.1.4). The samples were dark adapted for 45 seconds before the measurement. Fluorescence was measured with a pulse amplitude modulation fluorometer (Model PAM 101 Chlorophyll Fluorometer; H. Walz, Effeltrich, Germany). Light from a 500-w quartz lamp passed through an optical system containing computer controlled shutter, a water filter and glass interference filters passing a band around 550 nm (half band about 20 nm). The photo detector was a red sensitive photo multiplier and a narrow band 685 nm interference filter was used to isolate the fluorescence. The opening of a shutter introduced the actinic light onto the surface of the suspension quivete (which contained a dark adapted whole cells culture) and excited the fluorescence transients. The data was stored in a digital form, a graphic display of the fluorescence as a function of time was produced and induction time was then calculated.

The theoretical principle of this method is explained in Appendix 2.

2.2.3 Cell production determination-

A sample of cells taken from the Bioreactor was fixed by formaldehyde solution. Then counted by using bright line counting chamber, Hauser Scientific, under a light microscope (Nikon, Japan) at magnification of x 40.

2.2.4 Pigments extraction -

ß-carotene and Chlorophyll a and b determination was carried out by 80% acetone extraction of cells and then determined spectrophotometrically after Arnon (1949) and Ben-Amotz and Avron (1983).

Xanthophylls - Pigments were extracted from whole cells with ethanol:hexane (2:1,v/v) as described by Shaish, Ben-Amotz et al. (1991). HPLC column and solvent conditions, pigments identification and quantification were as described in Levy, Tal et al. (1993). The determination of the epoxidation state of xanthophylls was based on the following calculation, suggested by Demmig-Adams, (1990) :

$$(3) \quad \text{Epoxidation State} = (V+0.5A) / (Z+A+V)$$

Where V, A and Z are for Violaxanthin, Antheraxanthin and Zeaxanthin concentrations respectively.

2.2.5 Cbr Analysis -

The presence of Cbr in protein profiles of algae treated with different light regimes (both intensity and spectrum) and its daily response was detected by 15% Polyacrylamide SDS Gel electrophoresis (Laemmli, 1970) and immunoblot analysis using anti-Cbr antibodies as described in Levy, Gokhman et al. (1992).

3. Results

3.1 Daily cycle

In order to follow the daily photosynthetic response of *D. bardawil* to the changes in the sunlight spectrum and intensity during the daytime, both the sunlight and algal photosynthetic characteristics were monitored simultaneously. The relationships between the changes in the sunlight and photosynthetic production, pigmentation, Cbr accumulation and photosynthetic efficiency were determined.

In addition to the experiments which followed the natural daily cycles, a set of experiments was carried out manipulating the pattern of the daily change in the sunlight intensity. In these experiments, the daily response of algae exposed to a constant light dose throughout the day (i.e. treatment) was compared to the daily response of algae exposed to the natural cycle of the daily change in intensity (as shown in Fig 3.1). One set of the bioreactors (i.e. treatment) was exposed at an angle < 90⁰, such that light intensity at mid-day did not increase above that at 10:00 AM (time of optimum photosynthesis). The control set of bioreactors was situated at 90⁰ to the plane of the sunlight radiation such that it was exposed to the natural variations in intensities (see 2.1.3.2 for details).

Trends over the annual cycle are discussed separately below, but note that in spite of a seasonal trend, daily patterns were similar throughout the year.

3.1.1 The Sunlight daily cycle -

The distribution of the sunlight intensity with time has a bell shaped image (Fig 3.1), reaching a maximum around 800w/m^2 at midday and then decreasing until dark. This pattern was typically observed in bright days. The daily spectral distribution of the sunlight spectrum is shown in Figure 3.2. Most changes in the spectrum (due to the changes in the sunlight elevation angle) are in the blue region (400-490nm). Maximal values of blue/red light appear at midday when elevation angle and sunlight intensity are increased (see below).

Figure 3.3 shows possible relations between spectrum and intensity of the sunlight during the daily cycle. The 3 indexes which were chosen in order to characterize the daily spectral changes in the sunlight are blue/total, PAR/total and 480/650 nm (blue = 400 nm to 490 nm; total = 400 nm to 1000 nm and PAR is the photosynthetic active radiation range between 400 nm to 700 nm).

PAR/total intensity ratio seems to stay constant with time, while the 480/650 nm ratio shows sensitivity to the changes in intensity (Fig 3.3b). A weaker correlation was observed between the ratio of Blue/total and light intensity (not shown).
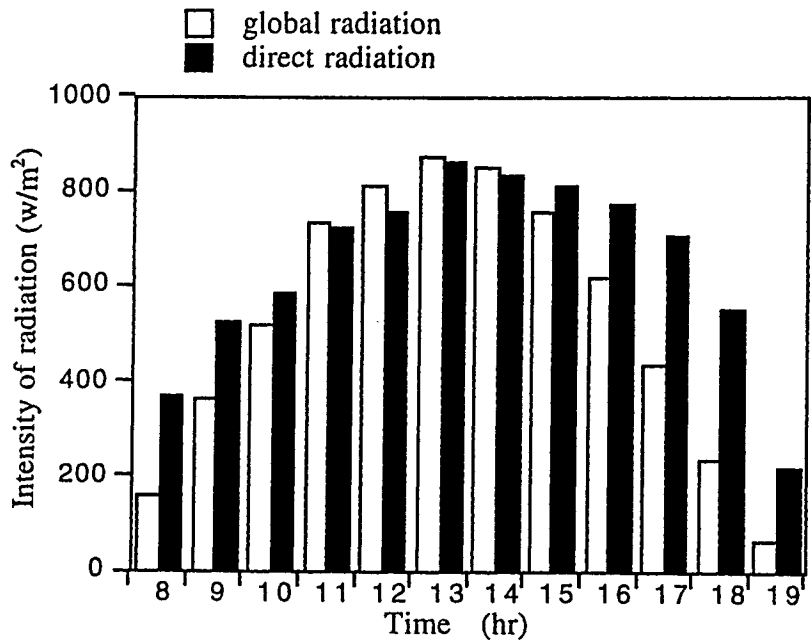
Figure 3.1: Distribution of sunlight radiation over a typical bright summer day, (the higher values of direct radiation relative to the global radiation values observed during the afternoon, is an artifact in the global results, due to the set up of the measurements equipment).
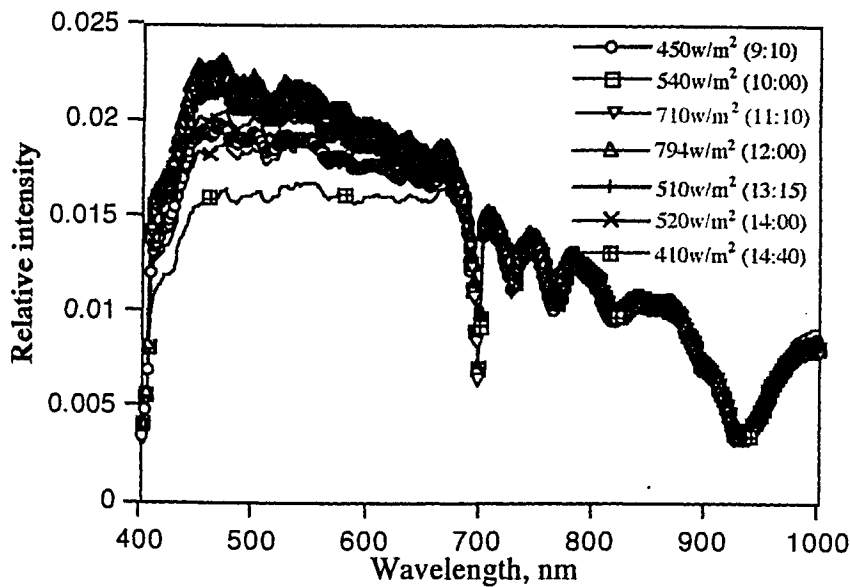
Figure 3.2: The spectral distribution of direct sunlight at different times of the day, the legends refer to the different sunlight intensities (in w/m²) measured at different times of the day. The sharp decrease around 700 nm is an experimental artifact due to exchange of the interference filters within the spectroradiometer.
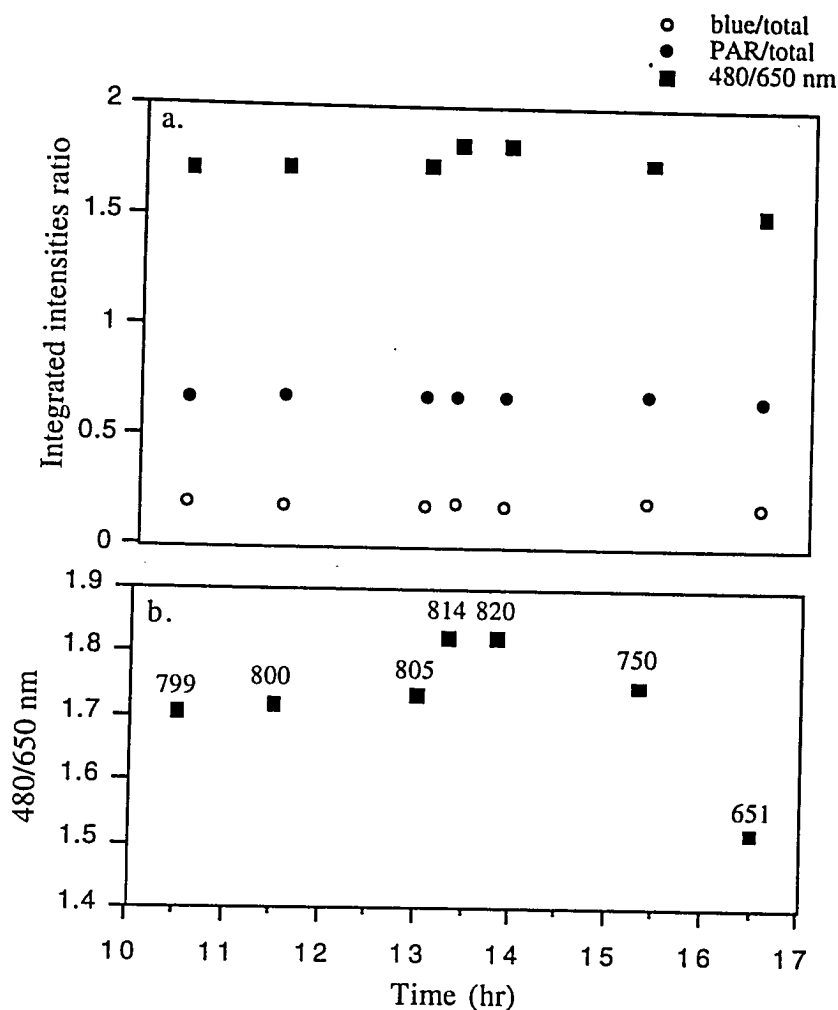
Figure 3.3: (a) The variations of sunlight spectrum with time, expressed by different spectral ratios.

(b) The ratio of 480/650 nm in an expanded y-scale (the values on the top part of the squares are the direct intensity in W/m^2 measured at that time).

Measurements before 10:00 am or after 17:00 pm could not be perform due to technical obsticles.

A linear correlation between the direct sunlight intensity and the ratio of 480nm/650 nm was observed (Fig 3.4). This correlation can be explained by Rayleigh's law. Rayleigh's law defines scattering entirely due to air molecules with a negligible contribution from dust and it states that the efficiency of scattering is proportional to $1/\lambda^4$, i.e. inversely correlated to the wavelength. Therefore shorter waves are scattered in a higher extent than the longer ones. This low holds for scattering by air molecules that are smaller than the wavelengths of solar radiation.

Therefore, at midday when the elevation angle of the sun increases, sun's radiation path length in the sky is minimal and the blue light is less scattered, this results in both maximal light intensity and increase in blue/red light ratio (the opposite occurs under conditions of a small elevation angle, then blue light is scattered significantly, causing a decrease in both light intensity reaching the earth and in the blue/red light ratio). It should be noticed that this pattern of the daily

change in the sunlight spectrum and intensity holds in clear days only. In cloudy days, the total sunlight intensity is reduced independently of wavelength (see 1.1 and 4.1).
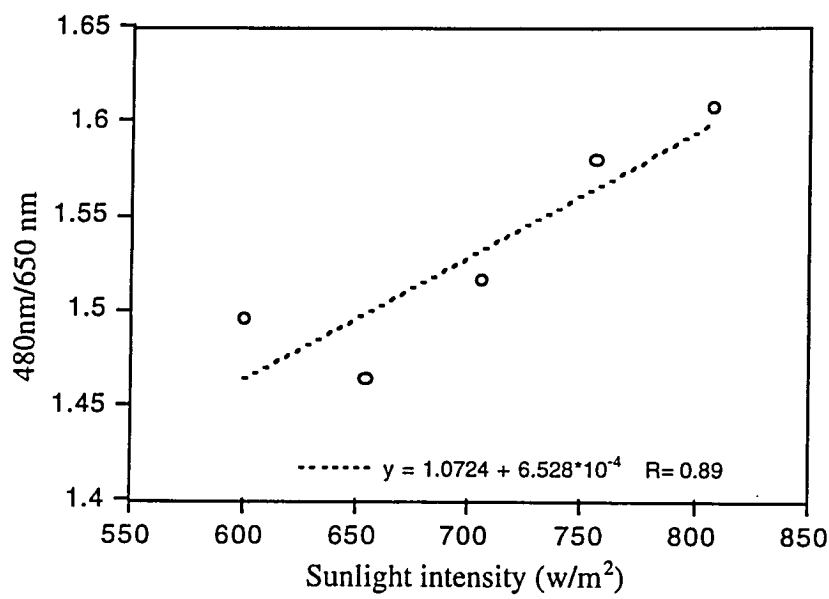
Figure 3.4: Linear correlation between sunlight intensity and the ratio of 480/650 nm.

3.1.2 Cell Production -

Typical division cycle of *D. bardawil* within the photobioreactor is shown in Figure 3.5. Cell division occurred mainly during the night time and doubling time was about 24 hr.

Although total cell yield varied during the year (as discussed in 3.2.2) the occurrence of division at night time was consistent.

3.1.3 Pigments-

Pigments concentrations (Figs 3.6-3.9a) were calculated on per cell and per ml basis, in order to expand the basis of information. While the concentrations per cell reflect the capability of the cell to synthesize pigments, the concentrations per ml give information about the optical density of the culture. The optical density of a culture is an important factor which indirectly affects the light spectrum and intensity reaching a single cell and therefore influencing the need for pigmentation by a single cell. Furthermore, monitoring pigments concentration per cell while comparing two different cultures avoids the effect of cells concentration. Therefore, the two parameters of per cell and ml concentrations must be both considered in order to include all the aspects of pigments synthesis.

ß-Carotene : The concentration of ß-carotene per cell (Fig 3.6) increased during the day time and then decreased at night time. This seems to be mostly due to cell division. Concentrations per ml show a gradual accumulation in a longer time scale (of days) until the third day afternoon (Fig 3.6 Nov. 26) when it reached a stable phase at night and then a slight decrease towards the next morning. This decrease indicates on degradation of ß-carotene which probably lies in the fact that at this stage, cells density was increased (see Nov. 26-27, Fig 3.5) and the need for ß-Carotene for protection against high light was reduced. This assumption is supported by the results of concentrations per cell which show a decrease from Nov. 26 in the afternoon.

The steady phase of ß-carotene concentration per ml observed on the third day night (and in other cases which are not shown here), indicate on the stability of ß-Carotene under dark conditions.

A similar pattern of accumulation was detected in *Haematococcus pluvialis* during a sunlight cycle by accumulating the red ketocarotenoid astaxanthin (Chaumont and Thepenier, 1995).
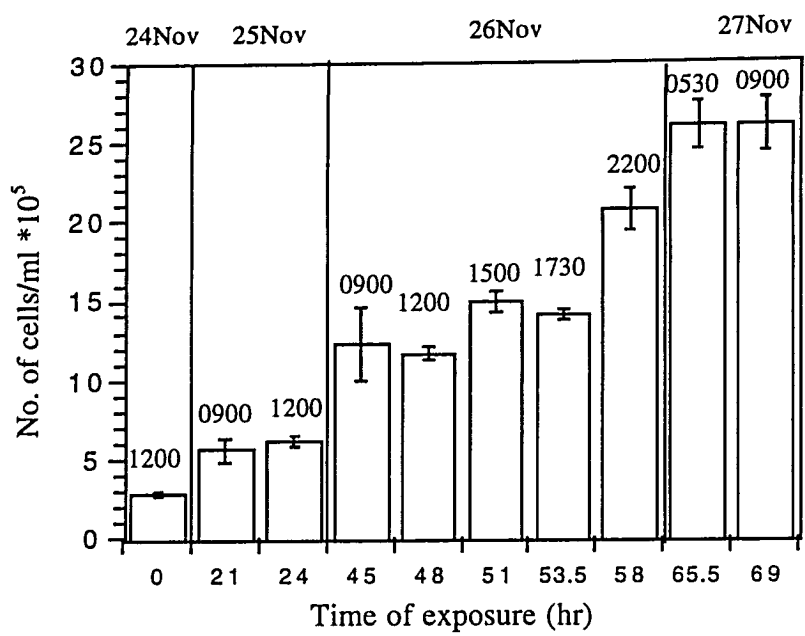
Figure 3.5: Cells production of *D. bardawil* in the photobioreactor during November, 24-27, 1997. (Values below the columns represent the time (in hr) from beginning of experiment, the values above represent the day time and the vertical lines separate the days of experiment).
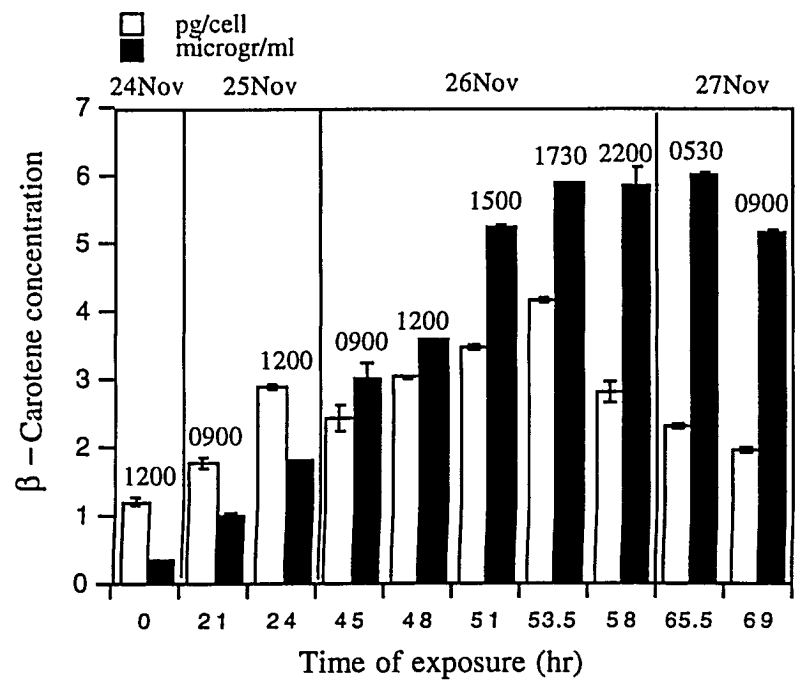
Figure 3.6: ß-carotene production by *D. bardawil* in the photobioreactor during November, 24 - 27, 1997 (empty columns are for concentration in pg/cell, full columns are in µgr/ml and the vertical lines separate between the days of experiment).

Chlorophyll : The pattern of the daily change in chlorophyll concentration is shown in Fig 3.7. The concentration of chlorophyll per cell versus time, seems stable during the first two days and then slightly increases on the third day. A decrease at night is observed between 26-27 in Nov. The decrease after dark was probably due to cells division. On a shorter term of hours, both concentrations per cell and per ml, decreased at noontime of the third day (26 Nov., fig 3.7) and then recovered in the afternoon. The decrease at noontime may reflect degradation of chlorophyll under high light intensity conditions, a phenomenon which was observed before in other systems (Pick, Gounaris et al., 1987) and others, and was explained mainly by chlorophyll b degradation (see also 4.3.2).

On a longer time scale of a few days, changes in the daily average concentration per cell were relatively small in the first 2 days of experiment while on the third day an increased average was observed. This is expected due to effects of self shading when cell density increases (observed also in ß-Carotene synthesis). Chlorophyll concentrations per ml increased moderately throughout the experiment. This is firstly due to synthesis per cell (every day), and secondly due to the increase in cell production. The decrease in concentration at noon time of the third day is observed also in concentration per ml supporting the assumption that chlorophyll was degraded at that time.
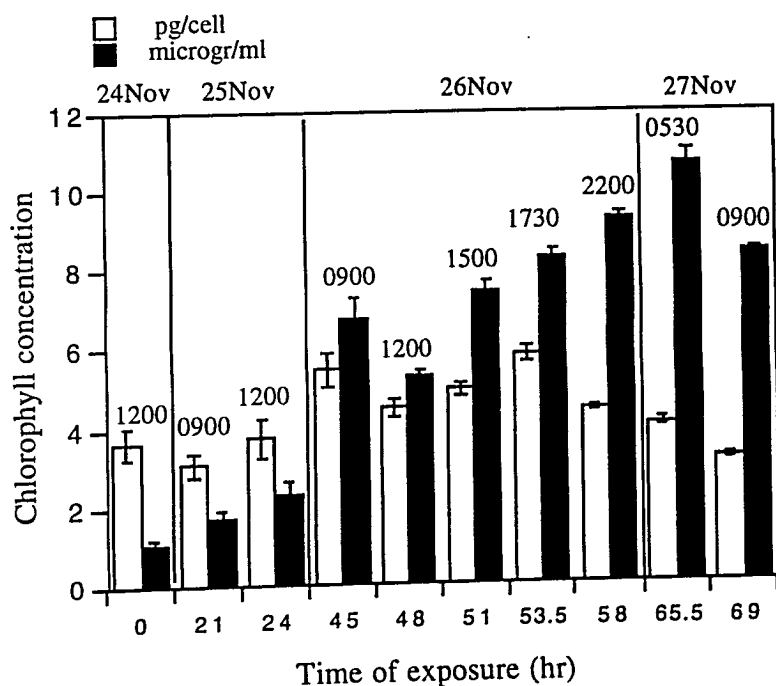
Figure 3.7: Chlorophyll production by *D. bardawil* within the photobioreactor during November, 24 - 27, 1997 (empty columns are for concentration in pg/cell, full columns are in µgr/ml and the vertical lines separate between the days of experiment).

Chlorophyll a/Chlorophyll b ratio : The diurnal changes in Chl a/Chl b ratio during 4 days in November are shown in fig 3.8. On the third day of experiment (Nov. 26) the ratio increased from the morning, accompanying the increase in sunlight intensity (and increasing blue/red peaks ratio) then decreasing again in the afternoon and at night time. This pattern of daily change is consistent with the daily change observed by total chlorophyll per cell (Fig 3.7, Nov. 26). Unexpectedly, in the longer time scale of days, the ratio of Chl a/Chl b at noontime (see 12:00 everyday, Fig 3.8) was inconsistent with total chlorophyll results (Fig 3.7). The slight increase in total chlorophyll was accompanied by a dominant increase in the Chl a/Chl b ratio, while inverse relations were expected. Nevertheless, such inconsistencies between total chlorophyll to Chl a/Chl b ratio are discussed in 4.3.2 and the trend of increased Chl a/Chl b ratio can be the response of acclimation to the higher light intensity existing outdoors compared to the conditions of growth before the experiment.
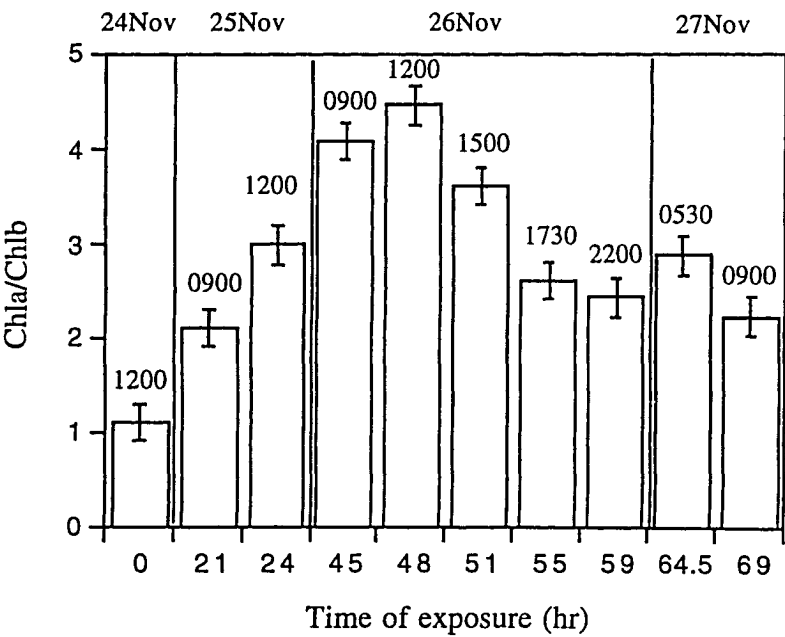
Figure 3.8: Chlorophyll a/b ratio analyzed from *D. bardawil* within the photobioreactor during November, 24 - 27, 1997 (the vertical lines separate between the days).

-Xanthophyll cycle: The dynamics of xanthophyll cycle is represented by the epoxidation state of xanthophylls (as formulated in 2.2.4) during the diurnal cycle. Figure 3.9a shows that the epoxidation state was sensitive to the daily changes in sunlight, showing higher values (indicating on low Zeaxanthin levels) in the morning time (when light intensity and blue/red light spectral ratio are low) and at night when there is no light stress. The low level of epoxidation which remains until sunset (on the second of April) shows that the reactions of the xanthophyll cycle lag behind the daily changes in sunlight, therefore relatively high epoxidation state still exists at 12:00 noontime when light stress is almost maximal, then decreasing during the afternoon and recovers again after sunset.

3.1.4 The daily cycle of Carotene Biosynthesis Related protein (Cbr) -

A clear diurnal cycle was observed in Cbr concentrations (Fig 3.9b). Cbr accumulated in the day time and degraded completely during the night. This may reflect unstability of Cbr compared to the pigments involved in the energy dissipation process. During the day time, the Cbr concentration reached a maximum in the afternoon and then degraded during the late afternoon until darkness. As in the case of the xanthophyll cycle, there seems to be a lag in the order of a few hours compared to the changes in the sunlight. There is a clear correlation between the level of epoxidation state and Cbr concentration. This correlation, between the stress pigment - Zeaxanthin and Cbr induction will be further discussed in 4.3.4.
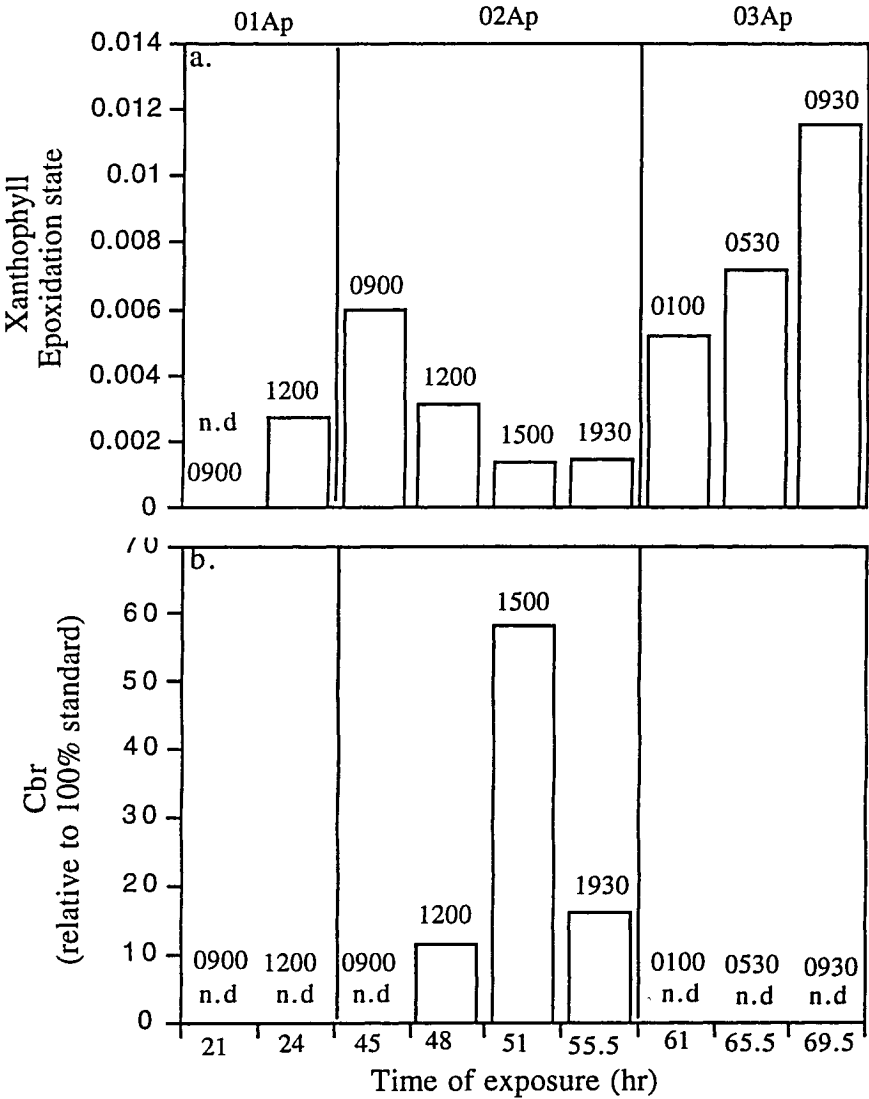
Figure 3.9 : The daily fluctuations in (a) The Epoxidation state of the xanthophylls (presented in dimensionless units). (b) The induction pattern of Cbr (Carotene Biosynthesis Related) protein (presented in percent per 100% standard of 50λ pure extraction of Cbr, n.d is below the detection range). The vertical lines separate between the days.

3.1.5 Antennae size and Photochemical efficiency of PSII estimated from fluorescence induction measurements -

3.1.5.1 Antennae size : The photosynthetic antennae size (Fig 3.10; 11Sep.) decreases at mid-day (when light intensity increases) and then recovers again during the afternoon. It therefore seems to be tightly related to the daily changes in the sunlight features (Figs 3.1-2). As expected, under high light conditions (Webb and Melis, 1995) existing at mid-day, "small antennae" is observed compared to the morning and afternoon.

An acclimation response on a longer time scale of days is also observed (Fig 3.10, during the 10 and 11 in Sep, compare values at 14:00). Since the culture was transferred from the lab (low light) to the field (high light) .
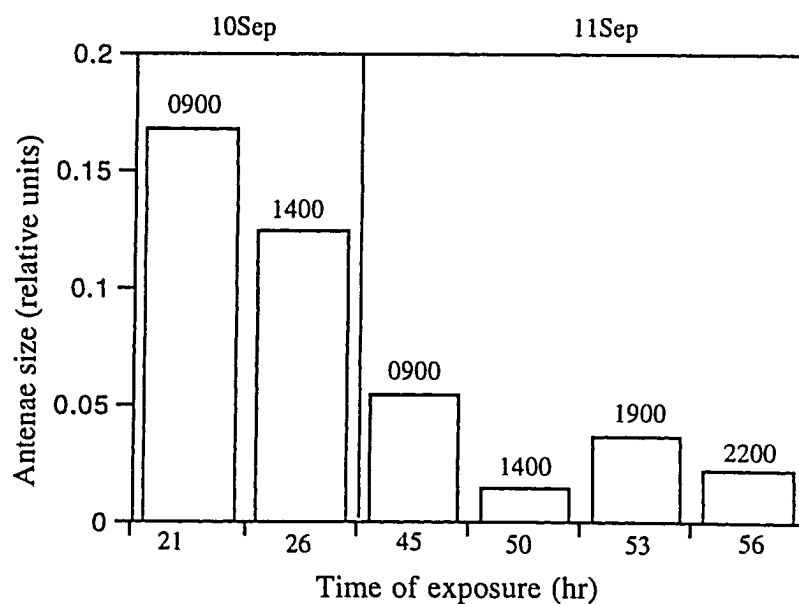
Figure 3.10 : Antennae size of RC II in *D. bardawil* as estimated from fluorescence induction measurements during a course of two days in September 97, (the relative units of the antennae size were accepted after normalizing the area above the fluorescence induction curve by dividing it to F_v, the value of Variable fluorescence, see also Appendix 2). The vertical lines separate between the days.

3.1.5.2 Photosynthetic efficiency : The efficiency of photosynthesis as estimated from fluorescence induction measurements (Fig 3.11a) correlates negatively with the light intensity. The low efficiency observed at midday is consistent with the phenomenon of a "midday depression" (Raschke and Resemann, 1986; Demmig-Adams, Adams III et al., 1989) observed in higher plants.

Such a decrease in photon efficiency at midday (when the given light dose exceeds the capacity of the photosynthetic machinery) may be the result of either damaging effects to the system, or of regulated processes leading to the dissipation of excess excitation energy within the photochemical apparatus (Demmig-Adams, 1990). Consequently, the observed decrease in the efficiency of photosynthetic energy conversion caused by high light, is often followed by recovery upon return to low light (as occurs in nature during the afternoon and early evening).

The increase in photosynthetic efficiency from Sep.10 to Sep.11 (Fig 3.11a) is consistent with the decrease in antennae size discussed above. After acclimation to the high light conditions (i.e. decreased antennae size in Sep. 11) an increase in photosynthetic efficiency can be expected, since damage by high light is decreased.

3.1.6 Quantum yield of photosynthesis estimated from Oxygen evolution technique-

The daily trend of the slope of the photosynthetic light response curve (Fig 3.11b), resembles the fluorescence induction results (Fig 3.11a, second day). The observed decrease in the quantum yield at midday and the afternoon recovery support the photosynthetic efficiency results. The possible reasons for this pattern of change are discussed in 4.4.

All the parameters above were shown to be affected by the day-night cycle and/or by the changes in the light environment during the day. It is already known that some of the acclimation processes occur due to changes in the light intensity. However, based on the daily interactions observed between the sunlight intensity and the blue/red light ratio (Figs 3.2-3.4), I hypothesized that not only sunlight intensity is involved in controlling the acclimation processes, but also - the changes in the blue/red light ratio, or principally blue light, play a direct role. This assumption was tested by artificially modifying the blue/red light spectrum and attempting to separate the spectral effect from variation in intensity over the day time. The results are shown and discussed in 3.3 and in the Discussion.
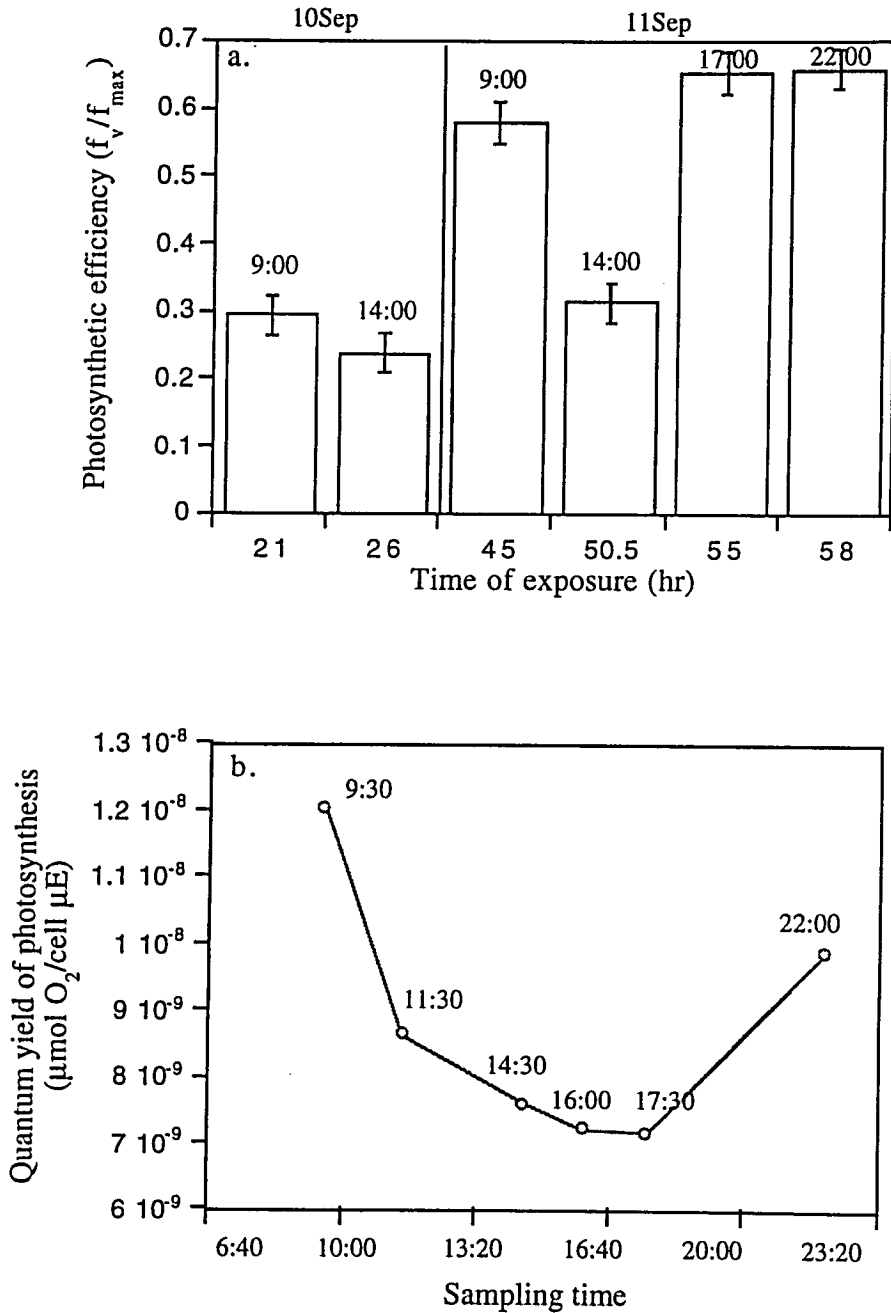
Figure 3.11 : Daily change in Photosynthesis performance. (a) Results of photosynthetic efficiency concluded from fluorescence induction measurements performed during a sunlight cycle of two days in September, (the vertical line is for separation between the two days). (b) Quantum yield of photosynthesis during a sunlight cycle of the third day of experiment in May, extracted from the initial slope of oxygen evolution measurements.

3.1.7 Manipulating the pattern of the daily change in the sunlight intensity -

In this set of experiments, the daily response of algae exposed to a constant light dose throughout the day (i.e. treatment) was compared to the daily response of algae exposed to the natural cycle of the daily change in intensity.

Two experiments of this kind were conducted, one in May and the other in July 1998. The inclination angle of the treated bioreactors was calculated in such a way that the sun daily orbit would give a constant value of light intensity through the day (see 2.1.3.2) which equals to it's value in the late morning. Control bioreactors were situated in an 90^0 angle to the plane of the sunlight daily apparent movement, so that the daily change in the sunlight intensity is parabolic (Fig 3.1).

The motivation for these experiments was based on two hypotheses :

- A decrease in the high light conditions measured at mid-day will avoid the phenomenon of the midday depression in photosynthetic yield observed in other experiments, therefore no photoinhibition at noon time will occur and overall efficiency of photosynthesis will be increased.

- Due to the fact that in both treatment and control, the daily changes in spectrum are similar while the intensity changes in a different manner, it is possible to isolate the component of the light spectrum from the light intensity. Therefore to follow the impact of each on the photosynthetic acclimation through the day time, and to estimate the spectral contribution to the daily photosynthetic adaptation processes.

3.1.7.1 Cell production : Cells production as well as growth rate results didn't differ significantly between treatment (inclined bioreactors) and control (results not shown).

3.1.7.2 Pigments : ß-Carotene - As seen in Fig 3.12, the concentration of ß-Carotene both per cell and per ml didn't differ significantly between control and treatment (except an unclear difference in concentration per cell which is observed at 18:00), also the pattern of change in the concentration during the day was similar in both treatment and control.

Chlorophyll - As seen in Fig 3.13 in both cases (per cell and per ml) chlorophyll concentration was significantly higher in the control culture. The difference was expressed also in the pattern of change. While the control concentration of chlorophyll seemed to gradually accumulate from noon time during the day, the chlorophyll concentration in the treated culture seemed to stay invariable during the afternoon.
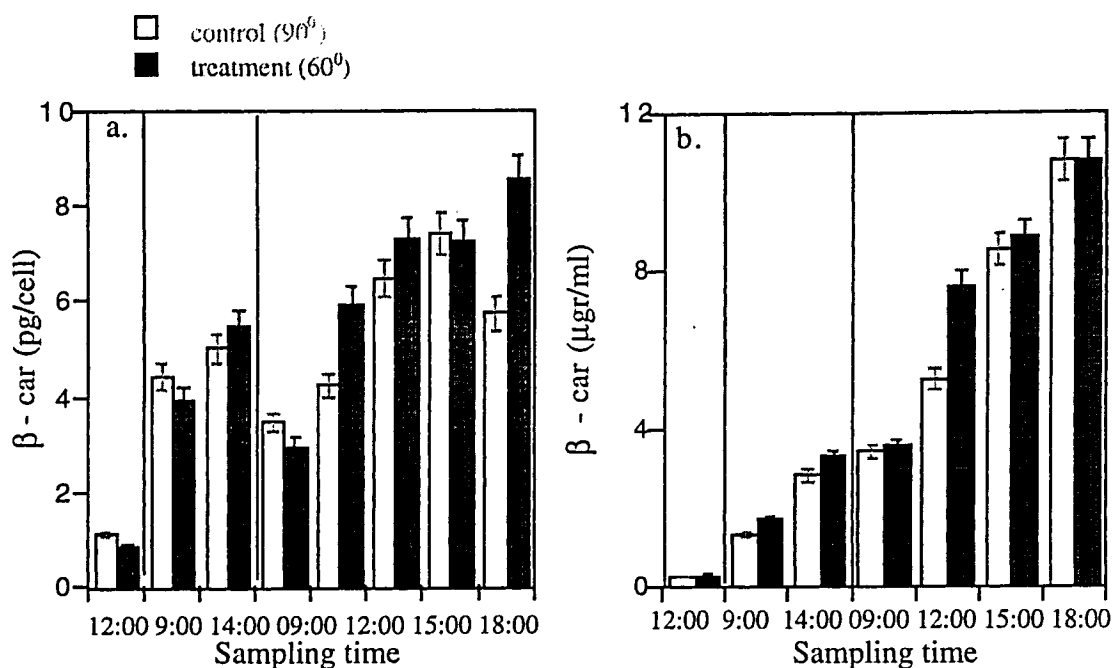
Figure 3.12 : Daily pattern of changes in ß-carotene concentration under different intensities of sunlight during 3 days in July. Full columns are for treatment (i.e. inclined bioreactors) of constant intensity of radiation (~ 550 w/m^2) during the day time, and empty columns are for control (receiving the original changes in sunlight intensity through the day time).

(a) Concentration of ß-carotene per cell.

(b) Concentration of ß-carotene per ml. The vertical lines separate between the days.
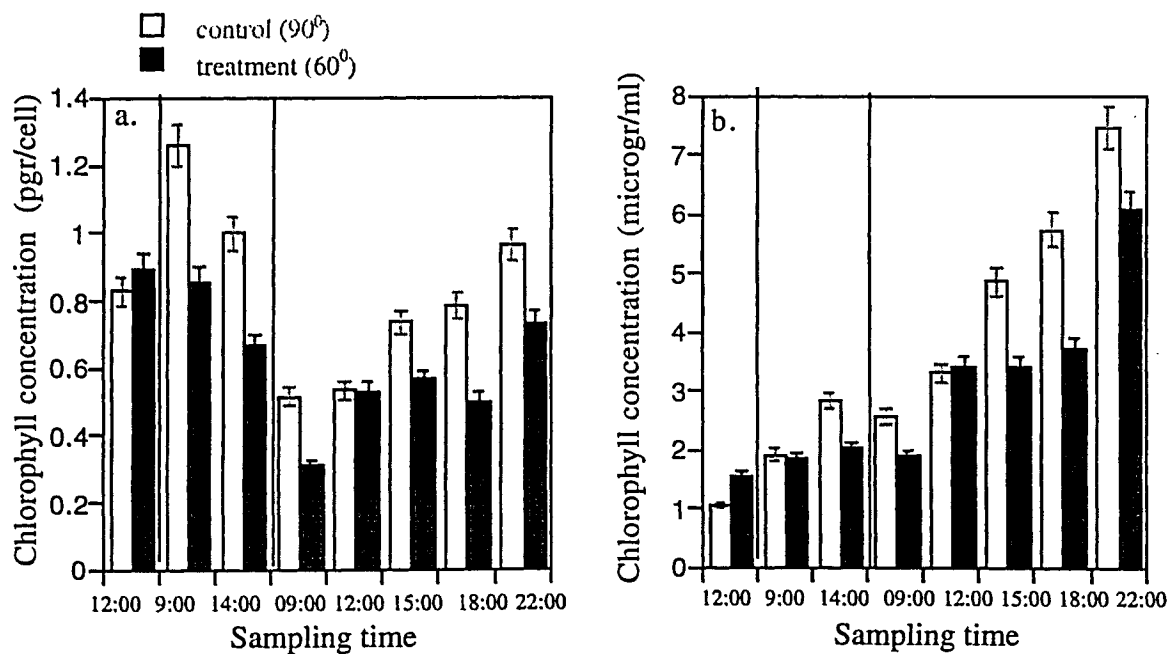
Figure 3.13 : Daily pattern of changes in chlorophyll concentration under different intensities of light during 3 days in July, full columns are for treatment of constant intensity of radiation (~550w/m^2) during the day time and empty columns are for control (receiving the natural changes in sunlight intensity through the day time).

(a) Concentration of chlorophyll per cell.

(b) Concentration of chlorophyll per ml. The vertical lines separate between the days.

ß-Carotene to Chlorophyll ratio - The car/chl. ratio is shown in Fig 3.14. The pattern of change during the experiment seems the same in both treated and control culture (i.e. gradual increase through the experiment and a decrease at 22:00 third day), nevertheless the absolute values differ. The higher values were found in the treated culture (i.e. receiving constant intensity of radiation through the day), compared to the control during the second and third day of experiment. This is mainly due to the significantly lower chlorophyll concentration which was detected at the treated culture (Fig 3.13). However, this is unexpected due to the fact that the integral daily light dose was lower by the treatment, (see also 4.3.2).

Chlorophyll a/ Chlorophyll b ratio - The Chl a/Chl b ratio during three days of experiments under two different positions to the plane of sunlight radiation (Figure 3.15) shows only a slight change in the first two days of experiment, and no difference between control and treatment. The difference between them started to appear on the third day's afternoon when the control's ratio increases and then diminishes back. Such a pattern of change in Chl a/Chl b ratio is expected under conditions of ordinary daily cycle. The ratio of Chl a/Chl b in the treatment (i.e. constant light intensity during the day) changes relatively little as expected under unvarying light intensity conditions. However, the reasons for the unexpected increase in Chl a/Chl b by the treatment towards 22:00, seem to be unclear.

3.1.7.3 Quantum yield - The pattern of change in controls' quantum yield during the third day of experiment (Fig 3.16) seems to exhibit a "midday depression" in respond to the high light intensity at midday and then a recovery in the afternoon (producing a negative correlation with the daily change in light intensity observed in Fig 3.1). On the other hand, there is only a minor change in the treatment's quantum yield during the daytime and the decrease observed at mid-day is less dominant than in the control.
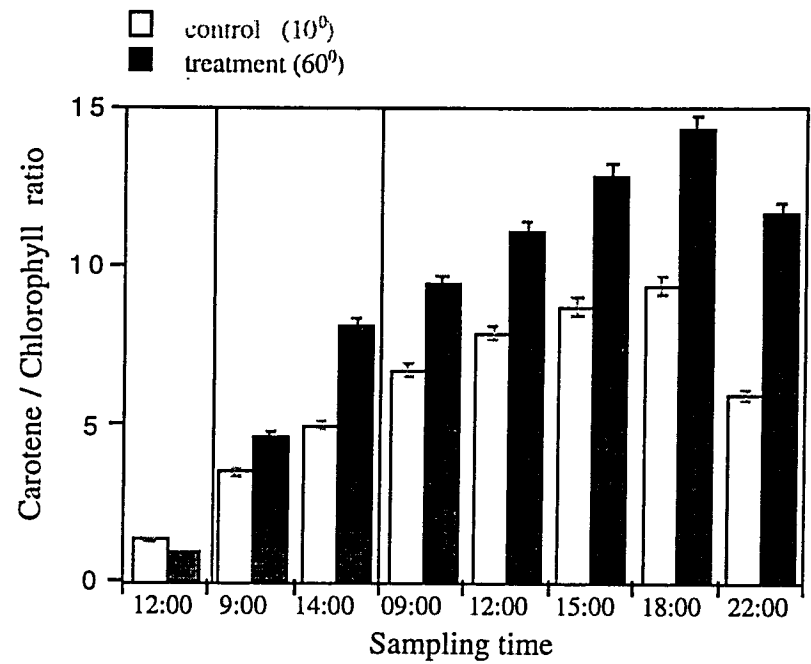
Figure 3.14 : Daily pattern of changes in the ratio of ß-carotene to chlorophyll under different light intensity regimes during 3 days in July. Full columns are for treatment of constant intensity of radiation ($\sim$550w/m^2) during the day time and empty columns are for control (receiving the natural changes in sunlight intensity through the day time). The vertical lines separate between the days.
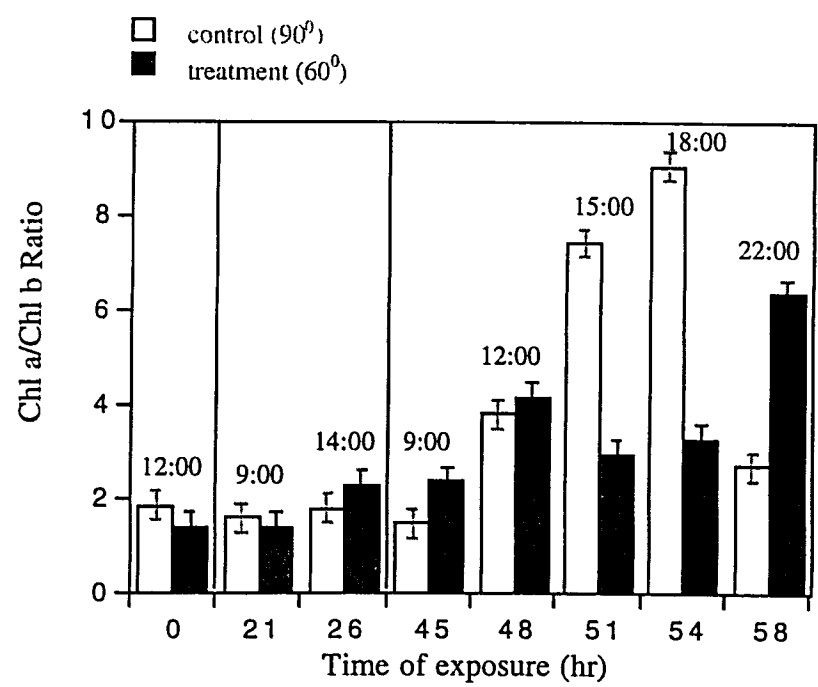
Figure 3.15 : Daily pattern of changes in chlorophyll a/b ratio under different intensities of light during 3 days in July. Full columns are for treatment of constant intensity of radiation ($\sim$550w/m^2) during the day time and empty columns are for control (receiving the natural changes in sunlight intensity through the day time).The vertical lines separate between the days.
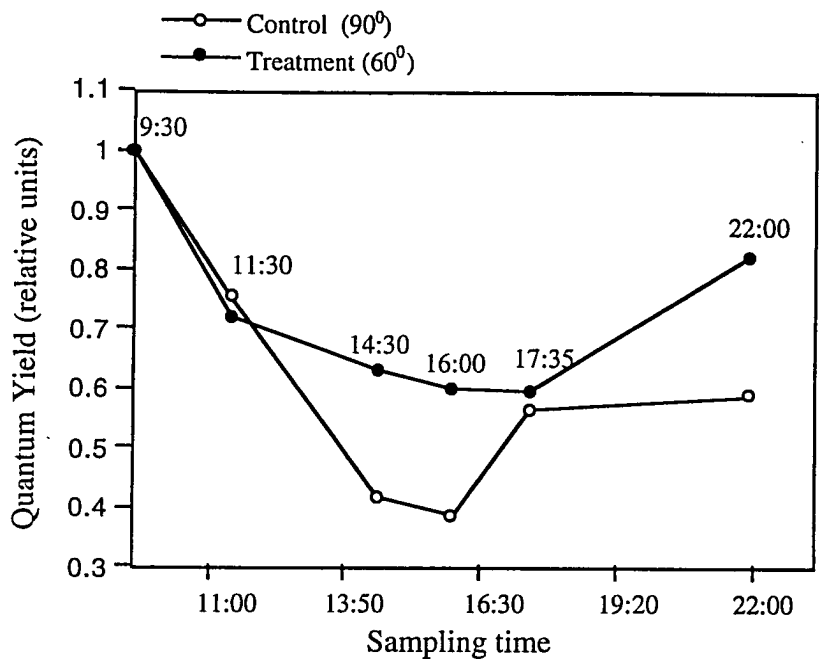
Figure 3.16 : Quantum yield of photosynthesis during the third day of experiment in July, extracted from the initial slope of oxygen evolution measurements, (each plot was divided to its maximal value which was $1.6*10^{-8}$ and $1.2*10^{-8}$ for control and treatment in order to normalize the data). Full circles are for treatment of constant intensity of radiation ($\sim$550w/m^2) during the day time and empty circles are for control (receiving the natural changes in sunlight intensity through the day time).

<u>3.2 The annual cycle</u>

In order to follow and to investigate the photosynthetic response to the annual changes in the sunlight regime (spectrum and intensity), measurements of the sunlight properties were carried out in parallel to measurements of cell production and pigments in different times of the year.

3.2.1 The Sunlight seasonal cycle -

The annual change in the sunlight position relative to the surface (at 32^0 north latitude) (see Fig 3.17a) and the record of sunlight intensity of radiation as measured at the solar tower in the Weizmann Institute of Science in Rehovot (same latitude, Fig 3.17b,c) seem to correlate positively as expected.

The annual distribution of sunlight intensity (the average noontime radiation (Fig 3.17c) as well as the integrated radiation throughout a whole daytime (Fig 3.17b)) is parabolic and correlates to the inclination angle of the sun in our area. It reaches maximum in the summertime (between May and July when inclination angle of the sun is maximal) as expected. There are no remarkable differences between the different years.

Figure 3.18 shows the diurnal change in sunlight spectrum measured through 1995 to 1997, at noontime. As in the case of the daily changes in the sunlight spectrum (3.1.1), the major differences through the year are observed in the blue range of the spectrum. The PAR over the total integrated spectrum range, seems to fluctuate randomly (Fig 3.18a) (implying that the relative <u>proportion</u> of energy source available for photosynthesis is more or less the same over the year). Both blue/total and 480 nm/650 nm intensity ratios (Fig 3.18 b,c) seem to increase during the summer months (observed mainly by the blue/whole ratio). Similar pattern of spectral change was observed in each year as well. The correlation between the proportion of blue light and intensity over the year is not high as observed in the daily cycle. The discrepancy is in the summer time when sunlight intensity is maximal (Fig 3.17 b, c) and we would expect to see maximal blue/red light ratio. Instead, there is a plateau in the summer time and two peaks in the spring and autumn. This phenomenon can be explained by the fact that there are many hazy days in the studied area during the summer time. The summer haze induces a farther scattering of the blue light, and a decrease in its intensity. Still, the values of blue light / total light observed in the summer (Fig 3.18b) are higher than at the winter time (when light intensity decreases).
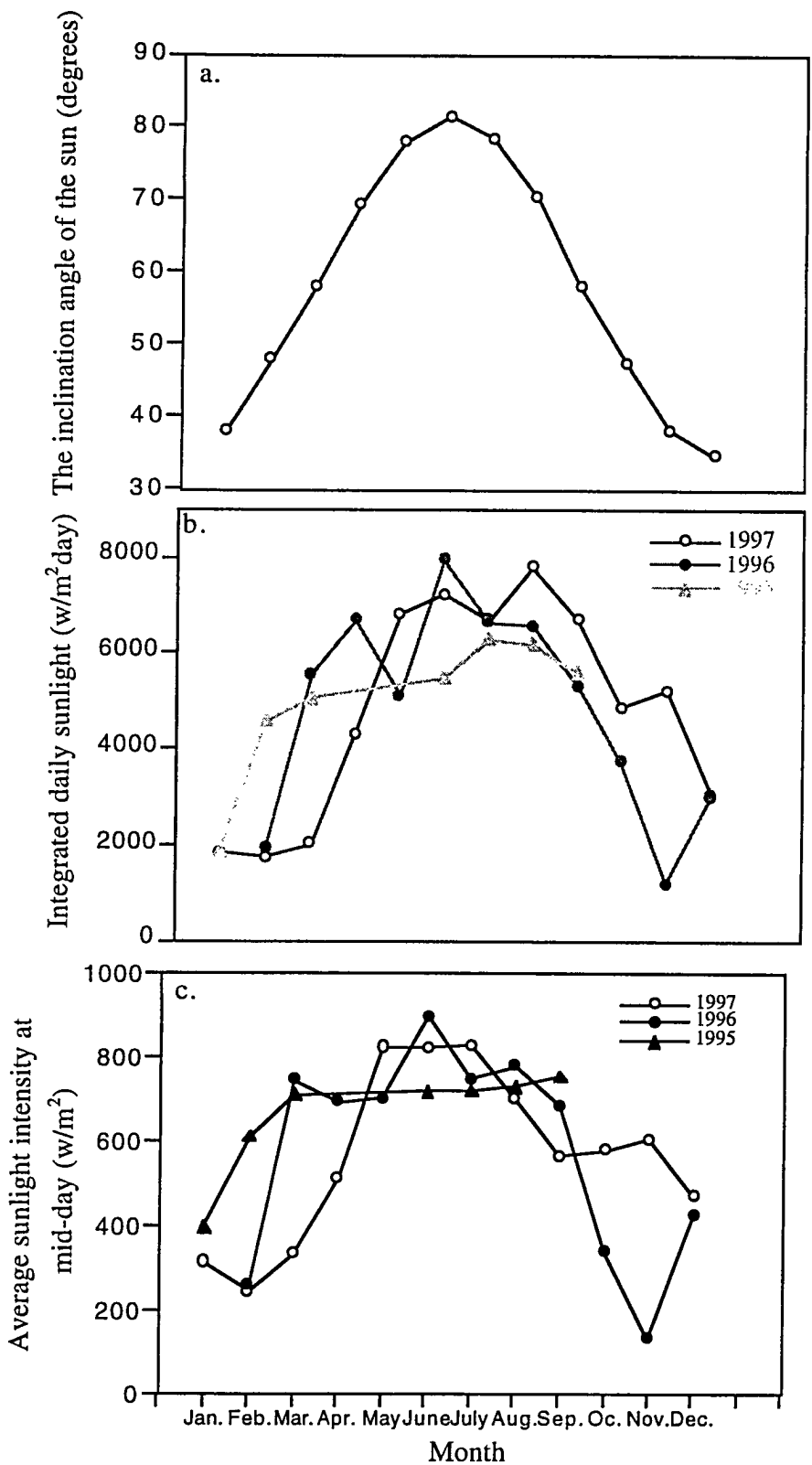
Figure 3.17 : The annual changes in :
(a) The solar position relative to the surface of the studied area expressed as the altitude angle of the sun at 12:00 (when azimuth is zero; (Plante, 1993)).
(b) The daily integrated sunlight radiation (averaged monthly).
(c) The average sunlight intensity at peak hr's of the day (from 11:00 to 15:00).
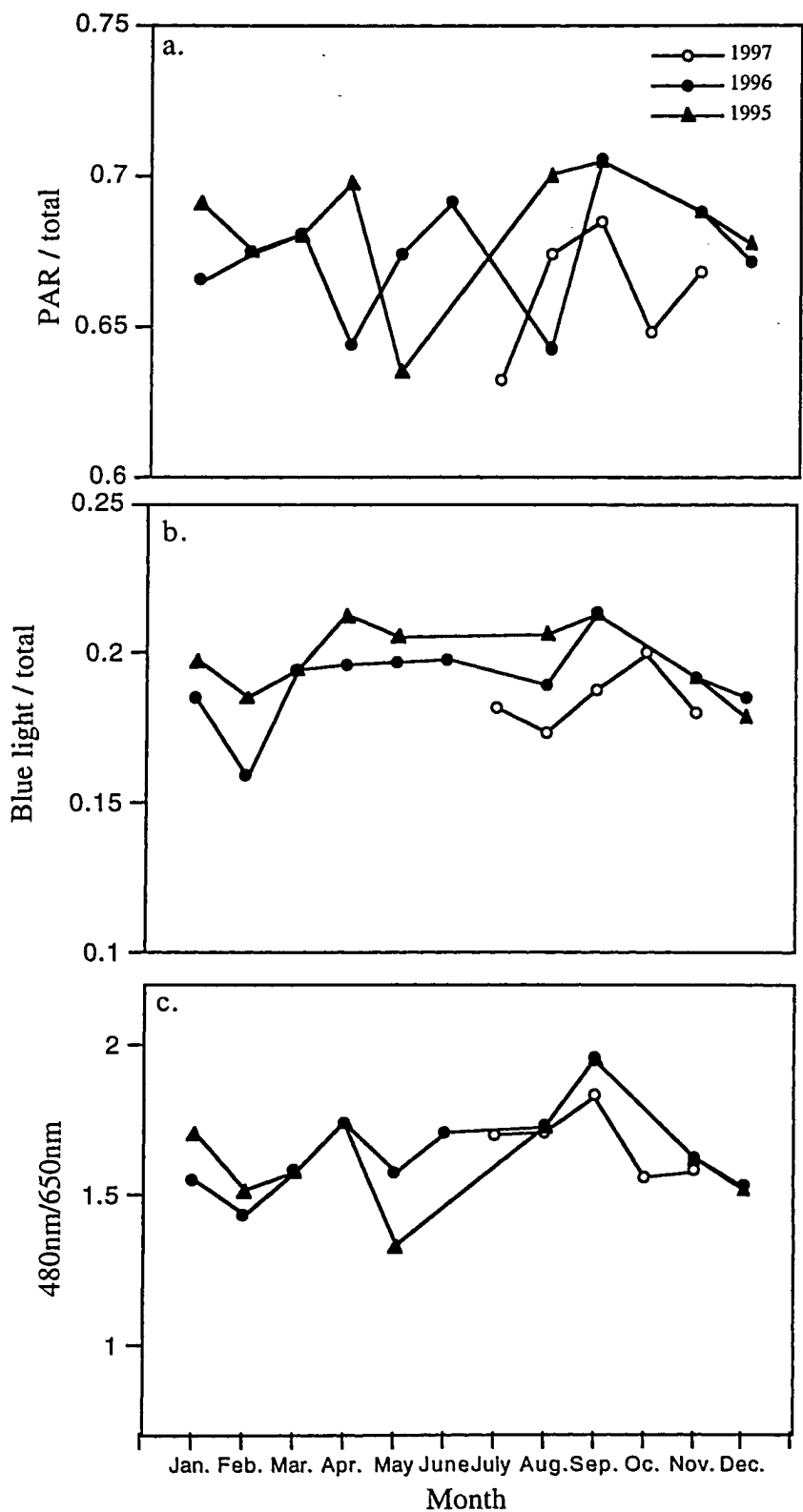
Fig 3.18 : Annual variations in the spectral features of the sunlight (measured at noontime through the year).

(a) Change in the ratio of PAR (photosynthetic active radiation, i.e. 400 to 700 nm) to the integrated intensity of the total measured spectrum (400 to 1000 nm).

(b) Change in the ratio of the intensity of blue light (400 to 490 nm) to the integrated intensity of the total measured spectrum (400 to 1000 nm).

(c) Changes in the ratio of the intensity at 480 nm to 650 nm.

3.2.2 Cell Production -

The Cells production and the rate of growth (per 24 hr's) on the third day of experiment over the years 1995-97 (Fig 3.19) are in correlation as expected.

In general, the shape of both figures resembles a mirror image of the annual distribution of sunlight intensity (Fig 3.17 b, c). On the surface, this implies a negative correlation between sunlight intensity and both cell production and the rate of growth.
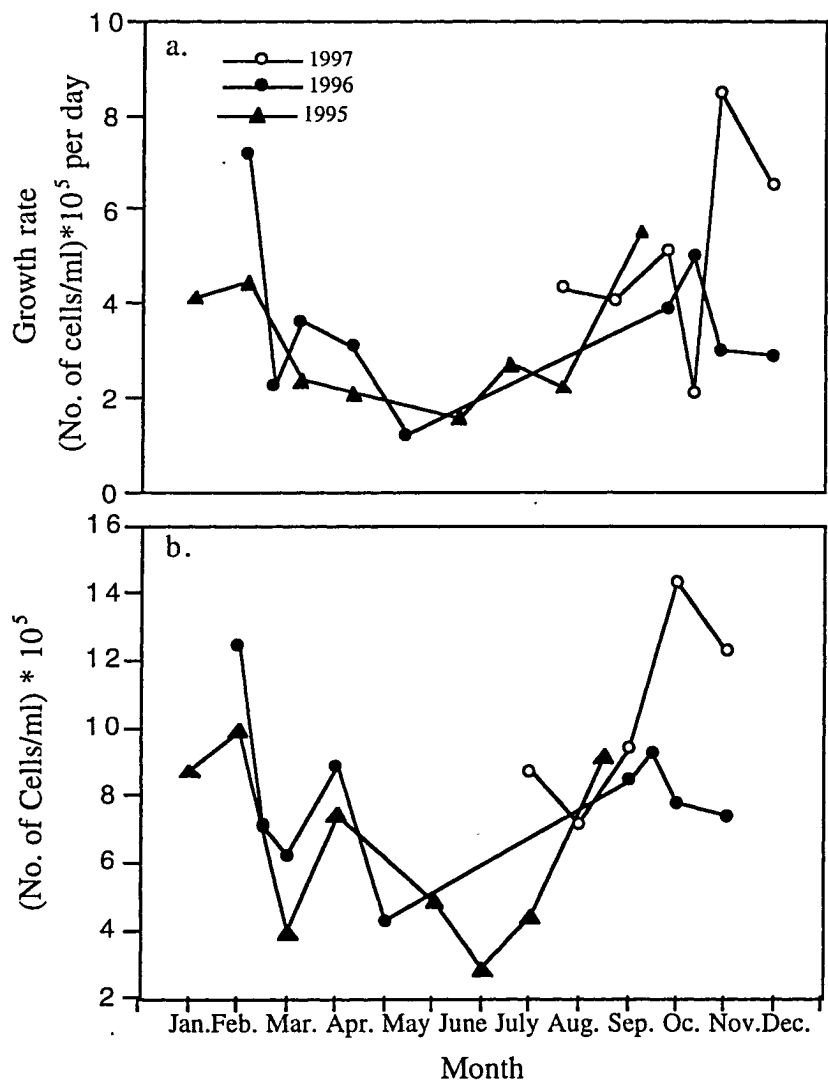
Figure 3.19: Annual Productivity.
(a) Growth rate through the logarithmic phase in units of no. of cells per ml. per day, (growth rate was calculated for the logarithmic phase of growth as the slope of the growth curve between second and third day of growth, (i.e. no. of cells at day (x) - no. of cell at day (x-1) divided by 1 day).
(b) Total number of cells measured on the third morning of experiments throughout the year.

As seen in figure 3.20, the results of division cycle and doubling time during the year showed differences in the timing for the beginning of doubling.

Figure 3.20a shows that during the summer time (when sunset occurs between 19:00 to 20:00) the process of cells division does not start before 22:00, while at winter time when sun set occurs around 18:00, cells division starts between 17:00 to 22:00 (Fig 3.20b).

Actually in November 1998 at 22:00 already third of the cells completed to divide (Fig 3.20b). These results not only emphasize the fact that cells division is induced in *D. bardawil* under natural conditions only after dark but might indicate different induction times for the beginning of division between summer and winter time (see discussion). The results of another set of experiments in which growth was monitored over the third day and night of experiment (not shown here) showed that no further division occurs after 4:00 in the morning independently of the season.
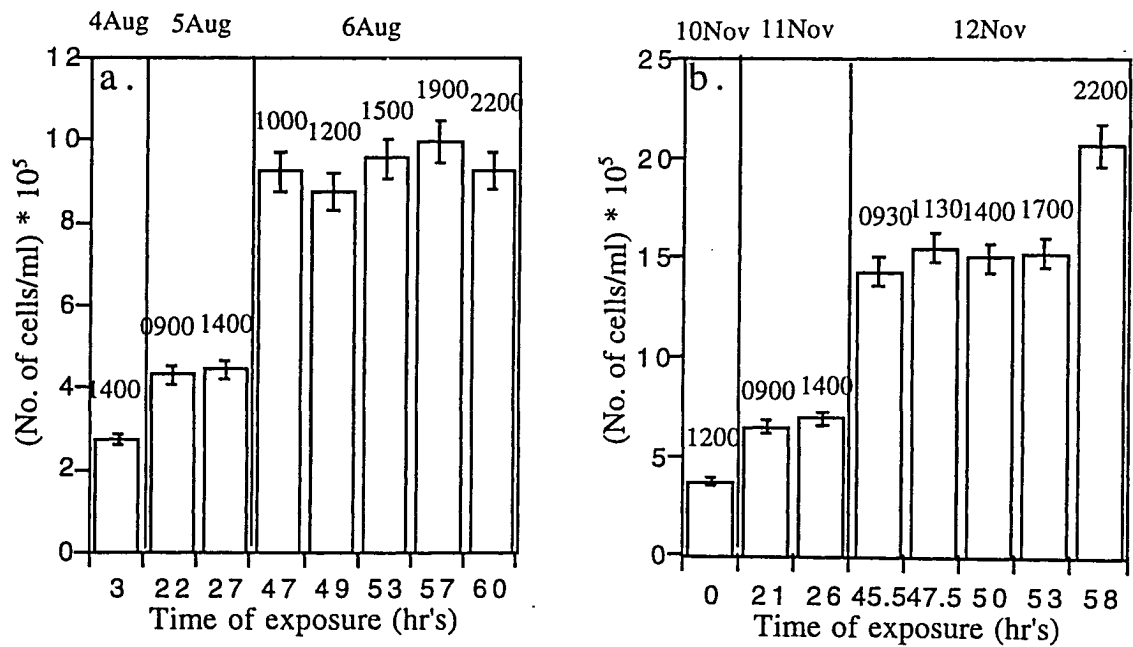
Figure 3.20: Cells Productivity during three days of experiment.
(a) Growth curve representative for experiments which were carried out during summer months.
(b) Growth curve representative for experiments which were carried out during winter months.
The vertical lines separate between the days.

3.2.3 Pigments-

In figure 3.21 ß-carotene was normalized on a chlorophyll basis, in order to avoid experimental artifacts which might be raised during 3 years of experiments.

In general, high ratio of ß-carotene to Chlorophyll is expected under stress conditions (Ben-Amotz and Avron, 1983) mostly under conditions of light stress (which might exist during the summer time in the studied area).

Surprisingly, there is no clear effect of seasonality on the ratio of car/chl observed in Fig 3.21. The exceptionally lower values observed at the end of 97 (October to December) may be explained by the high concentration of cells observed that winter (Fig 3.19b), the high cells density might have decreased light intensity reaching the culture as well as changed its spectrum during some of the time. Therefore, the chlorophyll concentration increased on one hand and ß-carotene concentration decreased on the other.

3.2.4 Quantum yield, antennae size and Photochemical efficiency of PSII estimated from fluorescence induction measurements.

The quantum yield of photosynthesis expressed as percentage of the maximal value measured through the year (Fig 3.22), seems to decrease during the summer months.

The annual pattern of change in quantum yield resembles the annual change in productivity (in Fig 3.19), reaching minimal values during summer time and then recovers back at winter time.

In addition, the parameters of photochemical efficiency and antennae size of PSII which were measured during September and November 97, support the quantum yield results, by exhibiting higher efficiency (F_v/F_{max} = 0.7 in Nov. vs. 0.3 in Sep.) and a larger antennae size (of 0.058 in Nov. relative to 0.005 in Sep, expressed in relative units after normalizing to F_v) in the winter relative to the spring and summer time. These results imply that the conditions of high light during the summer time induced a decrease in the photosynthetic capacity in the long term, firstly due to light stress and secondly due to the acclimation response of decreasing antennae size.
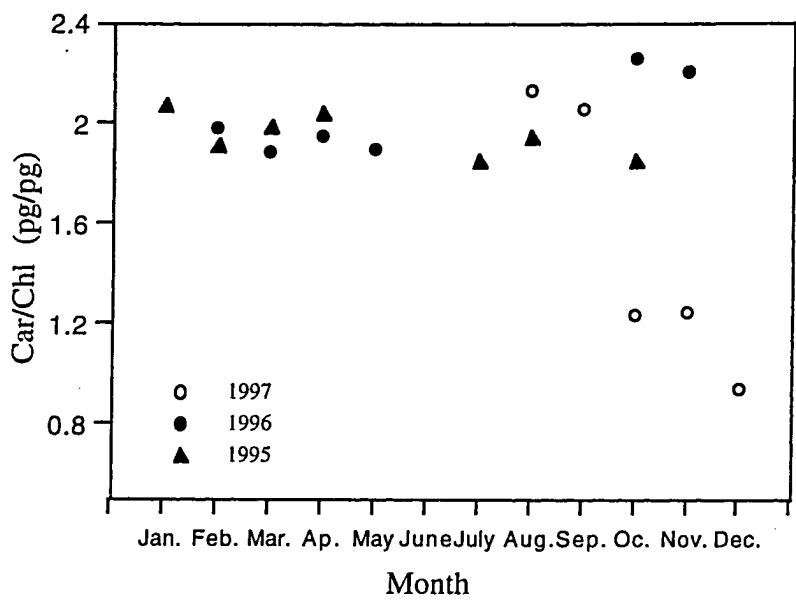
Figure 3.21 : The annual change in ß-car/chl ratio measured over the years 1995-97.
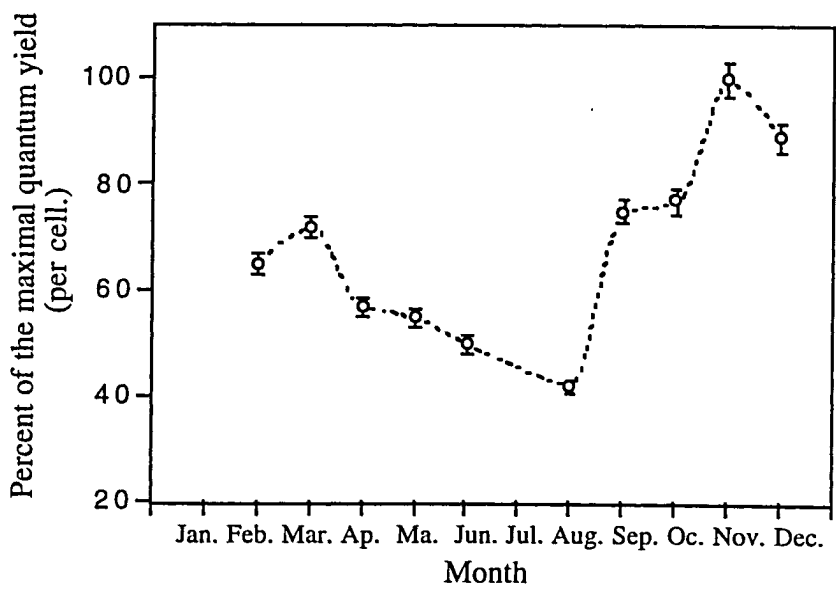
Figure 3.22 : The annual relative change in quantum yield was normalized to the maximal value of $3.9*10^{-8}$ µmol O_2 /quanta cell, measured in November.These results were recorded during 1996.

3.3 Spectral implications

In this section we try to characterize the role of the sunlight spectrum in the acclimation processes of the photosynthetic apparatus during the daily and annual cycles. Based on the correlation observed between blue light and the sunlight intensity during the daily and annual cycles and on the parallel photosynthetic acclimation responses, we tried to characterize the role of the blue light in those acclimation processes .

The sunlight spectrum was artificially modified in a way that influenced the blue/red light ratio. In addition, total sunlight intensity levels reaching the control (receiving the original sunlight spectrum) and treated bioreactors were adjusted to be the same. Therefore, any difference in the photosynthetic performance between treatment and control were supposedly due to spectral differences.

3.3.1 Manipulations of the sunlight -

Four optical filters were used in order to modify the sunlight spectrum: Potassium dichromate (Dich) and Copper Sulfate ($CuSO_4$) decreasing the penetration of the blue light, therefore inducing low blue/red light spectral ratio. A solution of Methylene Blue (MB) absorbing in the red range therefore increasing the blue/red light ratio, and a blue zelofan paper (Blue Z) which has a major absorption peak in the green and the red and a smaller one in the blue range. The blue zelofan paper increases the blue/red light ratio, but may also affect part of the "blue light processes". The absorption spectra of the filters which were used are depicted in Figure 3.23 (overlapping) or in Figure 2.3 (separated).
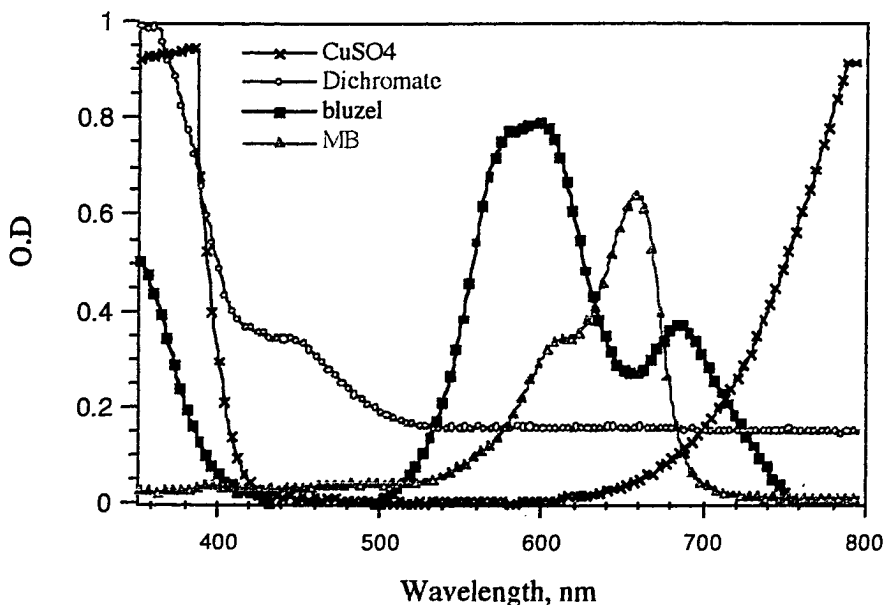
Figure 3.23 : Absorption spectra of the filters used in the modified sunlight spectrum experiments (the same O.D was used during the experiments).

The control bioreactors were covered with polyethylene paper in order to compare the light intensity impinging on treatment and control without modifying the original sunlight spectrum.

A discussion concerning the actual amount of light absorbed by a single cell growing under the different treatments, (which is just a fraction from the total light reaching the bioreactor) is presented in appendix 3.

The results of cells and pigments production are presented in Table 3.1.

Table 3.1. : Influence of modified sunlight spectrum on growth and pigments concentration in *D. bardawil*. (from third day of experiment at 15:00), normalized to control .
The data for the Growth rate was accepted by dividing the no. of cells at the afternoon of the third day by the no. of cells as measured 24 hr's before, the values in the table represent growth rate of treatment normalized to the growth rate of control.

	Dich/control	Blue Z/control	CuSO4/control	MB/control
cells per ml. *10^5	1.7 ± 0.2	0.9 ± 0.2	1.4 ± 0.2	1.1 ± 0.2
Growth rate per day	1.5 ± 0.1	1.0 ± 0.1	1.6 ± 0.1	1.2 ± 0.1
ß-car. (pg/cell)	0.7 ± 0.05	0.6 ± 0.05	0.8 ± 0.05	1.1 ± 0.05
(µgr/ml)	1.2 ± 0.05	0.5 ± 0.05	1.1 ± 0.05	1.2 ± 0.05
Chl. (pg/cell)	1.7 ± 0.05	1.0 ± 0.05	1.5 ± 0.05	0.8 ± 0.05
(µgr/ml)	2.7 ± 0.05	0.9 ± 0.05	2.3 ± 0.05	1.1 ± 0.05
Car/Chl (pg/pg)	0.4 ± 0.05	0.6 ± 0.05	0.5 ± 0.05	1.4 ± 0.05
Chl a / Chl b	0.8 ± 0.05	1.4 ± 0.05	0.7 ± 0.05	0.9 ± 0.05

Abbreviations -   Dich = Potassium Dichromate, Blue Z = Blue zelofan paper,
       CuSO4 = Copper Sulfate, MB = Methylene blue.

3.3.2. Cell Production and division cycle under modified sunlight spectrum -

3.3.2.1 Effects of the studied filters on Cell Production :

Under the blue light absorbing filters, cell production was increased by 70 and 40% in Dichromate and Copper Sulfate respectively. The higher cell production under dichromate and $CuSO_4$ compared to control was observed already in the second day of experiment. By using those filters, blue/red ratio was decreased artificially compared to the ratio in the original sunlight spectrum.

It should be noticed that although both Dich and $CuSO_4$ reduce the blue light fraction, their absorption spectra is different in away that light above 730 nm is being cut off by $CuSO_4$ on one hand, and a larger fraction of blue light (50% of the intensity between 420-480 nm) is absorbed by the Dich on the other. It should be therefore considered that any differences measured in the responses to these two filters might result from those spectral differences.

Under the blue zelofan and the Methylene blue filters, no significant effect compared to control was observed.

Growth rate was significantly higher in the Copper Sulfate and Dich treatments compared to control. The other two filters (MB and Blue zelofan) did not influence significantly on growth rate. Copper Sulfate and Potassium Dichromate clearly absorbed parts of the spectrum which might limit growth, therefore, we can conclude that there is a specific effect on growth mainly in the blue light range within the sunlight spectrum. While Blue Z did not influence on growth rate and Methylene Blue only slightly improved the growth rate of *D. bardawil* (see next section for the background of the MB results).

3.3.2.2 The influence of light quality on cell division cycle :

The timing of the cell division was changed after using MB and $CuSO_4$ (Fig 3.24a,b). Dich caused a gradual growth during the daytime compared to its control (Fig 3.24c; still, the most dominant division process occurred at night). The Blue Z didn't seem to influence the cell division cycle at all (not shown).

As seen in Fig 3.24a, there is no significant difference in growth between control and MB treated culture until the third day between 15:00 and 18:00, when MB treated cells almost doubled their number.

In the case of $CuSO_4$, cell division started between 18:00 to 22:00 compared to control algae which didn't start the division at least until 22:00 (as observed normally in summer time, see Fig 3.20a). Normally, cell division starts later at night (and seems to be dependent on the length of the day, see controls in Fig 3.24, and Figs 3.5, 3.20). The possible reasons for this anomaly are discussed in 4.2.2.
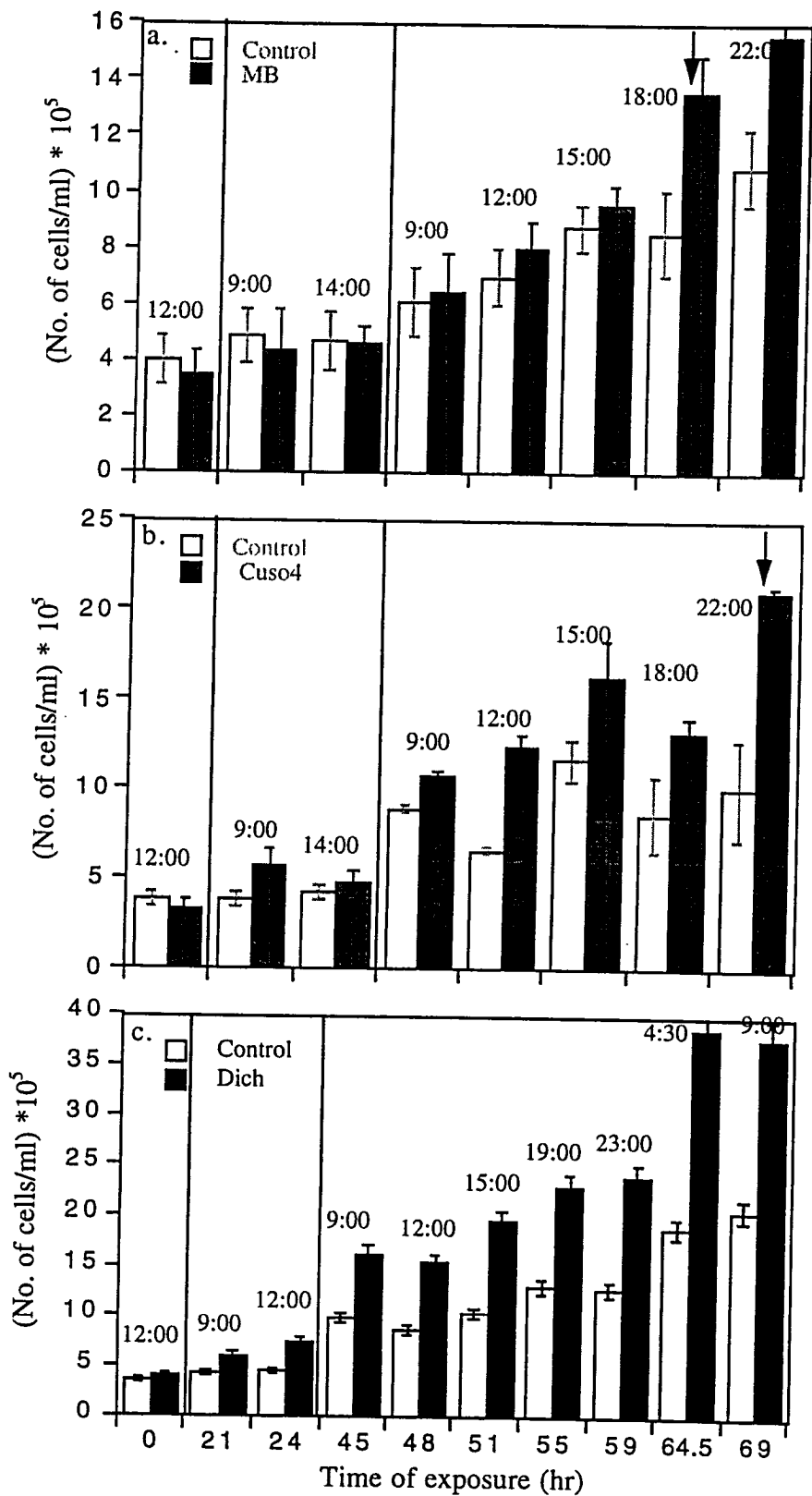
Figure 3.24 : Cell production of *D. bardawil* as measured during 3 days: (a) In July, treated with MB as a red light absorber. (b) In July treated with $CuSO_4$ as absorber of part of the blue and far red light. The arrows (in a and b) represent the point where cells production increased significantly at the treatment relative to control. (c) In September, treated with Dich as a blue light absorber. Empty columns represent the control (receiving the non-modified sunlight spectrum), full columns represent the treatments. The vertical lines separate between the days.

3.3.3 Pigments-

ß-Carotene : In all cases, except for MB (where concentration per cell was slightly higher than control), cells under modified light spectrum synthesized significantly less ß-carotene per cell than the control (Table 3.1). These results, together with the fact that only MB does not absorb in the blue light range (see Fig 3.23) might raise some speculations on blue light control of ß-Carotene synthesis and the possible involvement of a blue light photoreceptor in the induction for the synthesis, (see discussion).

Nevertheless, concentrations of ß-carotene per ml decreased to half of control in the Blue Z treatment (due to the combination of low cells concentration and low ß-carotene concentration per cell, Table 3.1) and increased by 20% over control in both Dich and MB. No significant differences were observed in ß-carotene per ml under CuSO4 treatment compared to control.

The increase in ß-carotene concentration per ml for the MB and Dich is the result of the higher cells production in those treatments.

Therefore, the results demonstrate that high ß-carotene production can be achieved not only by stress induced increase on a cell basis (Ben-Amotz and Avron, 1981; Ben Amotz, Katz et al., 1982; Ben-Amotz and Avron, 1983; Ben-Amotz and Avron, 1989a; Ben-Amotz, Shaish et al., 1989; Ben-Amotz, 1995) but also by enhancing cell yield (achieved by spectral manipulations of the light source for example; see also 3.4).

Chlorophyll : Dich and CuSO4 treated algae produced higher levels of chlorophyll per cell (1.7 and 1.5 times the control chlorophyll concentration, respectively, Table 3.1). The production of chlorophyll per cell by the Blue Z algae was not significantly different, and lower by 20% in the MB treated cells compared to control. The higher concentrations of Chlorophyll per cell appears under lower blue/red light ratio and the lower concentration was induced under high blue to red light ratio (MB). The high concentration of cells, together with the high production of chlorophyll by the Dich and CuSO4 cells are reflected in the high concentration of chlorophyll per ml compared to control cells. It can therefore be speculated that the low blue/red ratio (induced by the dichromate and CuSO4 filters) played a role in the increased chlorophyll synthesis.

In the Blue Z and MB treated cultures, concentration of chlorophyll per ml. did not differ significantly from control, (almost the same as in the case of concentration per cell). This may imply that subtraction of the red light does not interfere with chlorophyll synthesis process.

Chl a/Chl b ratio : The data presented in Table. 3.1, shows that chl a/chl b ratio was significantly lower in the Dich and CuSO4 treated cells (i.e. treated with low blue/red light ratio) compared to control. This is also consistent with the high levels of chlorophyll per cell detected under those treatments (see Introduction). On the other hand, higher chl a/chl b ratios were detected by the

Blue Z treated cells with no clear effect on total chlorophyll synthesis. However, in this case the high blue/red light ratio induced high chl a/chl b ratio.

Almost no effect was observed on chl a/chl b ratio at the MB compared to control.

However, the response of chl a/chl b ratio to high blue/red light under Blue Z treatment is similar to high light response reported in the literature.

On the other hand, the decrease in chl a/chl b ratio under low blue/red light ratio is reported as a low light adaptation process (Bjorkman, 1981; Leong, 1984a; Anderson and Osmond, 1987; Webb and Melis, 1995) this will be further discussed in the Discussion.

Xanthophyll cycle : Throughout the daytime, the epoxidation state of Potassium dichromate treated algae was in about 2 orders of magnitude larger than the control values (for example - 0.2 under dichromate versus 0.002 of control at midday). In both control and Dich (i.e. low blue/red light treatment), the epoxidation state was affected from the daily cycle (for control see Fig 3.9a), but to different extents. In general, xanthophyll pool size and zeaxanthin levels were higher in control then in the low blue/red light treatment throughout the experiment time.

3.3.4 Cbr biosynthesis-

Figure 3.25 shows the daily cycle of Cbr accumulation under Dich filter and control. Lower rates of accumulation and low concentrations of Cbr under the dichromate filter compared to control were measured. These results are in agreement with the low rates of ß-carotene synthesis observed by the dichromate culture (relative to control) and emphasize the specificity of blue light in Cbr and ß-carotene induction. As mentioned before (Introduction) Cbr is unstable, and degrades completely at night time. In the dichromate cells, no Cbr was detected 4 hours after sunset, compared to a diminished signal in the control. This phenomenon was reproducible and the reason for this is not clear. Cbr accumulation in both control and dichromate cultures responded to the diurnal cycle (see also 3.1.4) and was in positive correlation to the integrated light intensity over the day and negatively correlated to the epoxidation state of xanthophylls.
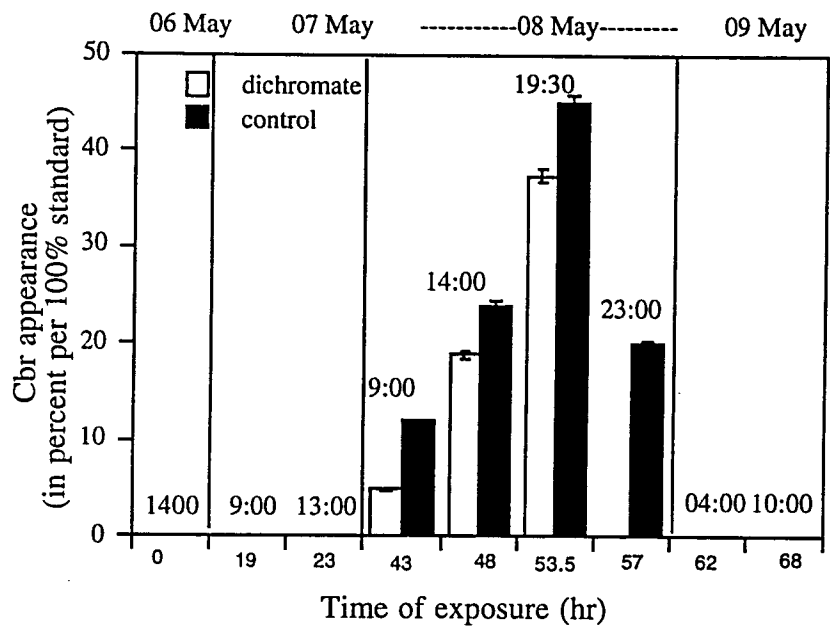
Figure 3.25 : The pattern of Cbr protein induction during the daily cycle influenced by Potassium Dichromate filter. The vertical lines separate between the days.

3.3.5 Antennae size and Photochemical efficiency of PSII estimated from fluorescence induction measurements-

During the daily fluctuations, the most dominant differences in the efficiency of photosynthesis and antennae size between the light treatments appeared around 14:00 in the afternoon. The results are summarized in table 3.2 .

Table 3.2 : Influence of modified sunlight spectrum on efficiency of photosynthesis and antennae size in *D. bardawil*, from the afternoon (14:00) of the third day of experiment, normalized to control.

	Dich./control	Blue Z/control	CuSO4/control	MB/control
Efficiency of photo'	*1.3 ± 0.1	*1.1 ± 0.1	**1.5 ± 0.1	**0.7 ± 0.1
LHCII antennae size	*3.4 ± 0.05	*0.7 ± 0.05	n.a	n.a

n.a = not analyzed
* determined by Fluorescence induction technique.
** determined by Oxygen evolution technique.

3.3.5.1 Antennae size affected by the sunlight spectrum:

Antennae size measured for Dich algae (i.e. under low blue/red light treatment) was 3.4 times the control value, while the antennae size of the Blue Z (high blue/red light treatment) culture reached only to 70% of control. These results indicate on a clear association between antennae size (which was known before as being regulated by light intensity (Malkin, Armond et al., 1981; Leong, 1984a)) and the fluctuations in blue/red light ratio.

3.3.5.2 Photosynthetic efficiency affected by the sunlight spectrum:

In the treatments of low blue/red light ratio (Dich and CuSO4) photosynthetic efficiency was shown to be higher by 30% and 50% respectively than the control value, while the photosynthetic efficiency of the MB culture decreased to 70% of control. Blue Z showed no significant changes in this case. These results indicate on a possibility that low spectral ratio of blue/red light diminishes the light stress conditions, an effect which results (as observed under Dich and CuSO4) in increased efficiency of photosynthesis as well as increase in the antennae size. The opposite process might also be considered, i.e. increase in the blue/red light ratio (by MB) might induce a situation of increase in light stress, therefore efficiency and antennae size are decreased (see also discussion).

3.4 Biotechnological Aspects-

One of the earlier objectives of this research, was to optimize and to enhance the photosynthetic production as well as to maximize ß-carotene yields. Basically, the photobioreactor was designed in a way that the sunlight (intensity and quality) was the only limiting factor and it could be artificially controlled and optimized to fulfill this objective. The results shown in Table 3.3 present a comparison of maximal cells and ß-carotene production achieved in different growth systems, sunlight was the light source in all growth systems with no spectral control.

Table 3.3 : Maximal cells concentration and β-carotene yields of *D. bardawil* grown in different growth systems, (DLB (= Double Lens Bioreactor) refers to the photobioreactor designed for this study, N.B.T is the commercial plant for ß-carotene production in Eilat. The data for the outdoor ponds was obtained at the Biochemistry Dep. at WIS., Rehovot. In all cases algae were grown in medium with full nutrients concentrations, in the case of the DLB the data are from the stationary phase after 7-8 days of experiment). The DLB results are not all from the same experiment.

	DLB	N.B.T (commercial ponds)	Outdoor Ponds (WIS.)
maximal cell conc. $*10^6$ per ml	4.4	0.5	1
maximal β-car conc. (pg/cell)	26	30	10
maximal β-car conc. (μgr/ml)	19	15	10

As seen in Table 3.3, the DLB optimized system, reached between 9 to 4 times the cells concentrations of the other systems, due to this fact also the β-car concentration per ml was increased significantly (19 μgr/ml compared with 10 and 15 μgr/ml in the Outdoor Ponds and N.B.T respectively, the DLB results represent the maximum results of different experiments).

After optimizing temporally and spatially the light intensity by the DLB as explained in section 2.1.3, the second step was to optimize light spectral conditions. As shown before (Table 3.1), the concentration of ß-carotene per ml increased by 20% under Dich and MB treatments compared to control (under unmodified sunlight spectrum, same light intensity as in treatments). At the Dich treatment, this increase in ß-carotene concentration (which reached 13.5 μgr/ml at a

cells concentration of $2.7*10^6$ on the third day of experiment) was obtained due to the significantly high cell production, in spite of lower ß-carotene per cell concentration. In the MB treatment, the reason was high production of ß-carotene per cell (presumably due to conditions of high blue/red spectral ratio). Both strategies of increasing cell production (therefore increasing ß-carotene concentration on a volume basis) by reducing blue light on one hand, or increasing ß-carotene concentration per cell (by increased light intensity and/or increased blue light fraction) on the other, can be considered.

4. Discussion

We investigated the role of the natural changes in the blue and/or in the red light range in the sunlight spectrum upon the *Dunaliellas'* acclimation strategies to different light regimes in the daily and seasonal cycles. We hypothesized that the mechanism for sensing the changes in the light intensity is related to the spectral changes in *D. bardawil*, particularly in the blue light range.

Testing this hypothesis was possible by using a new developed double lens Photobioreactor (DLB) that permitted spectral and intensity manipulations, while maintaining otherwise controlled conditions.

In the following sections I discuss the results and attempt to draw conclusions on the possible mechanisms underlying the response of *D. bardawil* to changes in the light environment under natural conditions.

4.1 Temporal changes in the sunlight properties

The association between the daily distribution of the sunlight intensity (which approaches maximum around 14:00) and the changes in the sunlight spectrum was resolved. It was observed that most spectral changes during the day occur in the blue light region and a linear correlation between the light intensity and the blue light fraction was established (Fig 3.4).

The main cause for the interaction between the daily change in the sunlight intensity and the radiation in blue light is the scattering of light by air molecules. Since the air molecules are much smaller than the wavelengths of solar radiation, the efficiency with which they scatter light is proportional to $1/\lambda^4$, in accordance with Rayleigh's Law (see also 1.1). Scattering of solar radiation is therefore much more intense at the short wavelength end of the spectrum.

While the differences in the sunlight spectrum during the day (i.e. in a shorter time scale) are dominated mostly by Rayleigh's Law (therefore the observation of the large daily attenuation in the blue light region), the seasonal differences in the sunlight spectrum are a consequence of a combination between Rayleigh's Law and Mie scattering.

Mie scattering is predominant when particles in the atmosphere are not sufficiently small relative to the wavelength of most of the solar radiation to obey Rayleigh's Law. Mie scattering (Kirk, 1983) is characterized by an angular distribution predominantly in the forward direction, and a much weaker dependence on wavelength, although scattering is still more intense at shorter wavelengths.

Measurements of the seasonal change in the sunlight intensity were (as expected) in correlation with the solar position (i.e. the inclination angle of the sun) relative to the surface of the studied area (Fig 3.17). The average light intensity is depended on the atmospheric pass length of radiation and was therefore lower in the winter time, higher during the summer and moderate in

the spring and autumn. However, during the winter in extremely bright days, higher value of direct sunlight intensity can be achieved compared to the summer maximal intensity of radiation (results are not shown). These conditions of relatively high light intensity can last for several hours. This phenomenon observed under clear winter sky conditions, is a result of a relative increase in the fraction of red light penetrating through the sky (due to a negligible influence of Mie scattering). The contribution of red light during the clear winter days (compared to the summer) lies in the fact that during the summer time the atmosphere is not clear and a larger fraction of the red light is scattered (by Mie scattering) and absorbed by dust particles and water vapor which almost do not exist during bright days in the winter. The lower values of blue to red light ratio measured during the winter, can be therefore a result of the larger fraction of red light penetrating through the clear atmosphere on one hand, and the lower fraction of blue light (due to Rayleigh's theory) on the other.

The atmosphere over any part of the Earth's surface always contains a certain amount of dust, the dust quantity depends on the area and the weather conditions (such as humidity, aerosols, winds etc.), therefore it varies with time at any given place. The seasonal differences in the clarity of the sky in the studied area is likely the reason for the low correlation between the blue light and the total light intensity during the seasonal changes (Fig 3.18b). The dusty, hazy days of the summer cause a larger extent of Mie scattering within the blue light range, therefore inducing a lower level of blue to total radiation ratio compared with spring and autumn time.

To sum up - A correlation between light intensity and the fraction of blue light was observed during the temporal changes in the sunlight position. This correlation is explained primarily by the intense scattering of light at the short wavelength end of the spectrum (Rayleigh's Law). While the correlation was high during the daily cycle, it seemed to be weak during the annual cycle. This is likely due to hazy days in the summer and therefore scatter of the blue light defined as Mie scattering.

In addition, the observed relationships between intensities and spectral composition may indicate that the response of *D. bardawil* to changes in light intensities can at least partly, be through sensing spectral changes.

4.2 The Diurnal and Seasonal changes in Cell production and Cell division cycle as affected by the natural changes in the sunlight features

4.2.1 Cell Production -

Since cell production is closely coupled with the cell division process, the same parameters affected both. It should be noticed that in our experiments all results of cells production refer to 3 days of growth. Therefore, they reflect growth during the logarithmic phase and not maximal cell production.

We propose that the higher yields of cells obtained during the winter time (Fig 3.19) is a result of a combination of the following causes - the seasonal change in the day length (which induced earlier start of the division burst during the winter shown in Fig 3.20b), the seasonal change in intensity (for example the high light intensities measured during summer time (Fig 3.17) which might have caused stress conditions that inhibited growth during this time of the year compared with the winter time) and the seasonal spectral attenuation in the blue light range (i.e. the low blue/red light ratio measured during the winter as observed in Fig's 3.18b, c).

The filter experiments' results of increased cell production (Table 3.1) under low blue/red light ratio (achieved by the Dich and $CuSO_4$ treatments), support the last speculation. The key for it lies in the fact that under low blue/red light ratio (as measured during the winter time), low light acclimation responses were observed (see 4.3-4.4 and Table 3.1) therefore high light stress is diminished resulting in higher cell productivity (during winter time as well as in Dich experiments).

As for the Blue zelofan and MB experiments, no real difference from control was observed in cells production (Table 3.1). We therefore suppose that the assumption made for Blue zelofan that green light (absorbed by this filter) does not affect cell division and that the amount of red light (transmitted through the blue zelofan) was sufficient for the division cycle signal transduction to operate (4.2.2), can be applied also here. As for the MB, the increase in growth rate occurred due to the earlier onset of the division burst. In this case, total cell production was measured just before this effect was observed and therefore no particular conclusions can be drawn.

4.2.2 Cell division cycle -

The precise mechanism controlling synchronization of the cell cycle in unicellular algae in general, and of *Dunaliella* specifically is not yet fully elucidated. Although the existence of genes involved in cell division process and the concept of "circadian clock" - controlled gene expression was proved in some of the cases (see for *Chlorella vulgaris* in Wakasugi, Nagai et al., (1997) and for a few other species (Oberschmidt, Hucking et al., 1995), the complete cell division signaling pathway is not yet known.

However, the endogenous rhythms which are known (Johnson, Golden et al., 1996) can be affected by daily cues in the environment. Some of these cues (see underlined bellow) were proved to be involved in triggering the cell division cycle:

Synchronization of cell division by a periodic light-dark regimen has been successfully tried with a number of phototrophic organisms (see Costas, GonzalezGil et al., (1996) and others). Among them was *Dunaliella parva* which could be synchronized in the lab by exposing it to light-dark rhythm of 14:10 h, under constant temperature and pH conditions (Brüggemann, Weiger et al., 1978). In another case, only the combination of light-dark rhythm together with temperature fluctuations could synchronize cell division cycle of *Dunaliella tertiolacta* (Wegmann and Metzner, 1971). The parameter of day length in nature (or the duration of light period under lab conditions) was also identified as an important factor in the synchronization of cells division (see Costas, GonzalezGil et al., (1996)) and the seasonal results of this study in Fig 3.20).

The results of this study showed that the timing for cell division in *D. bardawil* culture was adaped almost immediately after being transferred from continuous low light conditions (existing in the growing chamber) to the natural day-night cycle existing in the bioreactor. Figure 3.5 demonstrates a typical division cycle of the *Dunaliella* cells within the bioreactor. The division process took place at night time and in most cases (of control culture) a complete doubling of cells was achieved during one night, (in other cases, a complete doubling was achieved just during the second night of experiment). The occurrence of cell division process at night seems reasonable due to the fact that at night there are no light stress conditions which demand the energy for induction of dissipation or acclimation processes and at this time cell size was optimal for division.

In measurements which were conducted during the night time it was shown that cell division started about 2-3 hr's after dark and was completed between 4:00 to 5:00 in the morning, therefore it lasted for 6 to 8 hr's. These values are in agreement with the literature (see for example Brüggemann, Weiger et al., (1978)) where about 5.5 h are reported as typical time for division burst measured in *Dunaliella parva* .

In this study however, since division started after sunset, the duration of the division process under field conditions was depended on the day length and therefore on the season. It was shown that in winter time (when days are shorter) the division process started earlier compared to the summer time (see Fig 3.20). Therefore, it lasted for a longer period of time in winter nights. Since the shape of the annual cell production resembles a mirror image of the annual sunlight intensity (Fig's 3.19 and 3.17), the involvement of the parameter of light intensity in the cell division cycle must be also considered. It might be assumed that one of the reasons for the low summer yields is the high light intensities (photoinhibition). On the other hand, the results of Tekoah, (1994) which showed that an increase in light intensity by a factor of 1.2 and even more - did not inhibit growth, imply that the parameter of light intensity in this range, is not predominant in controling growth. In addition, winter sun intensity (see 4.1) was

measured to be under certain circumstances higher then the intensity measured in the summer. Another evidence for the dominance of day length over the light intensity on cells production are the results of the different angles experiments (3.1.7) where light intensity was different between treatment and control but day length and absolute spectral changes were the same. In this case cell production was measured to be the same in both control and treatment. The difference in timing for starting the division which results in longer duration of doubling in the winter nights, might be therefore part of the reason for the low yields of cells obtained during the summer compared to the winter time (Fig 3.19).

The influence of light quality on the cell division cycle was detected previously in different algae species. It was shown in lab experiments, that red light grown cells of *Dunaliella salina* were bigger and grew more rapidly than those grown in blue light. In addition, red light treated cells replicated their chl-DNA and divided their nucleoids before those grown in blue light (Zachleder, Kuptsova et al., 1989). Also the red algae, *Porphyra* showed higher growth rates under red compared to blue light (Figueroa, Aguilera et al., 1995), the difference was based on the higher photosynthetic rate measured at red light treated algae. The influence of red light on cultivation of *Chlorella vulgaris* was tested as well by using light-emitting diode with peak emittance of 680 nm (Lee and Palsson, 1996), the cycle behavior of *Chlorella* was altered in a way that the critical cell size for releasing autospores under red LED was smaller. However, in this case the final cell mass as well as the specific cellular growth rate didn't differ from cells grown under full spectrum light. Obviously, there are differences among the different taxonomic groups in the factors controlling cell division cycle.

Based on the well known phytochrome mediated morphogenic influence of far red (FR) light on growth in higher plants, Sánchez-Saavedra, Jiménez et al., (1996) checked the influence of FR light (~ 730 nm) on growth rate and maximal cells density in *D. bardawil*. Increased growth rate but a decrease in cells density was reported when white light together with FR light was applied compared to white light grown culture. Their conclusion was that FR appears to shorten the logarithmic phase of growth but reduces cell division in the exponential phase. Since changes in R/FR ratio are detected by phytochrome (which modulates growth in plants under natural conditions) and since it showed to affect the growth rate of *D. bardawil*, they proposed that phytochrome may be involved.

The results of the filter experiments performed in this study showed that light spectrum plays a role in the cell division cycle of *D. bardawil* under field conditions as well. In the Dich experiment (when blue light was diminished and therefore blue/red light ratio was reduced) growth rate increased (Table 3.1). Cell division under Dich proceeded gradually also during the day time (Fig 3.24 c) resulting in a 45% increase of the morning values. Therefore the Dich cells more then doubled themselves in a 24 hr's cycle (see 4.2.1). In general, the Dich results are in agreement with the observations under lab conditions which showed preferable growth under red light (Zachleder, Kuptsova et al., 1989; Figueroa, Aguilera et al., 1995). However,

unlike in the lab under artificial light source, it seems that in the natural sunlight cycle the blue light has a special role in preventing cells division during the daytime.

In the absence of red light (MB experiments) the process of cell division started already in the late afternoon instead of after sunset (Fig 3.24a). Based on the original daily changes observed in the sunlight spectrum (see 4.1 and 3.1) and on the Dich and MB cell division results (Fig 3.24 a,c), it can be speculated that under field conditions, when blue light starts to diminish and the red fraction increases towards the end of the day (resulting in a low blue/red light ratio), the cells start to prepare for division. It seems that as long as red light exists, division is inhibited. Therefore, in the MB experiment, late in the afternoon (~18:00) when blue light diminished adequately and red light was absent, the division could begin. Based on these observations I speculate that both blue and red light or a certain combination between them play a role in determining the timing of cell division process in *D. bardawil.*

The $CuSO_4$ results (Table 3.1 and Fig 3.24b) reflected the influence of both blue light and FR on the cell division process, since it cuts off below 420 nm and doesn't transmit beyond 700 nm (see absorption spectrum in Fig 3.23). Growth rate was increased by 60% compared to control culture. In addition, the $CuSO_4$ affected the timing of division : In the absence of FR light and part of the blue, the division started earlier that night (at 22:00) compared to the typical time observed in control during the summer time (Fig 3.24b).

Blue zelofan filter didn't have any effect on the division cycle, neither on the growth rate nor on the timing of the process. Therefore we can assume that the green light does not affect cells division under field conditions in *D. bardawil*, and it seems that the amount of red light transmitted through the blue zelofan was sufficient for the division cycle signal transduction to operate.

To sum up - Fast adaptation of cell division process was observed upon transferring *D. bardawil* to natural field conditions. The division process took place at night time, mostly reaching a complete doubling during one night.

Higher yields of cells were obtained in winter than during the summer time. This is explained by a combination of the following factors:

1) The low total light intensities observed during the winter, reduced the light stress conditions during the winter versus summer time. Therefore, allowing higher growth rates.

2) The shorter daylength in the winter, which caused earlier division burst therefore a prolonged duration of division during winter nights.

3) In addition to the clear influence of the effect of daylength and light intensity on cell division, the low blue/red light spectral ratio measured during the winter time seemed to advance the timing for cell division (was shown by $CuSO_4$ and Dich results which induced earlier division burst). In addition, it seems that the low blue/red light ratio diminished stress conditions and induced low light photosynthetic acclimation response (see pigmentation and Cbr results under $CuSO_4$ and Dich experiments) therefore cell production was enhanced during the winter.

4.3 The Diurnal and Seasonal Changes in Synthesis of Pigments and Cbr protein as affected by the natural changes in the sunlight features

4.3.1 ß- Carotene -

Several factors are known today to induce secondary ß-carotene synthesis in *D. bardawil*. Among them are - high light intensity and limiting conditions in growth (such as nitrate and sulfate deficiency, or high salt concentration etc.). The effect of light spectrum was tested as well in the study by Ben-Amotz and Avron, (1989b) where it was concluded that carotenogenesis in *D. bardawil* is independent of light quality, and by Sánchez-Saavedra, Jiménez et al., (1996) who stated that carotenoid synthesis by *D. bardawil* is stimulated both by increasing irradiance and by far red light. Both studies were conducted in the lab under continuos light conditions within range of intensities significantly lower than the sunlight.

The results of this study showed accumulation of ß-carotene on a culture volume basis through a three days of experiment independently of the season, (see Fig 3.6 for representative results of ß-carotene production in November). The concentration per cell was affected by cell division. It therefore decreased at night time after cell division. In general, it seems that the process of ß-carotene synthesis was positively correlated with the integrated light dose over the day, since all other factors known to induce ß-carotene synthesis in *D. bardawil* were optimized. However, the results of the different angles experiments (Fig 3.12), inducing different patterns of daily intensity distribution and integrated light dose over a day, showed that ß-carotene production was not affected significantly by the difference in intensity pattern. Therefore, we speculate that in the range of intensities beyond ~600 w/m^2 (which was the intensity impinging on the treated bioreactors) the response of ß-carotene induction is affected also by other parameters. It should be considered that the results showing linearity between the light intensity and ß-carotene accumulation (Ben-Amotz and Avron, 1989b) were obtained in considerable lower range of intensities (between 25 to 500 w/m^2). Still, ß-carotene concentrations measured in the summer time were higher compared with the winter values (reaching an average of 11 pg/cell at summer versus 3 pg/cell at winter time at the 5th day of experiment). This phenomenon can be the result of either the differing light spectrum (showing an increase in the blue/red light ratio during the summer and is discussed later by the filter experiments results) or the lower cells concentration observed in the summer time (allowing more light penetration per cell, compared to winter cultures) or a combination of both.

Based on the literature (Sánchez-Saavedra, Jiménez et al., 1996) and in order to complete the picture about the implications of the sunlight spectrum on ß-carotene induction under field

conditions in different scales of time, spectral influence on ß-carotene production was tested by the filters experiment.

In the absence of blue light (Dich filter) ß-carotene concentration (per cell) decreased by 30% (table 3.1) compared to control culture. These results are in agreement with the results of Tekoah, (1994) who used the same filter on *D. bardawil* in a similar bioreactor, and are also supported by the fact that blue light is known to be an important factor in inducing carotenoids synthesis in algae, fungi and bacteria (Rau and Schrott, 1987). The correlation observed between the blue light and the sunlight intensity (during the daily cycle as well as seasonal) together with the results of the Dich filter and the low levels of ß-carotene during winter time, support the hypothesis that also in the case of *Dunaliella*, blue light plays a major role in induction of ß-carotene synthesis. Furthermore, due to the fact that ß-carotene in this case is a stress pigment which absorbs blue light (therefore protects against it) and blue light fraction increases with light intensity we suggest that for the *D. bardawil*, blue light is functioning as a light stress "alarm". This idea will be discussed further below.

The low level of ß-carotene produced by cells treated with blue Zel. (Table 3.1) implies that either red, far red light (or a certain combination of those) and/or green light are essential for ß-carotene induction. Due to the fact that green light is not absorbed by the photosynthetic pigments or other known accessory pigments of the *Dunaliella*, the probability for a green light control is small. More reasonable conclusion, based on the speculations about the existence of phytochrome in *Dunaliella* (Ruyters, 1988) and on the $CuSO_4$ and MB filters results (discussed below), is that beside blue light, red light (around 700 nm) is important in some way for the ß-carotene induction. The MB results showed (Table 3.1) that ß-carotene concentration did not differ significantly from control, therefore we conclude that red light around 660 nm has no effect on ß-carotene synthesis. The filters results lead to the conclusion that in addition to blue light, red light or maybe a certain combination of them has a role in the ß-carotene induction pathway. Combining the filter results together with the daily and annual changes in the sunlight (reported above) leads to the conclusion that beside the influence of light intensity (which was demonstrated in the lab in the range below 500 w/m^2 (Ben-Amotz and Avron, 1989b)), ß-carotene synthesis is stimulated by the changes in the blue light and red light intensity which occur naturally in the sunlight. On the surface, our results differ from the results of Ben-Amotz and Avron, (1989b) which showed that accumulation of ß-carotene depends strongly on integrated light intensity but is independent of light quality. Since their experiments were conducted in completely different system (artificial continuos light source having intensity which is much lower than the sunlight) and part of the tested spectral ranges differed from those used in this study (they tested the influence of green light (546 nm + 578 nm, 578 nm), the range between 645 nm to 730 nm (i.e. red light) and white light up to 730 nm) there is in fact no contradiction. Nevertheless, those spectral treatments which were similar, proved to be unimportant for carotenogenesis in our study as well (like the effect of green light for example).

4.3.2 Chlorophyll -

The daily pattern of change in the chlorophyll concentration (extracted from *D. bardawil* during 3 days of growth in the bioreactor) shown in Fig 3.7, demonstrates accumulation of the pigment per ml of culture during the three days course. It seems that the synthesis proceeds also after dark (see concentration per ml), although in a lower rate compared to the daytime. However, a detailed analysis of the daily fluctuations of chlorophyll concentration shows a decrease at noontime of the third day of growth in both total chlorophyll (per ml) and per cell concentration, this decline is in negative correlation with the Chl a/Chl b ratio observed in Fig 3.8. The inverse correlation between Chl a/Chl b ratio and the total Chl concentration is typical during the acclimation process of photosystems of higher plants (Bjorkman, 1981) as well as in algae to changes in light intensity, and it was studied and observed under lab experiments in *Dunaliella salina* as well (Pick, Gounaris et al., 1987). However, the decrease in total Chl (possibly due to degradation of Chl b) which responds to the high sunlight intensity measured at noontime, is reversible and recovers again during the afternoon. A certain inconsistency between total chlorophyll and Chl a/Chl b ratio was observed during the second day of experiment (Figs 3.7 and 3.8) where the increase in the Chl a/Chl b ratio from morning to noon time was not accompanied by a decrease in total chlorophyll. Nevertheless, such a discrepancy (between chlorophyll concentration and Chl a/Chl b ratio) was noticed before in other species of *Dunaliella* (Falkowski, 1984; Pick, Gounaris et al., 1987) when rapid changes in Chl a/Chl b ratio were reversible and preceded any changes in the total cellular Chl content. This observation was based on a proposed strategy of redistributing the chlorophyll (among LHCII and PSII reaction centers) so as to optimize the ratio between the PSII reaction center concentration and the level of antennae chlorophyll (Pick, Gounaris et al., 1987). Therefore under these circumstances a small decrease in the Chl a/Chl b ratio is not necessarily a result of *de novo* Chl b synthesis but due to a direct conversion of Chl a to Chl b and *vice versa*.

The influence of the daily distribution of light intensity (as well as integrated intensity over the day) on chlorophyll adaptation was observed in the different angles experiment (Fig 3.13). In contrast to the pattern of accumulation of chlorophyll (observed after noontime of the third day of experiment) by the control culture, the chlorophyll level at the treatment (receiving daily constant light dose) remained almost constant through the light period. However, while apparently the daily fluctuation in chlorophyll concentration responded to the daily change in light intensity, the absolute levels of chlorophyll concentration didn't seem to adapt to the light intensity. In contrast to the usual acclimation scenario of decreased chlorophyll concentration under high light regime and *vice versa*, in this experiment the lower values of chlorophyll were detected under lower integrated light intensity (see treatment in Fig 3.13). On the other hand, the change in Chl a/Chl b ratio as well as its order of magnitude observed in Fig 3.15 seem to respond to the different treatments as expected (i.e. higher Chl a/Chl b ratio under higher light, and steady low values under the low constant light regime).

These inconsistencies can be resolved by calculating the ß-carotene to chlorophyll ratio (Fig 3.14), which was used before in other studies of *D. bardawil* (Ben-Amotz and Avron, 1981; Jiménez and Pick, 1993; Tekoah, 1994), and proved to be a better "stress index" in this case. The reason for this lies in the fact that the balance between ß-carotene to chlorophyll plays a role in determining the effects of light regime (both in intensity and quality) which actually is reached and absorbed by the cell. In addition, since the accumulation process of ß-carotene by the *D. bardawil* reflects light stress and the changes in chlorophyll concentration reflect adaptation to the light environment (and it's concentration is supposedly negatively correlated with light stress), the ratio between them should reflect the state of stress conditions of the algae. The results presented in Fig 3.14 imply that the treated culture which received constant lower light during the day, showed to be under stress (presenting higher levels of car/chl ratio) compared to the control culture (which received natural sunlight in a parabolic distribution over the day). The possible explanation for these results lies in the fact that the lower chlorophyll concentration (detected by the treated culture) enabled higher levels of light to penetrate in to the culture (especially in the spectral range which is absorbed by chlorophyll), relative to the control. Therefore, the increased levels of ß-carotene to chlorophyll ratio (Fig 3.14) by the treated culture express the situation of a light stress. Still, the basic reasons for the low chlorophyll concentration observed under the treatment of constant daily levels of lower light are not clear.

In addition to the known effects of light quality on chlorophyll synthesis in both higher plants and algae (see Introduction and Wallen and Geen, 1971; Senger and Bauer, 1987; Dring, 1988; Senger, Humbeck et al. 1992; López-Juez and Hughes 1995; Sánchez-Saavedra, Jiménez et al., 1996), in order to complete the picture about the role of changes in light quality through the natural cycles of the sunlight on chlorophyll production by *D. bardawil* in particular, the filters experiments were conducted. Reducing blue and FR light (blue light by Dich and part of blue + FR light by $CuSO_4$), induced an increase in chlorophyll synthesis per cell (also observed by Sánchez-Saavedra, Jiménez et al., (1996)) which was accompanied by a decrease in Chl a/Chl b compared to control (Table 3.1).

Since the responses of increased chlorophyll concentration and a decrease in Chl a/Chl b ratio are known as low light acclimation responses (Leong, 1984a; Leong, 1984b; Anderson and Osmond, 1987; Webb and Melis, 1995) we suggest that reducing blue light (under these two filters) mimicked the conditions of low light for the *D. bardawil*. As for the influence of FR ($CuSO_4$) on chlorophyll adaptation, no clear conclusion can be made since the $CuSO_4$ is not a specific absorber for the FR range. Reducing the red light fraction around 660 nm (MB) induced a slight decline in total chlorophyll concentration per cell, while Chl a/Chl b ratio did not change significantly. In this case, the ratio of blue to red light was increased and induced a slight increase in ß-carotene production. This resulted in an increase in ß-carotene/chlorophyll ratio which indicates on a typical high light response. The absence of green and red light (~ 700

nm, absorbed by the Blue Z treatment) did not induce any change in total chlorophyll but caused a 40% increase in Chl a/Chl b ratio. Such an inconsistency between changes in total chlorophyll level and in Chl a/Chl b ratio was observed and discussed earlier in this section. Nevertheless, the results obtained by Wallen and Geen, (1971) showed that green light induced a decrease in total chlorophyll while the Chl a/Chl b ratio was not affected in *D. tertiolecta*. Since in that study they used a lower light intensity as well as different conditions, the results are not comparable.

To sum up for the relation between chlorophyll synthesis and light quality, our results show a clear interaction between blue light (or the ratio of blue to red light) and chlorophyll adaptation (either by changes in concentration or/and by changes in Chl a/Chl b ratio). Furthermore, the treatments inducing low blue light or low ratio of blue to red light proved to stimulate low light adaptation processes, such as higher chlorophyll concentration, lower Chl a/Chl b ratio and lower car/chl ratio. Based on these results and together with the fluctuations of the blue/red light ratio occurring in nature and it's correlation to the light intensity, we suggest that in nature, at least part of the sensing mechanism of *Dunaliella* for different light intensities is stimulated through the changes in the blue/red light ratio.

4.3.3 Xanthophyll Cycle-

The high light driven de-epoxidation of the Xanthophyll Violaxanthin (Viola) to Zeaxanthin (Zea) is widely conserved among plants and algae (Demmig-Adams, 1990). Zea which is accumulated mostly in the antennae of both photosystems is proposed to participate in mechanisms which protect the photosynthetic apparatus against photo destructive damages. The Xanthophyll cycle was investigated under lab as well as under field conditions intensively and it was shown to respond very fast to the changes in the light conditions. Nevertheless, not much is known concerning wavelength dependence of the Xanthophyll cycle. Lee and Yamamoto, (1968) indicated that de-epoxidation occurred in spinach under red and blue light but not under green light. Banet, (1997) investigated the influence of light spectrum under lab conditions on Xanthophyll cycle in *Dunaliella*. It was shown that both *D. salina* and *D. bardawil* responded similarly to all wavelengths but the intensity threshold for de-epoxidation in *D. bardawil* under blue light was higher compared to *D. salina*. The results of this study (Fig 3.9a, expressed as Epoxidation state) show a daily response of the epoxidation cycle to the changes in sunlight features, by showing high values (i.e. low Zeaxanthin levels) under low light conditions (occurring in the morning and late afternoon) and *vice versa*. However, it was shown that the reactions of xanthophyll cycle slightly lag behind the daily changes in sunlight. The Dich results show the same daily pattern of change in epoxidation state as in the control, but higher values in 2 orders of magnitude. This indicates that in the absence of blue light, there was a decrease in the need for protection mechanism. These results agree with the results of (Banet, 1997) and moreover, they indicate a possible connection between light stress and blue

light signal in inducing photo-protective mechanisms such as the Viola de-epoxidation in *D. bardawil.*

4.3.4 Induction of the Cbr protein -

The daily change in Cbr content fluctuated in accordance with the daily changes in the light intensity and its spectrum (Fig 3.9b). Lab experiments (using artificial light source) conducted by Banet, (1997) showed that blue light radiation was the most efficient in inducing Cbr synthesis in *D. bardawil* . In addition, our results showed lower levels of Cbr in the absence of blue light (Fig 3.25, Dich results). Based on these results we speculate that the high blue/red spectral ratio which occurs under high sunlight intensity (typical to noontime for example) plays a role in addition to the effect of high light in induction of Cbr synthesis during the daily cycle. Most striking is the correlation between the Cbr and the Xanthophyll cycle. Figure 3.9 shows that the epoxidation state negatively correlates with Cbr levels, (i.e. positive correlation between Zea and Cbr levels). This correlation is consistent with the proposed function of Cbr (Levy, Tal et al., 1993) in binding Zeaxanthin to form photo protective complexes within the light harvesting antennae under light stress conditions.

Moreover, the results for the influence of Dich on Cbr induction are consistent with the low rates of ß-carotene synthesis per cell which was observed by the Dich culture (relative to control) and are in agreement with the hypothesis that low blue/red light simulates low light conditions in *D.* bardawil. Therefore, the results emphasize the specificity of blue light in Cbr and ß-carotene induction.

To sum up - Pigments and Cbr synthesis is clearly influenced by both light intensity and blue/red light ratio. Although it is generally expected that ß-carotene will correlate with the light intensity, our results in the different daily light intensity distribution experiments show that increase in sunlight above about 550 W/m^2 did not increase ß-carotene yield. The expected correlation may therefore hold only at intensities below that level.

Chlorophyll, Cbr and xanthophyll epoxidation state changed with intensity as expected. Low chlorophyll, high chl a/chl b ratio, low epoxidation state and high Cbr levels were associated with high light intensity. These effects were observed on both daily and seasonal cycles.

High light intensity is associated with high blue/red light ratio (and *vice versa*), here we show that reducing the blue light, induced low ß-carotene, Cbr and Zeaxanthin levels together with high levels of chlorophyll (accompanied with low chl a/chl b ratio). These reactions are known as low light responses. This is consistent with our suggestion that there is a connection between high blue light signal and conditions of light stress (see also López-Juez and Hughes, 1995, and 4.5 in this study).

Also in the case of Cbr, it was shown that the blue/red light spectral ratio plays a role in addition to the effect of high light in induction of Cbr synthesis. The correlation observed between Cbr and the Xanthophyll cycle is consistent with the proposed function of Cbr in

binding Zeaxanthin to form photo protective complexes within the light harvesting antennae under light stress conditions.

4.4 Acclimation of Antennae Size and Photosynthetic yield to the Diurnal and Seasonal changes in the sunlight features

Antennae size of PSII as was estimated by fluorescence induction measurements (Malkin and Kok, 1966) showed a remarkable sensitivity to the changes in the sunlight regime (Fig 3.10). The massive decrease in antennae size observed between the first and second day of measurements was a consequence of the new light environment (which differed from the limited lab conditions in intensity, spectrum and the diel rhythm from the previous growth conditions). In addition, the fluctuations in antennae size observed during the second day (Fig 3.10) seemed to follow the daily changes in the sunlight features (both intensity and spectrum) by reducing antennae size at peak hr's of radiation (when intensity and blue/red light ratio were increased). The antennae size response to changes in the light intensity was studied previously in *Dunaliella* (Falkowski, 1984; Pick, Gounaris et al., 1987; Smith, Morrissey et al., 1990) and other species (Bonnie and Krauss, 1970).

The response of antennae size to changes in the sunlight spectrum was also followed in the filters experiments carried out in this study, (Table 3.2). It was found that there is a spectral effect on PSII antennae size. A blue light effect on the adaptation of antennae size was clearly seen by the Dich results, showing that in the absence of blue light - antennae size was dramatically increased (i.e. low light response). These results support our hypothesis that blue light plays a role in adapting to changes in the light intensity. On the other hand, a decrease in antennae size (which is known as a high light response) was observed in the absence of green and red light (around 700 nm, see Blue Zel. Table 3.2). Furthermore, by using the Blue Zel filter - blue/red light ratio was increased resulting in typical high light acclimation (i.e. decreased antennae). The significance of green light can not be estimated in this case due to the lack of a specific green filter.

The daily fluctuation in photosynthesis efficiency (Fig 3.11) evaluated from fluorescence induction and oxygen evolution techniques, showed signs of the phenomenon of a "midday depression" (i.e. a markedly reversible decrease in the rate of photosynthesis during midday). The same event was observed as well in higher plants in nature (Demmig-Adams, Adams III et al., 1989), where the midday depression is partially a consequence of diurnal changes in temperature, humidity and stomatal conductance.

In the case of *Dunaliella* grown in our bioreactor, the "midday depression" can be explained by the diurnal change in sunlight intensity (which sometimes at midday exceeds levels around $900 w/m^2$, which is more then is utilized for photosynthesis, and therefore might generate

photoinhibition) as well as by changes in the sunlight spectrum. The latter can potentially induce the occurrence of adaptation or protection mechanisms that cause a reduction in photosynthetic efficiency. As expected, the "midday depression" detected in *D. bardawil*, was accompanied by a parallel increase in energy dissipating processes such as Cbr and Zeaxanthin formation (see above) and high light acclimation mechanisms (such as increase in Chl a/Chl b ratio or a decrease in PSII antennae size). The afternoon recovery in the photosynthetic efficiency following the depression is apparently due to repair of the damage (in the case of photoinhibition) or due to a relaxation of dissipation processes, or both. Since some of these dissipation and adaptation processes showed to be spectrally depended (especially upon blue light), we tested the spectrum influence on the efficiency of photosynthesis (Table 3.2). The results showed that in the absence of blue light (treatments of Dich and $CuSO_4$) photosynthesis efficiency was increased. The increased efficiency of photosynthesis under those two filters is consistent with the high cells production and growth rate achieved in these cases (Table 3.1). On the other hand, increasing the blue to red light ratio (MB) resulted in reduced efficiency of photosynthesis. By that, and relying on the correlation observed in nature between light intensity and the blue light, we can conclude that, indirectly, part of the midday depression is induced by the spectral daily changes in the sunlight.

The light intensity effect on photosynthetic efficiency was separated from the spectral effect by the angles experiments (Fig 3.16). The treated culture (under a constant daily light dose) roughly showed a midday depression in photosynthesis compared to the control. Therefore, it seems that a combination between the two parameters of light spectrum and intensity is involved in affecting, indirectly (by controlling the adaptation and protection mechanisms) the daily change in the efficiency of photosynthesis .

Clearly, both dissipation and acclimation processes contribute to the observed "midday depression" in photosynthesis, however, it is difficult to estimate the relative contribution of each. The rapidity of the recovering process can be helpful for the interpretation of underlying causes of depressions in photochemical efficiency (Demmig-Adams, Adams III et al., 1989). However, it must be noticed that the speed of the reversal change in energy dissipation processes has been shown to be highly variable, from as fast as minutes to as long as days. Similar differences may exist for the rapidity of repair processes.

The annual change in quantum yield of photosynthesis is presented in Fig 3.22 and resembles the pattern of change in growth rate and cell production (which were negatively correlated to the annual sunlight intensity curve). On the surface, the low quantum yield measured during the summer time relatively to the high winter yields, seems to be a direct result of photoinhibition (i.e. a consequence of high light). Based on our previous conclusion with respect to the indirect effect of blue light (playing a role as a signal for the light intensity) on photosynthetic efficiency and on the relative high values of antennae size measured during the winter (3.2.4), we suggest that the annual changes in the sunlight spectrum (Fig 3.18 b,c) and especially the seasonal variations in the blue light fraction play an important role also in this case. Therefore, during

the summer when blue light fraction increases concomitantly with the increase in sunlight intensity - processes of energy dissipation as well as high light acclimation are generated and induce a reduction in the efficiency of photosynthesis, the opposite scenario occurs during the winter time resulting in higher photosynthetic efficiency.

To sum up - In addition to the clear interaction of antennae size of PSII with the light intensity, it was found that there is a spectral effect on the antennae size as well. In conditions of reduced blue light, the antennae size was dramatically increased and *vice versa*.

The daily pattern of change in photosynthetic efficiency showed the signs of "midday depression" which was accompanied by a parallel increase in energy dissipating processes (i.e. increase in Cbr, Zeaxanthin and ß-carotene concentration) and high light acclimation mechanisms (i.e. a decrease in antennae size and in total chlorophyll concentration accompanied by a decrease in chl a/chl b ratio). Spectral light treatments showed that reduced blue light lead to an increase in the photosynthetic efficiency. Therefore it seems that a combination of light intensity and spectrum indirectly affects (by controlling the adaptation and protection mechanisms) the daily changes in the efficiency of photosynthesis. However, it is difficult to estimate the relative contribution of each.

The annual change in quantum yield of photosynthesis resembled the pattern of change in both growth rate and cell production (i.e. lower in summer time compared to the winter). We suggest that in addition to the response to the light stress in the summer time, the increase in the blue/red light ratio during the summer play an important role also in this case (see 4.5).

4.5 The role of Blue light in the response of *D. bardawil* under natural conditions

We tested the hypothesis that the variations in the blue light intensity (or in the blue to red light ratio) as part of the natural cycles of the sunlight (daily and annual), play a role in sensing the changes in the light intensity by the photosynthetic machinery of *D. bardawil*.

We conclude that the correlation between the blue light fraction and the sunlight intensity found in nature, provided evolutionary selection pressure for the development of such a blue light - sensitive mechanism in higher plants as well as in the *D. bardawil*. This concept can be supported by the results of López-Juez and Hughes (1995), which suggested that the photoreceptor responsible for light acclimation which is capable of distinguishing low *versus* high light fluence rate, is sensitive preferentially to blue light. On the other hand, it seems that our results (showing mainly low light acclimation response of the *Dunaliella* in the absence of blue light) are inconsistent with Wallen and Geen (1971) and Senger, Humbeck et al. (1992), which claimed that blue light mimics shade conditions for green algae. However, they tested

only a few kinds of deep water green algae (such as *Scenedesmus oblique* and *Chlorella fusca*) and the light intensity used in their experiments was much lower compared to the sunlight intensity used in this study. Generalization in this case can therefore be misleading. Still, we agree with Senger and Bauer (1987) who stated that the specificity of the species as well as purity and intensity of the spectral light and the development stage of the culture have to be considered in investigating the acclimation dependence on the light quality. In addition, since *D. bardawil* is unquestionably a "sun habitat" species, growing in areas of high irradiance in the upper surface of the water column, it perceives the natural changes during the sunlight cycle. Therefore, it can not be compared to other deep water green algae (which receive a completely different sunlight spectra due to the absorption and attenuation of light by the water body and microorganisms).

Based on the specificity of the blue light effects demonstrated in this study, and on pervious studies with *D. bardawil* (Ruyters, 1988), which proposed the existence of a UV/blue - light photoreceptor in *Dunaliella*, we suspect that blue light photoreceptor is involved in the acclimation responses observed in this study. It should be considered that high blue light plays a double role for the *Dunaliella*. First, it stimulates high light responses, and second, it seems to be the major source of damage under high light conditions. This was shown in the results for Dich, while reducing the blue light under high light conditions induced low light response, but also resulted with the highest cell production and photosynthetic efficiency (Tables 3.1, 3.2; Figs 3.24c, 25) . These results are consistent also with those for $CuSO_4$ filter which reduced a smaller fraction of the blue light and resulted with a smaller increase in cell production and low light responses compared to Dich, under high light (Tables 3.1, 3.2; Fig 3.24b). It is important to notice that in our experiments, we refer to high intensities of blue light (in the hundreds of W/m^2 range) which are different from the intensities of blue light range, required for development (phototropism, stomatal opening, inhibition of hypocotyl elongation etc.) in higher plants.

4.6 From the lab to the Field - Innovative Aspects

The concept of a field study is of main importance in this study. Although it includes many complications (such as the experimental set up and the variability in conditions), a field experiment contributes information which sometimes can not be obtained in the lab. Nevertheless, these difficulties can be solved partly by increasing replication, as well as by operating a "controlled field conditions" experiment like in this study.

One of the main factors in carrying out field studies of photosynthesis is the use of the natural sunlight as the light source. As shown, the sunlight spectrum and intensity and the temporal changes in its features are a dynamic system which is very difficult to imitate in the lab. However, these natural changes in the sunlight are important in plant acclimation responses.

Another important parameter in outdoors experimental systems is the possibility of following the photosynthetic response under different time scales (from daily to annual changes in the same system).

Lastly, on a practical term, the possibility of up scaling the size of the growth chamber in outdoors systems is an advantage compared to the limited size of growth chambers in the lab.

Clearly, both approaches of field and lab experiments are important in addressing scientific problems. Frequently, a lab experiment can answer questions which can not be addressed to under field condition and *vice versa*, therefore the cooperation between lab and field is essential. Still, it is important to consider the differences between the experimental conditions when the results are being related. The use of the double lens photobioreactor (DLB) in this study enabled us to integrate two basic ideas :

I. The detection of changes within the photosynthetic system in response to the natural changes in the sunlight intensity and spectrum on "real time".

II. The separation between effects of the sunlight spectrum and intensity and monitoring them.

Both I and II were conducted under optimal temperature, pH (CO_2) and nutrients supply.

On commercial terms, using blue light absorbers similar to Dich or $CuSO_4$ in outdoors mass cultivation of *D. bardawil* can significantly improve yields of cells (by about 70%) and ß-carotene (as shown in Table 3.1, 3.3 and Sec 3.4, 4.5 by 20-30%). The novel Bioreactor used in this study (DLB) offers the possibility to use such blue light absorbers (or any other optical manipulations).

5. Summary

The efficiency of photosynthesis depends on several limiting factors such as the availability of water and nutrients, ambient temperature, concentration of CO_2 and O_2 in the ambient air, pH and the quality and quantity of light.

The light quality, intensity, and their temporal variations significantly affect photosynthesis and plant growth. Plants' systems of receptors and pigments have adapted to the unique structure of the sun's spectral output. The response of photosynthesis and primary productivity to quantum flux density and to the spectral conditions has been the subject of intensive investigation in the recent years (Bjorkman, 1981; Falkowski, 1984; Anderson and Osmond, 1987; Senger and Bauer, 1987; Dring, 1988; Demmig-Adams, Adams III et al., 1989; Smith, Samson et al., 1993; Chamovits and Deng, 1996; Thiel, Döhring et al., 1996 and others).

Various properties such as the composition, structure and function of thylakoid membranes, pigments composition and the overall rates of photosynthesis are known to be adaptive to changes in the light intensity and spectrum. Moreover, it was indicated in several cases that some of the responses which are typical to changes in the light intensity are in fact stimulated by changes in the light spectrum (Buschmann, Meier et al., 1978; Lichtenthaler, Buschmann et al., 1980; Lichtenthaler, Kuhn et al., 1982; López-Juez and Hughes, 1995). In general, it was concluded that for higher plants, blue light was more effective in producing high light response. There is however, no general concept regarding algae. Furthermore, in most cases, the interrelation between the light spectrum and intensity and the photosynthetic acclimation response was investigated under lab conditions using artificial source of light and usually low light intensities.

The main objective of this study was to investigate the role of the natural changes during the daily and annual cycles within the sunlight spectrum, upon the acclimation strategies of the unicellular green algae *Dunaliella bardawil* to differing light conditions.

Accomplishment of the main objective is expected to enable enhanced photosynthetic productivity of *D. bardawil* by optimizing light conditions in the filed.

We chose *Dunaliella* as the model organism since it is predominant in the upper few centimeters of the water column (Borowitzka and Borowitzka, 1988) and therefore more directly exposed to variations in sunlight intensity and spectrum. Furthermore, the *Dunaliella* has a high potential in dissipating excess light energy and in acclimating to changes in the light environment (1.2.4).

We grew *D. bardawil* in a newly developed bioreactor (double lens bioreactor, DLB, which was composed of 2 concentric pyrex tubes). This bioreactor enabled the control over temperature, pH and nutrients levels. In addition, it improved the spatial and temporal distribution of light within the bioreactor, and enabled to manipulate the sunlight spectrum and intensity. The spectral control was achieved by using either filter solutions in the space between the 2 concentric tubes or by coating the bioreactor with colored zelofan paper.

We followed cell production, Xanthophylls concentration, Chlorophyll and ß-carotene production, the accumulation of Cbr, antennae size and the photosynthetic efficiency of *D. bardawil*. In parallel, the spectral and intensity changes in the sunlight were measured through the daily and seasonal cycles.

The results of sunlight measurements showed a clear positive correlation between the sunlight intensity and the fraction of blue light. This correlation was more significant during the daily cycle as compared with the annual cycle.

The measured photosynthetic characteristics of the *Dunaliella* grown in the bioreactor responded to the natural daily and seasonal fluctuations in the blue light and in the total light intensity. For example, during the winter time (when low blue/red light ratio was measured compared to the summer) high yields of cells and higher efficiency of photosynthesis were detected. During the daily cycle, chlorophyll concentration as well as Chl a/Chl b ratio fluctuated with the relative intensity of blue light (i.e. total chlorophyll decreased by 20% at noon time under high blue light, while Chl a/Chl b ratio increased by 12%). Also the photosynthetic antennae size was decreased by 60% at mid-day compared to the mornings' value. Cbr concentrations increased at mid-day (under increasing blue light intensity) by 80%, and then decreased again in the late afternoon in response to the natural decline in the blue light fraction. A similar pattern of daily changes was observed also by Zeaxanthin concentrations.

A decline of 37% and 45% from the morning's values was observed at mid-day in quantum yield and photosynthetic efficiency (F_v/F_m) respectively in the *Dunaliella* . This reduction in efficiency at mid-day can be the result of either energy loss by dissipation and acclimation processes, or the result of photoinhibition, or both.

In a suit of experiments, we separated the effects of total sunlight intensity and blue/red light ratio on photosynthetic characteristics. Results of artificial reduction of the blue light from the sunlight spectrum by using Potassium Dichromate (Dich) filter (absorbs most of the blue light below 480 nm), and $CuSO_4$ filter (absorbs below 420 nm) showed an increase in cell production by 70 % and 40% respectively compared to control. An increase in efficiency of photosynthesis (F_v/F_m) by 30% under Dich and 50% under $CuSO_4$ was observed as well.

The low blue light treatments (Dich and $CuSO_4$ filters) were carried out under high sunlight intensity, and seemed to affect the acclimation of the *Dunaliella*. Chlorophyll concentration per cell was increased by 70% and 50% under Dich and $CuSO_4$ respectively, and antennae size was increased by a factor of 3.4 under Dich compared to control. The increase in chlorophyll concentration was as expected, accompanied by a decrease in the Chl a/Chl b ratio by 20% under Dich and by 30% under $CuSO_4$. The induction of ß-carotene (known to accumulate under stress conditions) in *D. bardawil* was reduced under Dich and $CuSO_4$ by 30% and 20% respectively.

The production of Cbr (Carotene biosynthesis related protein, associated with stress conditions in *D. bardawil*), was decreased under reduced blue light treatment (Dich) by ~20%. Xanthophyll pool size and Zeaxanthin levels decreased by about two orders of magnitude under reduced blue light (Dich filter) compared to control.

We found that blue light is important in the cell division cycle as well. Both Dich and CuSO4 filters resulted with changes in the patterns of cell division. The more efficient blue light absorber (Dich) induced a gradual cell division throughout the daytime, while the less efficient blue light filter (CuSO4) only advanced the timing of the division burst.

Experiments designed to increase blue/red light spectral ratio within the sunlight, were carried out using treatments of reduced part of the red and/or green light (Blue Zel filter with maximal O.D around 600 nm and a small absorption peak below 400 nm, and MB filter with maximal O.D around 660 nm). These treatments did not affect cell production or the daily growth rate compared to control. A reduction in the ß-carotene production per cell by 40% was observed under blue Z (absorbs under 400 nm and around 600 nm) and an increase of 10% was observed under reduced red light (MB). Unexpectedly, both chlorophyll concentration and Chl a/Chl b ratio decreased by 20% and 10% respectively under reduced red light (MB) compared to control. This may be explained by a proposed strategy optimizing the ratio between the PSII reaction center concentration and the level of antennae chlorophyll (Falkowski, 1984; Pick, Gounaris et al., 1987). Reduction of the sunlight spectral range below 400 nm and around 600 nm (Blue Z filter) did not affect total chlorophyll concentration, but increased the ratio of Chl a/Chl b by 40%.

The following conclusions were drawn :

I. Based on the induction of low light response of the *Dunaliella* under conditions of decreased blue light level under high light intensities, and the correlation between blue/red spectral ratio and intensity in the sunlight, we concluded that blue light serves as indicator for light intensity in *D. bardawil*.

II. Based on the enhanced productivity observed under the conditions of high light intensity and low blue light, we concluded that the high intensity of blue light was also the cause of high light stress effects.

Thus, blue light has dual but distinct roles of indicating the light intensity level on one hand, and producing stress effects on the other.

The conclusion regarding the dual effect of the blue light in *D. bardawil*, helps to explain the phenomenon of a mid-day depression and the decline in photosynthetic production in the summer compared to winter. In both cases (mid-day and summer time), algae are exposed to high blue light in nature.

Based on our results, we further propose that a blue light photoreceptor must be involved in the acclimation of *D. bardawil* to light conditions. This concept is supported by the proposed existence of a UV/blue-light photoreceptor in *Dunaliella* (Ruyters, 1988) and by the results of López-Juez and Hughes, (1995) which suggested that the photoreceptor responsible for light acclimation in higher plants which is capable of distinguishing low *versus* high light fluence rate, is sensitive preferentially to blue light. Naturally, the photoperception discussed here must respond to blue light at high intensities.

III. Manipulations of the sunlight spectrum had a clear effect on the pattern of cell division. We concluded that both blue or red light, or likely a certain ratio of them, determine the timing of cell division in *D. bardawil*. It seems that low blue/red light ratio (such as occurs at sunset) induces preparation for division. High blue light during the day, may inhibit division. In addition, the seasonal variations in day length influenced timing of cell division (earlier division burst in shorter days). This is also likely due to associated changes in the daily spectral changes which depended on the season.

IV. Based on the results of increased cell and ß-carotene production by 70% and 20-30% respectively, we conclude that using blue light absorbers (similar to Dich or $CuSO_4$) in outdoors mass cultivation of *D. bardawil* can enhance the mass production of *D. bardawil* and its by-products, such as ß-carotene.

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Declaration

I declare that this thesis is the result of my own investigations and that no part has been submitted for any degree other than that of Doctor of Philosophy at the Weizmann Ins. of sciences

Roni Ashkenazi

1999

Appendix 1 Background for the Optical principles of the bioreactor

In order to define the optimal tubes dimensions so that all the incident light at the outer surface will reach the inner tube's surface, we have to look for the right radiuses ratio of the outer and inner tubes.

The ratio of r/R (with R equals to the radius of the external tube and r equals the radius of internal tube) can be presented by formula 1, which is based on simple trigonometry of the triangle ABC in figure 1.

$$(1) \qquad r/R = \sin(\beta)$$

(β is the refraction angle of light reaching the surface of the outer tube).
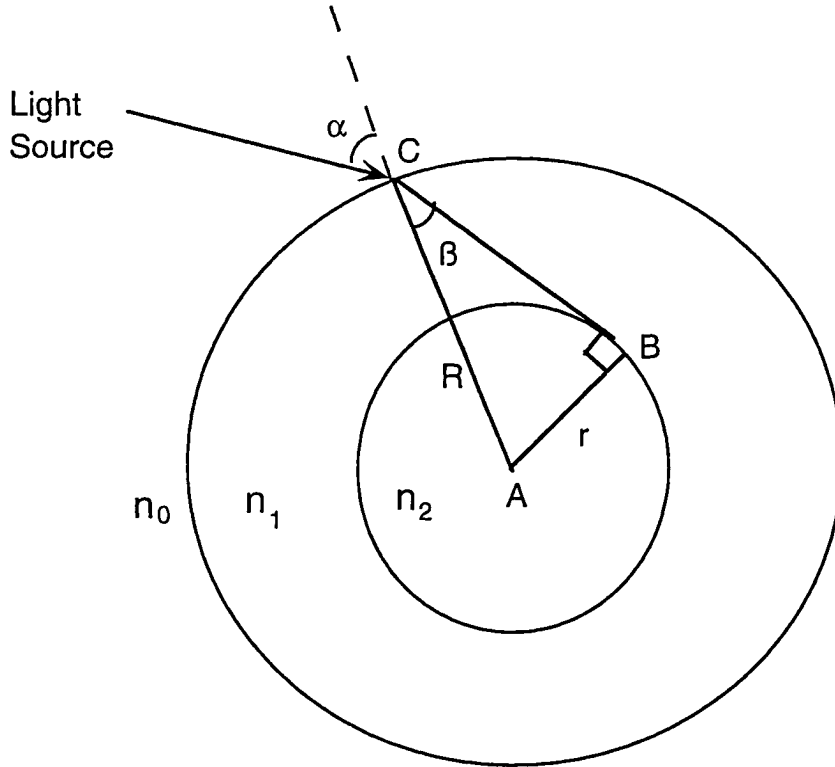
Figure 1: A geometrical description of the light intercepted by the wall of a bioreactor based on the double wall lens concentrator principle. The triangle ABC is combined of the small radius r, the tangent to the inner tube's surface (BC, which is perpendicular to r) and the radius of the outer tube (R). α is the angle between the light source and the perpendicular to the plane of the outer tubes' surface. $n_{0,1,2}$ are the refractive indexes of the media outside, in-between and inside the bioreactor walls respectively.

β is defined as the critical angle, i.e. rays of light refracting in an angle larger than β, will not reach the inner tube's surface and vice versa. By using Snell's low (in formula 2) we can find the optimal α (the angle in which the light "should" hit the outer surface of the bioreactor and then after refraction will reach the inner tube).

$$(2) \qquad n_0 \, \mathrm{Sin}\alpha = n_1 \, \mathrm{Sin}\beta$$

In our case, n_0 equals to the refractive index of air ($n_0 = 1$) and therefore $\mathrm{Sin}\beta$ can be expressed in the following way:

$$(3) \qquad \mathrm{Sin}\beta = \mathrm{Sin}\alpha \, / \, n_1$$

Since in our system, the light reaches the bioreactor from all angles between 0^0 to 90^0 to the surface (due to the large size of its source - the sunlight) the demand for 100% of the light reaching the inner tube can be fulfilled if we substitute α to be 90^0:

$$(4) \qquad \mathrm{Sin}\beta = 1 \, / \, n_1$$

and by substituting the value of $\mathrm{Sin}\beta$ (from formula 1) we get:

$$(5) \qquad r/R = 1 \, / \, n_1$$

$$(6) \qquad R/r = n_1$$

The conclusion is that in an ideal concentrating system (i.e. with all the light hitting the outer tube's surface reaching the inner tube, see fig 2), the ratio between the radiuses of the outer/inner tubes should be equal to the value of the refractive index of the tube and the solution inside the tubes, and a concentration power equals to R/r is approached.
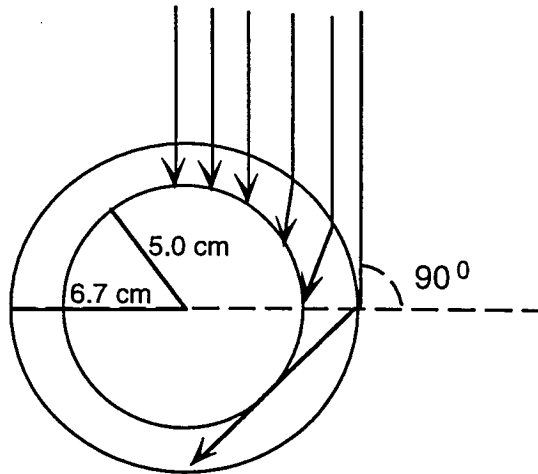

Figure 2: A Cross section of an ideal bioreactor (where all the incident light at the outer surface reaching the inner tube surface.

Appendix 2 Fluorescence Induction method

Chlorophyll a fluorescence instantly monitors the balance of the competing decay processes from the excited state and can be used as a measure of the photochemical conversion. The light induced of Chl a fluorescence from a dark adapted initial value (F_0) to a maximum value (F_{max}), a phenomena called fluorescence induction is believed to reflect the conversion of the primary acceptor (Q_A) of PSII from an oxidized to a reduced form (Q_A-) by the light induced primary charge separation reactions. In the presence of DCMU, (3-(3,4-dichlorophenyl)-1,1-dimethylurea) no further electron transport from (Q_A-) is possible and the reaction is limited to the conversion of Q_A to Q_A- alone.

The fluorescence intensity and the photochemical activity complementary relationship is shown in eq. 1:

$$(1) \quad \Phi_p + \Phi_f + \Phi_d = 1$$

Where Φ is the quantum yield and the subscripts are for photochemistry (p), fluorescence (f), and nonradiative dissipation (d).

As a consequence of this relationship it was shown that the complementary area above the fluorescence induction curve is a valid measure of the number of active PSII centers in the presence of DCMU.

Therefore, the electron acceptor pool can be computed directly from the fluorescence induction time (t) and the absorbed light intensity (Malkin and Kok, 1966; Malkin, Armond et al., 1981). From these considerations, it follows that for a sample unit area incident to a parallel beam of exciting light, the quantity of RC_{II} (in mol) is given in eq. 2:

$$(2) \quad RC_{II} = \alpha_2 \, \phi_2 \, I_{abs} \, t \quad \text{(Malkin and Kok, 1966)}$$

where α_2 is the fraction of light which is absorbed directly by PSII, $\phi_2 \, (= f_v \, / \, f_{max})$ is the maximal efficiency of photo chemistry in PSII (for open reaction centers) and I_{abs} is the flux of light absorbed by the sample (expressed in Einstein per unit area and unit time).

Since I_{abs} and α_2 are assumed to be constants (for each sample), just ϕ_2, t and their product (i.e. the "antennae size") are calculated.

Appendix 3 Estimating the actual amount of light absorbed by the _Dunaliella_ culture during an experiment

In order to estimate the actual amount of light absorbed by a single cell growing within the bioreactor, we should consider the following optical properties :

1. The fraction of chlorophyll absorption spectra within the photosynthetic active radiation range (PAR) : The efficiency by which light may be absorbed by a leaf, depends on the content of chlorophyll per unit leaf area. The dependence is described by Beer Lambert's law (presented below in formula 1) implying that the fractional absorptance is less than proportional to increase in pigment content. Bjorkman, [1982] claims that glabrous leaves with normal chlorophyll content of 400-600 mg chl/m^2 absorb about 80 to 85% of the daylight in the waveband of PAR. This order of magnitude was proposed also by others (walker, 1990). I assume that for unicellular green algae (like the _Dunaliella bardawil_ with the same chlorophyll content), the same order of magnitude for efficiency of light absorbed by the cells can also be established.

2. The gradient of light within a cross section of the bioreactor (of a 5 cm diameter) which is affected by absorption and scattering by pigments and cells growing under the different light treatments: Due to the significant role of blue light in concern with the results presented in this report, together with the fact that blue light is mainly absorbed by ß-carotene (which is accumulated by the _Dunaliella_) as well as by chlorophyll, the absorbency correction due to pigments concentration and culture's density was accounted for the blue range of the light spectrum. The general pattern of the light intensity gradient within the cross section of the bioreactor is described by a logarithmic decay by Beer Lambert's law :

$$(1) \quad (O.D)\lambda = (\varepsilon)\lambda \, [C] \, L = \log [I_0/I_t]$$

Where O.D is the optical density of a solution (termed also as absorbency, it is depended on λ, the wavelength which was determined to be 480 nm in this case).

ε is the extinction coefficient of the solute (chlorophyll and/or ß-carotene in this case).

L is the optical path length (2.5 cm, the bioreactor radius).

I_0 and I_t are the intensity of the incident and transmitted light respectively.

Since pigments concentration was not constant through the day, I chose to calculate the fraction of light transmitted by the algal culture at noontime on the third day of growth (when differences among treatments were more significant and also the blue light intensity was high). The averaged amount of light absorbed at 480 nm (i.e. optical density, O.D) measured at the pigments extracted from dichromate treated algae (within a sampling quivete of 1 cm optical

path length) was 0.41. This number was multiplied by the bioreactor radius length (2.5 cm) to give the fraction of light which is absorbed through the center of the bioreactor tube. The resulting O.D was 1.02 indicating (by using Beer Lambert's law) that 10% of incident light at 480nm was reaching the center of the tube. Same procedure was repeated for the other light treatments and their corresponding controls, the results are presented in the following table.

	Dich	control	Blue Z	control	MB	control	CuSO$_4$	control
O.D$_{480\ nm}$ for L=1.0 cm	0.41	0.38	0.14	0.24	0.17	0.12	0.22	0.13
percent of light transmitted for L=2.5 cm	10.00	11.00	43.00	25.00	38.00	50.12	28.18	47.90
cells density*10^5 per ml	19.25	11.32	14.08	15.52	8.03	6.98	12.33	6.57

The intensity of light reaching the center of the internal bioreactor's tube after considering the optical density of the algae culture at 480 nm was about the same in dichromate and it's control. The light intensity penetrating through the Blue Z culture was higher by 1.7 from control, and reached to about only 70% in the MB and CuSO$_4$ treated cultures compared to control.

The light attenuation due to scattering by the Dunaliella cells (assumed as spherical particles in this case) can be derived theoretically according to a theory developed by Mie (1908), in this case the particles are not small enough to obey Rayleigh's law (see 3.1.1) and therefore there is only a slight dependence on the wavelength. Mie scattering is known to occur predominantly in the forward direction, consequently any differences in the amount of light attenuation due to differences in cells density, has to be considered only in the circumference of the bioreactor (i.e. the peripheral layer of algae close to the tube's wall). The light which is further scattered inside the tube is preserved. Due to these considerations and based on the fact that the amount of light which is lost by scattering at the boundary layer is depended on the cells concentration and their refraction index, it can be assumed to be correlated linearly to the cells concentration. It shows a major difference in the Dich and CuSO$_4$ of about 2 folds compared to control culture, and was not significantly different between Blue Z, MB and their controls.

Therefore, after considering both absorption by pigments and scattering by whole cells, the intensity of light reaching the cells in the center of the inner tube was about 50% in the Dich, 30% in the CuSO$_4$, 75% in MB and 170% in the Blue Z compared to 100% of control.

However, it should be considered that the cultures are being stirred constantly so that every individual cell "sees" an averaged amount of light which is representative of the overall light

regime within the bioreactor. This representative value is a combination of the mean light regime within the cross section of the tube, including the original light irradiation impinging on the tube's surface. As was mentioned above, the table represents differences which appeared just on the third day of growth. Naturally, these differences did not exist on the first day (when the culture was identical in both treatment and control) and were still minimal through the second day of the experiment. Since these differences in the optical characteristics of the culture only started to appear on the third day of the experiment they might have cause further photosynthetic responds on the following days but their influence before was minimal. Therefore we can conclude that these differences are a result of the light regime which was dictated by the different filters in use and .

In order to monitor any differences in the daily integral amount of light reaching the treated and control culture due to the daily changes in blue/red light ratio and its interactions with the absorption spectrum of polyethylene and the filter used we should superimpose the filters spectrum on the daily changes in sunlight intensity and spectrum and compare it to the daily integrated amount of light after passing through the polyethylene layer.

3. Differences in the integral of daily sunlight intensity between polyethylene and dichromate treated algae - The Dichromate filter (which cuts off the light below 430 nm, see fig 2.3a) subtracts a small fraction from the blue range of the sunlight spectrum which does not seem to vary significantly with the daily fluctuations in sunlight, therefore any differences in the total daily amount of light between the Dich and control algae can be neglected. The same treatment can be referred also to $CuSO_4$ (fig 2.3b) where the main peaks of absorption are located in the red range (which does not change in the sunlight through the day time), and in the range which is lower than 420 nm. Under MB the conclusion is that it does not cause artificial fluctuations in the diurnal change in sunlight intensity as well, due to the fact that it absorbs in a range which does not fluctuate through the day time (see fig 2.3d). Therefore, light intensity transmitted by these filters and the polyethylene layer was equal throughout the day time.

Differences in the total daily sunlight intensity between polyethylene and Blue Z (see absorption spectrum in fig 2.3c) treated algae were estimated by comparing daily integral amount of light intensity after passing through the polyethylene layers (which reduced in the case of Blue Z series of experiments the total amount of light by 40% independently of the daily spectral changes in the sunlight) to the daily integral amount of light passing through the Blue Z filter paper - The theoretical basis for the comparison is presented bellow:

The daily integral amount of light energy (E_t) is expressed by the integral of intensities (I) over a day (eq. 2):

$$(2) E_t = \int_{day} I(t)dt$$

The integrated amount of light energy (Eλ) within the range of 400 to 500 nm (which is transmitted by the Blue Z) during the day time is expressed by the integral of light intensities (Iλ) between 400 to 500 nm :

$$(3) E_\lambda = \int_{400}^{500} I(\lambda)d\lambda$$

Therefore the daily overall light dose (E) will be equal to $0.4\int I(t)\, dt$ in the control and to the combination of eq. 2 and 3 at the Blue Z treated cells culture :

$$(4)\ E = \int I(t,\lambda)\, dt\, d\lambda$$

Calculations results (for days in different times of the year) showed that -

* Throughout the day time, higher blue/red light ratio was kept under the Blue Z treatment compare to control.

* The addition of around 9% in the total daily amount of light reaching the control algae compared to the Blue Z treated algae was detected during winter time. This difference decreased during the rest of the year when the blue/red light ratio in the sunlight spectrum is more pronounced therefore allowing the Blue Z to transmit a larger fraction of the sunlight spectrum radiation.

II. בהתבסס על העלייה בפרודוקטיביות שנצפתה בתנאים של אור גבוה ונתח ספקטרלי כחול בעל עצמה נמוכה, אנו מניחים שלאור כחול בעצמה גבוהה, תפקיד מרכזי ביצירת תנאי עקת אור בתנאי אור גבוה (high light stress). לפיכך, היגענו למסקנה שהאור הכחול הוא בעל תפקיד כפול בתהליך ההסתגלות של הדונליאלה לסביבת אור משתנה – היותו גורם ראשי ביצירת מצב עקה מחד, והיותו סמן לתנאי אור גבוה מאידך, בשני המקרים מדובר על אור כחול גבוה. מסקנה זאת, תורמת להבנתנו את התופעה של הירידה ביעילות הפוטוסינטתית בצהרי היום ("mid-day depression") ובפרודוקטיביות בעונת הקיץ (בהשוואה לחורף). בשני המקרים, התרבית נחשפה באופן טבעי לעוצמות גבוהות של אור כחול.

בנוסף לכך, הצענו שבתהליכים אלו של הסתגלות/תגובת האצה לעצמת האור, קיימת מעורבות של קולטן ספציפי לאור כחול (blue light photoreceptor) בדונליאלה בארדאוויל. סימוכין להצעה זאת ניתן למצוא בעבודות קדמות, כאשר בעבדתו של Ruyters, (1988) מניחים את קיומו של UV/blue-light photoreceptor בדונליאלה, ואילו עבדתם של Lopez-Juez and Hughs, (1995) מציעה שקולטן כחול הוא האחראי במנגנוני צמחים עילאיים ל"הבחנה" בין תנאי אור חלש לאור גבוה. כמובן שבמקרה זה כמו במקרה שלנו, הכוונה היא לקולטן המגיב לאור כחול בעצמה גבוהה.

III. נראה שלמניפולציה של ספקטרום אור השמש היתה השפעה גם על תהליך חלוקת התא. על פי התוצאות, נראה כי יחס מסוים בין נתח האור הכחול והאדום קובע את זמן תחילת החלוקה ב ד. בארדאוויל. בעוד שנראה שיחס נמוך של אור כחול לאדום (כמו שנמדד בזמן השקיעה) מסמן לתחילת ההכנה לקראת חלוקה, יחס גבוה יותר ככל הנראה מעכב חלוקה לאורך היום. בנוסף, ניכרה גם השפעת השינוי העונתי באורך היום על זמני חלוקת התא (חלוקה מוקדמת בימים קצרים ולהפך), יתכן שגם במקרה זה הסיבה האמיתית היא השינויים הספקטרליים הכרוכים בשינוי העונתי.

IV. בהתבסס על תוצאות הפרודוקטיביות הגבוהה של מספר התאים ושל כמות ה ß–Carotene ב 70% וב 20-30% בהתאמה, בנסיונות של אור כחול מופחת, אנו מסיקים ששימוש בפילטרים הבולעים בתחום הכחול (כמו KH_2Cr_2 ו $CuSO_4$) בגידול תעשייתי של ד. בארדאוויל עשוי להשפיע באופן דומה על התוצרים.

הכללי של כלורופיל ירד בשיעור 20% בשעת צהרים (בה נמדדו בדרך כלל עוצמות אור שמש גבוהות בכלל ושל אור כחול בפרט) בהשוואה לבוקר. הירידה בריכוז הכלורופיל לוותה בעליה של 12% ביחס Chlorophyll a/b. מגמה דומה נצפתה גם בגודל האנטנה הפוטוסנתטית כאשר נמדדה ירידה של 60% בשעת הצהרים ביחס לבוקר. ריכוז Cbr עלה ב 80% בצהרים וירד שוב בהמשך היום, כפי הנראה כתגובה לירידה הטבעית בעוצמת האור הכללי והאור הכחול בשעות אחה"צ. ההשתנות היומית כפי שנצפתה בריכוז זאקסנטין (Zeaxanthin) היתה בקורלציה לריכוזי Cbr.

ירידה של 37% ו 45% ביעילות הקוונטית וביעילות הפוטוסנתטית (F_v/F_{max}) בהתאמה, נמדדה בצהרים. תופעה זו תיתכן כתוצאה מפוטואינהיביציה (photoinhibition) או כתוצאה מאיבוד אנרגיה בתהליכים של הסתגלות (acclimation) ו/או דיסיפציה (dissipation) של עודף אנרגיית אור הנוצרת לעיתים בשעת צהרים. סביר להניח ששלושת התהליכים גם יחד היו מעורבים והובילו לירידה ביעילות הפוטוסנתזה במשך הצהרים. תוצאות ניסיונות בהם הפרדנו באופן מלאכותי בין השפעות עצמת השמש לבין השפעת הספקטרום על התגובה הפוטוסינתטית של הדונליאלה, הראו עלייה של 40% עד 70% בפרדוקטיביות (כתלות בפילטר בו השתמשנו) תחת טיפולים של אור כחול נמוך ועצמת שמש גבוהה. כמו כן, נצפתה עלייה שבין 30% ל 50% ביעילות הפוטוסינתטית במקרים אלה. טיפולים אלה בהם הופחתה עצמת האור הכחול (בעזרת שימוש בתמיסות $CuSO_4$ ו KH_2Cr_2 כפילטרים כחולים לאור השמש) השפיעו ככל הנראה על כל תהליכי ההסתגלות של הדונליאלה לסביבת אור משתנה. מסקנה זו מתבססת על תוצאות של עליה של 50% עד 70% בריכוז כלורופיל לתא (שלוותה בירידה של 20%-30% ביחס Chlorophyll a/b), גידול בפקטור של פי 3.4 בגודל אנטנה פוטוסינטטית וירידה של 20%-30% בריכוז ß–Carotene כל זאת תחת עצמת אור גבוהה ($800w/m^2$~) ואור כחול נמוך ($CuSO_4$) או בהעדרו (KH_2Cr_2) בהשוואה לקבוצת ביקורת (בעלת אותה עצמת אור, ואור כחול בעוצמה גבוהה בהתאם).

כמו כן נמדדה באותם תנאים של אור כחול מופחת, ירידה בריכוז החלבון Cbr (שמצטבר בדרך כלל ביחס ישר לתנאי עקת אור) ובריכוז זאקסנטין בהשוואה לקבוצת הביקורת.

גם בתהליך חלוקת התא נראתה מעורבות של האור הכחול, כאשר בהיעדרו נראתה חלוקת תאים גם במשך שעות היום ואילו בטיפול בו הופחתה עוצמתו, הוקדמה שעת תחילת החלוקה לשעות אחר הצהריים המאוחרות, כל זאת בהשוואה לסינכרוניזציה של חלוקת ליית שנצפתה בקבוצת הביקורת.

בניסיונות בהם יצרנו באופן מלאכותי יחס גבוה בין כחול לאדום בספקטרום אור השמש (בעזרת שימוש בנייר פילטר כחול הבולע סביב 600nm ומתחת ל 400nm ותמיסת צבען מתילן כחול, MB, Methylene Blue; הבולעת קרינה סביב 662nm) לא הושפעו קצבי גידול או פרודוקטיביות תאים בהשוואה לביקורת. מאידך, נצפתה ירידה של 40% בריכוז ה ß–Carotene בטיפול הפילטר הכחול ועלייה של 10% בקבוצה שטופלה ב MB.

שלא כצפוי לגבי היחס בניהם, ריכוז כלורופיל והיחס Chlorophyll a/b ירדו ב 20% וב 10% בהתאמה, בטיפול של MB (כלומד יחס גבוה של כחול לאדום בהשוואה לספקטרום של קבוצת הביקורת). הסבר אפשרי לתופעה זאת שנצפתה גם בעבודות אחרות הוא אססרטגיה של ויסות היחס האופטימלי בין ריכוז הכלורופיל במרכזי הריאקציה של PSII לבין ריכוז הכלורופיל שבאנטנה ולאו דווקא יחס הפוך בין ריכוז כלורופיל כללי והיחס Chlorophyll a/b (Falkowski, 1984; Pick, Gounaris et al., 1987).
מובאות בזאת המסקנות הסופיות של העבודה :

I.‏ בהתבסס על העובדה שבתנאי אור כחול נמוך ועצמת אור חזקה, הדונליאלה הראתה תגובה לאור נמוך ובהתבסס על הקורלציה שנמצאה בטבע בין עצמת אור השמש לבין היחס הספקטרלי שבין אור כחול לאדום הגענו למסקנה שהאור הכחול משמש כסמן, (indicator) לעצמת השמש עבור האצה ד. בארדאוויל.

תקציר העבודה

יעילות תהליך הפוטוסינתזה תלויה במספר גורמים מגבילים, כגון חומרי הזנה (נוטריינטים), מים, pH,
טמפרטורה, ריכוזי O_2 ו-CO_2 , מקור אור, עצמתו ותכונותיו הספקטרליות.

לשינויים בעצמת ובספקטרום האור תפקיד משמעותי בתהליך הפוטוסינתזה ובהתפתחות צמחים. מערכות
הרצפטורים והפיגמנטים הצמחיים הסתגלו במשך האבולוציה לתכונות האור הנפלט מן השמש. יעילות
תהליך הפוטוסינתזה והתגובה הפוטוסינתטית לשינויים בעצמה ובספקטרום האור נחקרה במשך השנים
האחרונות.

(Bjorkman, 1988; Falkowski, 1984; Anderson and Osmond, 1987; Senger and Bauer, 1987; Dring,
1988; Demmig-Adams and Adams III et al., 1989;Smith, Samson et al., 1993; Chamovits and
Deng, 1996; Thiel, Dohring et al., 1996 and others).

הרכב הפיגמנטים, מבנה ותפקוד ממברנת התילקואיד, ויעילות תהליך הפוטוסינתזה נתונים לשינוי בהתאם
לתנודות בהרכב הספקטרלי של מקור האור ובעצמתו. במספר מקרים הוכח שחלק מן התגובה הפוטוסינתטית
לשינוי בעצמת האור נבעה כתוצאה משינויים ספקטרליים.

(Buschmann, Meier et al., 1978 Lichtenthaler, Buscmann et al., 1980; al., 1982; Lichtenthaler,
Kuhn et al., 1982; Lopez-Juez and Hughes, 1995).

בעוד שלגבי אצות לא קיימת מסקנה כוללת, בצמחים עילאיים, הגיעו למסקנה שלאור הכחול תפקיד
דומיננטי בהשריית תהליכי הסתגלות לאור גבוה. בכל מקרה, רוב העבודות הבוחנות את יחסי הגומלין בין
ספקטרום ועצמת מקור האור לבין תהליכי הסתגלות פוטוסינתטים (אקלימציה), נעשו בתנאי מעבדה תחת
אור מלאכותי בעצמה נמוכה בהרבה מזו של השמש.

המטרה העיקרית של עבודה זאת, היא לחקור את השפעת השינויים הטבעיים ביחס הספקטרלי של האור
הכחול והאדום באור השמש על מנגנוני ההסתגלות של האצה החד-תאית דונליאלה בארדאוויל (*Dunaliella*
bardawil) לתנאי הארה המשתנים, כל זאת במשך המחזוריות היומית והעונתית של הטבע. השגתה של מטרה
זו, עשויה לאפשר שיפור ביעילות הפוטוסינתזה ועלייה בפרודוקטיביות, זאת, על-ידי אופטימיזציה של תנאי
ההארה בשדה.

הבחירה ב דונליאלה כמודל במקרה זה, נעשתה מסיבת יכולת ההסתגלות הגבוהה שלה לתנאי סביבה
קיצוניים (1.2.4), וכמו כן מהסיבה שבית הגידול הטבעי שלה הוא החלק העליון בעמודת המים (1988
Borowitzka and Borowitzka,) עובדה שגורמת להיותה חשופה באופן ישיר לשינויים באור ובעצמת השמש.
במהלך הניסיונות, גידלנו תרבית של ד. בארדאוויל בביוראקטור חדשני (Double lens Bioreactor, DLB)
שאיפשר בקרה על דרגת טמפרטורה, pH, צורת ספקטרום השמש המגיעה לתרבית ודיכוך נוטריינטים בתרבית.
בנוסף, מבנהו המיוחד של הביוראקטור שיפר את תנאי ההארה על התרבית לאורך היום וכן את אופן פיזורו
המקומי. הפרמטרים הנבחנים היו – פרודוקטיביות תאים, ריכוזי פיגמנטים (כמו קסנטופילים, כלורופיל ו-ß
(Carotene, ריכוז החלבון Cbr האופייני לדונליאלה תחת תנאי אנטנה, גדל אנטנה ויעילות פוטוסינתטית.
במקביל, נמדדו השינויים בעצמה ובספקטרום השמש במשך המחזוריות היומית והעונתית.

ממדידות ספקטרום השמש ניתן היה להבחין בשינויים דומיננטיים בעצמת האור הכחול וכן במנה הספקטרלית
שבין אור כחול לאדום (Blue/Red light ratio) לאורך המחזוריות היומית והעונתית. הוסק יחס ליניארי בין
עצמת השמש לעצמת האור הכחול, היחס היה מובהק יותר לאורך השינוי היומי ובמידה פחותה במחזוריות
העונתית.

המאפיינים הפוטוסינתטיים שנמדדו, הגיבו לשינויים היומיים והעונתיים באור הכחול ובעצמה הכללית של
השמש. לדוגמא, במשך תקופת החודף (כאשר נמדד יחס נמוך של אור כחול לאדום בהשוואה לקיץ) נמדד
שעור גבוה של יבול תאים ושל קצבי פוטוסנתזה. כמו כן, ריכוז כלורופיל והיחס בין כלורופיל a וכלורופיל
b (Chlorophyll a/b ratio) השתנו בהתאם לשינוי היחסי באור הכחול במשך המעגל היומי. למשל, הריכוז

בהנחיית פרופסור דן יקיר ופרופסור אמנון יוגב.

Roni Ashkenazi

רוני אשכנזי

תגובת האצה דונליאלה בארדאוויל לשינויים
הטבעיים בספקטרום ובעוצמת השמש

The Response of *Dunaliella Bardawil* to the Natural Changes in the Sunlight Spectrum and Intensity