Handbook of Microalgal Culture: Biotechnology and Applied Phycology

Edited by

Amos Richmond

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Preface

An introduction into the state of the art

Over 15 years have elapsed since the previous Handbook addressing mass cultivation of microalgae (CRC Press, 1986) was published. At that time, it was already evident that the original concept viewing microalgae as a future agricultural commodity for solving world nutrition needs has no basis in reality. Photosynthetic efficiency in strong sunlight falls far short of the theoretical potential resulting in low yields which are the major culprits for the forbiddingly high production cost of algal cell mass. Economically, therefore, outdoor cultivation of photoautotrophic cell mass is inferior to conventional production of commodities such as grains or soybeans. At this stage of our experience with mass production of photoautotrophic microalgae, it is indeed evident that certain very ambitious roles that have been suggested for large-scale microalgaeculture – e.g. reduction of global carbon dioxide using large areas of unlined, minimally mixed open raceways – are unrealistic, being based on unfounded assumptions concerning, in particular, maintenance costs and the expected long-term productivity. Notwithstanding, schemes for local reduction of carbon and nitrogen emissions from, e.g. power plants, using intensive microalgal cultures in efficient photobioreactors, may have economic prospects based on winning valuable environmental credits for the polluting industry and provided such environmental treatments are, in effect, subsidised by State laws in which strict demands for reducing combustion gases within a definite period are imposed.

Similarly, the grand idea of using algal systems for the sole purpose of industrial energy production, such as hydrogen or methane (unlike the bacterial–algal systems meant to produce these chemical energies coupled to processes of waste clearance), is simply unrealistic: Technologies by which to harness solar energy, e.g. wind machines, photovoltaic systems or a whole array of solar collectors, are much closer to becoming an ongoing economic reality than microalgal cultures bent on producing, with dismal efficiency, bio-hydrogen.

One unique grand scheme, however, sea nourishment to augment phytoplankton growth, is worthy of critical examination. Several land and ocean areas on our planet are exhibiting low productivity due to lack of factors required for plant growth and large ocean expanses are essentially barren due to an acute shortage of some mineral element, e.g. nitrogen or iron. The productivity of such desert oceans could be readily improved by a small, judicious addition of the growth-limiting factor and, there are experimental indications showing this idea to be feasible. The growing world population in certain areas of this planet mandates urgent efforts to achieve a substantial increase in local food production, and barren oceans may be regarded as an

extension of land in which rather extreme manipulations of the natural environment for the purpose of food production have been acceptable for years. Such schemes naturally arouse intense criticism based on fears of evoking unknown deleterious environmental consequences. Nevertheless, adding small amounts of a growth-limiting nutrient to desert oceans carries the prospects of benefitting from both carbon dioxide sequestration and fish productivity. A reassuring aspect of this scheme rests on the fact that ocean nourishment may be quickly modified or altogether stopped if the results are judged to bring about negative environmental consequences.

A development which may soon lead to massive production scale of microalgae stems from the fact that production of heterotrophic microalgae has significant economic advantages over photoautotrophic production. The recent successful attempts to convert the trophic level of strictly autotrophic species (e.g. *Porphyridium cruentum*) into that of heterotrophic producers represent, therefore, a landmark in microalgal biotechnology. It is conceivable that once efficient trophic conversions become readily available for practical use, several photoautrotrophic microalgae will be grown commercially in very much the same simple and effective mode by which bacteria, yeast or fungi are commercially produced. Indeed, if the requirement for light is eliminated, microalgae could be grown in accurately controlled, very large-culture vessels of a few hundred thousands liters, holding cell densities higher by about two orders of magnitude above the optimal for an open raceway. A cut of perhaps one order of magnitude in the cost of production, compared with that of photoautrotrophic microalgae, has thus been envisioned.

Presently, the most important endeavor unfolding in commercial microalgaculture is the use of heterotrophic microalgae for a whole line of new products to supplement animal and aquacultural feed, as well as human nutrition. The first production lines so far developed by MARTEK, USA, concerns long chained polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic acid (DHA). Soon to follow will probably be production facilities of microalgal feed for animal husbandry, particularly for aquaculture. It is significant that the first truly large-scale industrial production of microalgae in a photobioreactor, the 700 000 l tubular reactor (divided into some 20 subunits), constructed and run by IGV Ltd in Germany which is producing *Chlorella* as a food additive for poultry, is based on a mixotrophic mode of nutrition.

Is then the strictly photoautotrophic production mode in commercial microalgaculture on the verge of phasing out? Despite the imminent onslaught of trophic conversions of several microalgal species, which would to some extent undermine phototrophic production, photoautotrophic microalgae do have a rather safe future for several specific purposes, most prominent of which are in aquaculture, bioactive compounds, water clearance for a sustainable environment as well as fresh water supplies, nutraceuticals regarded as healthfood and finally, as a basic human food.

Since most artificial substitutes are inferior to live microalgae as feed for the critical stages in the life cycles of several aquacultural species, a growing demand for microalgae will go hand in hand with the expected growth of aquaculture throughout the world. Presently, most aquacultural enterprises

produce (albeit with only limited success in many cases) their own supply of microalgae. Since the algal cultures can be often fed directly to the feeding animals, eliminating thereby the necessity for harvesting and processing, such rather small scale on-site production makes economic sense. Centralized microalgal facilities which sell (for a high price) frozen pastes or highly concentrated refrigerated stock cultures cover at present only a small part of the aquacultural demand for live microalgae. Once heterotrophic production is established and inexpensive microalgal feed becomes widely available, it seems certain that centralized production of microalgae for aquaculture will receive a strong impetus. Nevertheless, costs of local, in situ production of microalgae could be greatly reduced through improved implementation of practical know-how in mass cultivation giving cause to expect that on-site production of photoautotrophic microalgae carried out presently in many hatcheries, will at least to some extent, maintain its ground.

Wastewater clearance represents another important niche in which photoautotrophic microalgae are prominent. Using photosynthetic microalgae to take up the oxidized minerals released by bacterial action and, in turn, enrich the water with oxygen to promote an aerobic environment and reduce pathogens, makes good practical sense and could be well used in suitable locations the worldover. An interesting and promising variation on this general theme may be seen in land-based integrated systems, in which microalgae together with bacteria play a role in clearing aquacultural wastes, becoming in turn feed for herbivores and filter-feeders. These systems well integrate with the environment and will probably become widespread in favorable locations the world over.

Ever since the inception of commercial mass cultivation of microalgae in the early 1950s, the mainstream of product development has been diverted to the nutraceutical and health food, markets. There are good reasons to believe this trend will continue, considering the growing economic affluence the world over as well as the growing interest in the western world in vegetarian eating modes. The collection of pills and powders made from *Chlorella*, *Spirulina* (or *Arthrospira*) and *Dunaliella* is being enriched by a promising newcomer, *Haematococcus pluvialis*. Originally meant to produce the carotenoid Astaxanthine for fish and shrimp pigmentation, astaxanthine was discovered to be an outstanding antioxidant with antiaging potential, so the present primary production target is focused on the usual nutraceutical venue.

Concerning this trend, it is my opinion that a gross mistake has been made by the microalgal industry in focusing all marketing efforts on health foods and the like. It is a lucrative market, but is naturally rather small and cannot stir a large demand for microalgae. This marketing focus may be as culpable in impeding progress of industrial-scale microalgal culture, as high production costs, by curbing potential demand. It is an erroneous approach in that it overlooks the fact that several microalgae (such as *Spirulina*, *Chlorella*, *Dunaliella*, as well as other species such as *Scenedesmus*) when correctly processed have an attractive or piquant taste and could be thus well incorporated into many types of human foods, greatly expanding demand for microalgae. I thus believe the microalgal industry would much benefit from

a closer interaction with the food industry, employing food technology methods to create a myriad of possible new food products. Incorporating suitably processed microalgae into nearly all food categories would add not only nutritional value, but also new, unique and attractive tastes to such food items as pasta, pretzels, potato and corn chips, soup mix and seasonings, an assortment of dairy products, and even an assortment of candies, and ice-creams, to mention but a few obvious possibilities.

Much effort has been expended on the search for new compounds of therapeutic potential, demonstrated in microalgae of all classes, possessing antibacterial, antifungal and anticancer activities. Indeed, there are many promising prospects for new chemicals reported in recent years, the most prominent of which are carotenoids of nutritional and medical values, new polysaccharides and radical scavengers, as well as a whole array of unique chemicals in cyanobacteria, and in the vast diversity of marine microalgae. Considering the untapped resources with which it may be possible to enrich the pharmaceutical arsenal, it seems safe to predict that the search for photoautotrophic microalgal *gold mines* will continue for years to come. The prospects for generating bioactive products using photoautotrophic cultures, however, would unfold only if alternative sources, i.e. an inexpensive heterotrophic production mode or chemical synthesis of the active substances, will not present a more economically attractive venue.

Photobioreactor design was the subject of much research in recent years, yet little real progress was accomplished. Meaningful improvements in this field would no doubt strengthen the economic basis of commercial photoautotrophy by reducing production costs. The tubular design seems to have gained popularity at present, yet it is questionable whether it represents the optimal design for strictly photoautotrophic production. Small tube diameters do not go hand in hand with very high cell densities, for which fast, turbulent flows are strictly mandatory. Flat plate reactors (without alveoli), which facilitate cultures of very high cell densities devoid of oxygen accumulation in greatly reduced optical paths together with the required turbulent streaming, may be readily scaled-up. Well suited for utilizing strong light, plate reactors offer hope for obtaining a significant increase in productivity of cell mass, once the growth-physiology of very high cell concentrations (mandatory for efficient use of strong light) will be sufficiently understood, so as to prevent or control the growth-inhibition effects, which unfold in cultures of ultra-high cell densities, barring at present industrial use of such cultures.

It is well to note that the type of reactor used has a profound effect on the cost of production of cell mass and cell products, considering the investment, as well as the running costs. Much of the future of the photoautotrophic mode of production depends on success in greatly reducing these costs. The rather simple, less expensive techniques involved in mass production in open tanks and raceways have, under certain circumstances, advantages in this respect, well seen in many hatcheries as well as commercial plants. Most algal species, however, cannot be long maintained as continuous, monoalgal cultures in open systems, which in addition may not be suitable for general use as human food.

Some 50 years of experience, the world over, with microalgal mass cultures have witnessed an exciting canvass of successes as well as some failures reflected in this Handbook to which leading authorities in their respective fields have contributed. The accomplishments, during this period, in addressing the various aspects of mass microalgal production seem somewhat overshadowed by the outstanding achievements the pioneers of this biotechnology who were active in the '50 and '60, had attained in laying out, with great intuition, the basic physiological principles involved in mass cultivation of photoautotrophic microalgae outdoors.

It is, therefore, somewhat surprising that an output rate of some 70 g dry cell mass m^2 (ground) day^{-1} was envisioned at that time as a practical goal for open systems which could be well reached and surpassed. This daily output rate of protein-rich cell mass represents an annual yield of some 250 t ha^{-1}, i.e. several times that of any agricultural commodity. Such expectations were, in effect, translated into a firmly held premise, enthusiastically perceiving outdoor mass cultivation of microalgae as a means by which to avert hunger in a fast growing humanity. Today, this prospect is justifiably regarded as nothing but a dream.

Were the early pioneers, then, completely wrong? This is not as easy to answer as it may seem, for the future will unfold possibilities that presently border on sheer fantasy. The methodology of genetic engineering which already facilitates such feats as effective trophic conversions and combating Malaria by use of microalgae incorporated with bacterial toxins lethal to the mosquito larvae, are but the harbingers of vast future opportunities in microalgal culture. The future could well see greatly improved, fast-growing microalgal species with significantly improved capabilities to carry out effective photosynthesis utilizing strong sunlight, and photoautotrophic microalgal culture may yet become an economic alternative for provision of food and feed in the sunny, more arid, parts of our planet.

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The strenuous task of editing this multi-author Handbook was much relieved due to the patience and encouragement given to me by my wife, Dahlia, whom I thank most heartily.

Amos Richmond

Part I

The Microalgae: With Reference to Mass-Cultivation

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1 The Microalgal Cell

Luisa Tomaselli

1.1 What is the meaning of *microalgae* in applied algology?

Phycologists regard any organisms with chlorophyll *a* and a thallus not differentiated into roots, stem and leaves to be an alga (Lee, 1989). Cyanobacteria are included in this definition, even though they are prokaryotic organisms. Therefore, in applied phycology the term microalgae refers to the microscopic algae *sensu stricto*, and the oxygenic photosynthetic bacteria, i.e. the cyanobacteria, formerly known as Cyanophyceae.

The interest for these two groups of phototrophic organisms lies in their potential utilization, in a similar way to heterotrophic microorganisms, to produce biomass for food, feed and fine chemicals, using solar energy. The origins of applied phycology most probably date back to the establishment of a culture of *Chlorella* by Beijerinck (1890). Even today *Chlorella* takes up the first place in the commercial use of these microorganisms.

Microalgae are found all over the world. They are mainly distributed in the waters, but are also found on the surface of all type of soils. Although they are generally free-living, a certain number of microalgae live in symbiotic association with a variety of other organisms.

1.2 Structural and morphological features of microalgae

1.2.1 Microscopy: examining fresh material; making permanent slides

Examination of fresh material can be directly performed on a drop of liquid sample, or after the solid sample has been mixed with distilled water or saline solution. In presence of motile cells the sample should be mixed with a weak acid, such as acetic acid. Settling, centrifugation or filtration can be used to concentrate the living or preserved material. To minimize changes in the composition of the samples after collection, fixation using formaldehyde, Lugol's solution and glutaraldehyde should be carried out quickly, or the sample should be cooled and stored in total darkness to ensure a low activity rate.

Permanent slides can be simply prepared, placing the cell suspension on a coverslip and drying over gentle heat. The sample is then inverted onto a slide with a mounting medium of suitable refractive index. Canada Balsam is commonly used (Reid, 1978). Sometimes the removal of free water from the cells requires dehydration procedures, which are carried out using gradually increasing concentrations of an alcohol series. Staining techniques are

used to distinguishing some special features, such as sheath and specific organelles (Clark, 1973). Finally, the coverslip with the sample in the mounting medium is sealed to a glass slide usually using clear nail polish.

1.2.2 Types of cell organization: unicellular flagellate, unicellular non-flagellate (motile, nonmotile); colonial flagellate, colonial non-flagellate; filamentous (unbranched, branched)

Microalgae may have different types of cell organization: unicellular, colonial and filamentous. Most of the unicellular cyanobacteria are nonmotile, but gliding and swimming motility may occur. Baeocytes, cells arising from multiple fission of a parental cell, may have a gliding motility. Swimming motility occurs in a *Synechococcus* sp., even if flagella are not known. Unicellular microalgae may or may not be motile. In motile forms, motility is essentially due to the presence of flagella. The movement by the secretion of mucilage is more unusual. Gametes and zoospores are generally flagellate and motile. Some pennate diatoms have a type of gliding motility, as well as the red alga *Porphyridium* and a few green algae.

Cyanobacteria with colonial cell organization have nonmotile colonies (e.g. *Gloeocapsa*). In microalgae motile flagellate cells may aggregate to form motile (e.g. *Volvox*) or nonmotile colonies (e.g. *Gloeocystis*). Nonmotile cells may be organized into coenobic forms with a fixed number of cells in the colony (e.g. *Scenedesmus*), or into non-coenobic forms with a variable number of cells (e.g. *Pediastrum*). Many filamentous cyanobacteria may have gliding motility often accompanied by rotation and by creeping (e.g. *Oscillatoria*), but others may be motile at the stage of hormogonia (e.g. *Nostoc*). Microalgae, with unbranched or branched filamentous cell organization are nonmotile, zoospores and gametes excepted. Siphonaceous and parenchymatous cell organization occur mostly in macroalgae.

1.2.3 Cellular organization: prokaryotic; eukaryotic: uninucleate, multinucleate (coenocytic)

The DNA of prokaryotic Cyanobacteria and Prochlorophytes is not organized in chromosomes, lies free in the cytoplasm together with the photosynthetic membranes, and is not surrounded by a membrane. Moreover, the prokaryotes have no membrane-bounded organelles (Fig. 1.1). The eukaryotic microalgae possess a true membrane-bounded nucleus, which contains the major part of the genome distributed on a set of chromosomes, and the nucleolus. They have cytoplasm divided into compartments and membrane-bounded organelles (Golgi body, mitochondria, endoplasmic reticulum, vacuoles, centrioles and plastids) devoted to specific functions (Fig. 1.2). Many microalgae are uninucleate, those with multinucleate cellular organization (coenocytic) usually have a peripheric cytoplasm containing nuclei and chloroplasts, which are the most important plastids.

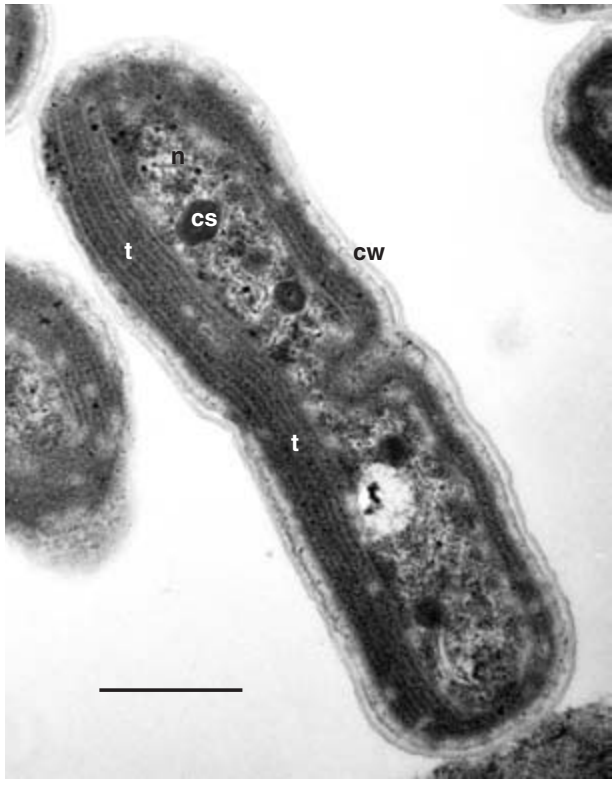

Fig. 1.1. Electron micrograph of a dividing cell of *Synechococcus* sp. in longitudinal section. Abbreviations: **cw** – cell wall, **t** – thylakoids, **cs** – carboxysomes, **n** – nucleoplasm with DNA fibrils. Scale = 0.5 μm (Courtesy of M.R. Palandri).

1.2.4 Colony features: orderly (e.g. netted) or random; shape and investments

Different shapes of colonial organization occur: flat, spherical, cubic, palmelloid, dendroid, flagellate, and non-flagellate. The cells are held together by an amorphous (e.g. *Microcystis*) or microfibrillar polysaccharide envelope (e.g. *Gloeothece*). Inside the colony the cells may be orderly or irregularly arranged in the mucilage (e.g. *Microcystis*). Both colonies with orderly (e.g. *Pediastrum*) and irregularly arranged cells (e.g. *Palmella*) occur in microalgae. Moreover, nonmotile (e.g. *Coelastrum*) and motile colonies formed of flagellate cells, embedded in a mucilage, are common (e.g. *Gonium*). The polysaccharide investment may be amorphous or laminated with a microfibrillar structure; depending on its consistency, it may be called sheath, glycocalyx, capsule, or slime. Cyanobacteria sheaths may contain pigments functioning as sun-screen compounds (Garcia-Pichel *et al.*, 1992), or UV-A/B-absorbing mycosporine-like amino acids (Ehling-Schulz *et al.*, 1997). Capsule and slime envelopes are particularly abundant in many species (*Cyanospira capsulata*).

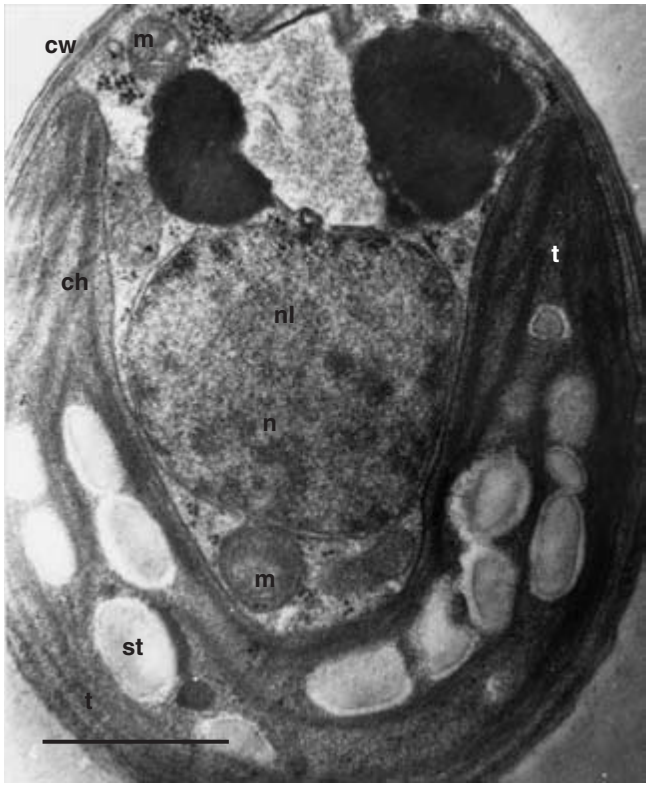

Fig. 1.2. Electron micrograph of a cell of *Chlorella vulgaris* in longitudinal section. Abbreviations: **cw** – cell wall, **ch** – cup shaped chloroplast, **t** – thylakoids, **st** – starch grains (leucoplasts), **n** – nucleus, **nl** – nucleolus, **m** – mitochondria. Scale = 1 μm (Courtesy of M.A. Favali).

1.2.5 Morphological adaptation: specialized cells (spores, heterocysts, hormogonia), pili, flagella, light shielding and flotation structures

Specialized cells as akinetes, heterocysts, hormogonia, and pili or fimbriae occur in many cyanobacteria. Akinetes, or spores, are cells with thick walls and granular content, which originate from vegetative cells under unfavourable conditions and germinate when favourable conditions for growth are restored. Heterocysts are unique cells where nitrogen fixation takes place. They have thick wall and rarefied cytoplasm, characterized by two polar nodules of cyanophycin. Hormogonia are short trichome pieces or development stage of filamentous cyanobacteria. They usually have gliding motility, smaller cell size, and/or gasvacuolation. Gas vacuoles are specific subcellullar inclusions that appear highly refractile in the light microscope. They are composed of elongated gas vesicles with pointed ends, which may function in light shielding and/or buoyancy. Pili or fimbriae are non-flagellar proteinaceous appendages protruding from the cell wall.

Spores and flagella may occur in microalgae. The spores, or resting cells, have thick walls and like akinetes are formed under unfavourable conditions. Resting cells of *Botryococcus braunii* may accumulate in the cell wall, a hydrocarbon up

to 70% of its dry weight (Knights *et al*., 1970). Flagella are locomotory organs with a complex structure consisting of an axoneme of nine peripheral double microtubules surrounding two central microtubules; the whole structure is enclosed by the plasma membrane. The flagella may be smooth or hairy, and are inserted in the outer layer of the cytoplasm *via* a basal body.

1.3 Ultrastructure and cell division

1.3.1 Prokaryotes

1.3.1.1 Cell wall

Cyanobacteria and Prochlorophytes have a four layered cell wall which is of the Gram-negative type; the structural part consists of a murein (peptidoglycan) layer, outside which there is a lipopolysaccharide layer. The high digestibility of cyanobacteria cells, due to the lack of cellulose, unlike the majority of algae, facilitates their use for human consumption (e.g. *Spirulina* – health food). Mucilaginous envelopes may surround the cell wall (sheaths, glycocalix, capsule or slime). The cell wall may be perforated by small pores and may also have appendages such as fimbriae and pili.

1.3.1.2 Plasma membrane

Beneath the cell wall there is the plasma membrane, or plasmalemma. It is a thin unit membrane of about 8 nm thickness.

1.3.1.3 Thylakoid arrangement

Thylakoids are the most evident membrane system occurring in the cyanobacterial cell; they lie free in the cytoplasm and contain the photosynthetic apparatus. Thylakoids appear as flattened sacs showing phycobilisomes attached to the protoplasmic surface in regularly spaced rows. The phycobilisomes contain the phycobiliproteins that are widely used as fluorescent tags (Glazer, 1999); phycocyanin from *Arthrospira* is commercialized as natural pigment (linablue). Thylakoids may be arranged in concentric rings, in parallel bundles, dispersed, etc. They are not present in *Gloeobacter*, which possesses only a peripheral row of phycobilisomes. Phycobilisomes are absent in the prochlorophytes, which possess an extensive membrane system with stacked thylakoids.

1.3.1.4 Cell inclusions

The most common cell inclusions of cyanobacteria are the glycogen granules, cyanophycin granules, carboxysomes, polyphosphate granules, lipid droplets, gas vacuoles, and ribosomes. The glycogen granules (α-1,4-linked glucan) lie between the thylakoids and represent a reserve material, such as the

cyanophycin granules, polymer of arginine and aspartic acid. Carboxysomes, containing the enzyme ribulose 1,5-bisphosphate carboxylase-oxygenase, lie in the central cytoplasm. Poly-hydroxybutyrate granules, appearing as empty holes, represent unusual inclusions and a potential source of natural biodegradable thermoplastic polymers (Suzuki *et al.*, 1996). Ribosomes are distributed throughout the cytoplasm. In the planktonic forms there are gas vacuoles.

1.3.1.5 Cell division

Cell division may occur through binary fission, with constriction of all the wall layers that grow inward, or invagination of the plasma membrane and peptidoglycan layer without involvement of the outer membrane. Cell division may also occur by multiple fission leading to the formation of baeocytes. A very particular type of cell division, similar to budding, occurs in *Chamaesiphon*. Cyanobacteria also reproduce by fragmentation (hormogonia). Moreover, some filamentous genera produce akinetes. Although the cyanobacteria have no evident sexual reproduction, genetic recombination by transformation or conjugation may occur.

1.3.2 Eukaryotes

1.3.2.1 Cell wall, outer investments

A microfibrillar layer of cellulose, which may be surrounded by an amorphous layer, generally composes the microalgal cell wall. The cell wall is secreted by the Golgi apparatus. It may be silicified or calcified, and it may be strengthened with plates and scales. Some species are naked, lacking the cell wall. Outside the outer amorphous layer there may occur a laminated polysaccharide investment. The nature of the outer cell wall layers supports polysaccharide production (alginates, agar and carrageenans) from various macro algae as well as from the microalga *Porphyridium* (Arad, 1999).

1.3.2.2 Plasma membrane, periplast, pellicle

The plasma membrane is a thin unit membrane that bounds the cytoplasm. The Chryptophyta do not possess a cell wall but there is an outer cell wall covering the cytoplasm, called periplast. In the Euglenophyta the proteinaceous outer covering is called pellicle.

1.3.2.3 Cytoplasm, nucleus, organelles

The cytoplasm contains the nucleus and different kinds of organelles and compartments formed by invagination of the plasma membrane and endoplasmic reticulum. Among the organelles there are: chloroplast, Golgi apparatus, endoplasmic reticulum, ribosomes, mitochondria, vacuoles, contractile vacuoles, plastids, lipid globules, flagella, and microtubules. Chloroplast and cytoplasmic lipids represent an important source of polyunsaturated fatty

acids, such as eicosapentaenoic, docosahexaenoic and arachidonic acids (Pohl, 1982). The nucleus is bounded by a double nuclear membrane; it contains the nucleolus and several DNA molecules distributed among the chromosomes, and undergoes division by mitosis.

1.3.2.4 Chloroplast

The chloroplast contains a series of flattened vesicles, or thylakoids, containing the chlorophylls, and a surrounding matrix, or stroma. Thylakoids also contain phycobiliproteins in phycobilisomes in the Rhodophyta, whereas in the Cryptophyta the phycobiliproteins are dispersed within the thylakoids. Thylakoids can be free or grouped in bands. Pyrenoids can occur within chloroplasts. In many motile forms there is an orange-red eyespot, or stigma, made of lipid globules. A double membrane envelops the chloroplast; in some algal division besides this double membrane one or two membranes of endoplasmic reticulum are present.

1.3.2.5 Cell division and reproduction

Vegetative reproduction by cell division is widespread in the algae and related, in many species, to an increase in cell or colony size. Other types of asexual reproduction occur by fragmentation and by production of spores, named zoospores if flagellate and aplanospores or hypnospores if non-flagellate. Autospores are also produced by various algae and are like aplanospores lacking the ontogenetic capacity for motility.

Although sexual reproduction occurs in the life-history of most of the species, it is not a universal feature in algae. It involves the combination of gametes, often having different morphology and dimension, from two organisms of the same species (isogamy, anisogamy or oogamy). Five schematic types of life-histories are recognizable: (i) predominantly diploid life history with meiosis occurring before the formation of gametes (haploid part of life cycle); (ii) predominantly haploid life history with meiosis occurring when the zygote germinates (zygote only diploid part of life cycle); (iii) isomorphic alternation of generation (alternation of haploid *gametophytic* plants bearing gametes with diploid *sporophytic* plants bearing spores); (iv) heteromorphic alternation of generations (alternation of small haploid plants bearing gametes with large diploid plants bearing spores, or large haploid plants alternating with smaller diploid plants); (v) triphasic life cycle, in red algae, consisting of haploid gametophyte, diploid carposporophyte and diploid tetrasporophyte.

1.4 Cell growth and development

1.4.1 Cell growth

Growth is defined as an increase in living substance, usually the number of cells for unicellular microoganisms or total mass of cells for multicellular organisms. The most used parameter to measure change in cell number or cell

mass per unit time is the growth rate. Cell growth is treated in detail elsewhere in this volume (Chapter 4).

1.4.2 Cell cycle

In unicellular microalgae the cell size generally doubles and then the cell divides into two daughter cells which will then increase in size. The cell cycle in eukaryotic algae involves two phases: mitosis and interphase. During the interphase the cell grows and all cellular constituents increase in number so that each daughter cell will receive a complete set of the replicated DNA molecule and sufficient copies of all other constituents and organelles. During the mitosis the nuclear division occurs.

1.4.3 Cell decline

Microbial growth is influenced by several chemical and physical conditions. As the substrate concentration or other factors become limiting, or toxic metabolites accumulate, the growth rate decreases. In this growth phase, the production of secondary metabolites often takes place. As long as there is consumption of storage material the organism remains viable. When energy is no longer produced for cell maintenance, the cell declines and finally dies. In some cases this process is accompanied by the formation of a few spores or similar structures, which may survive and overcome adverse conditions giving rise to new individuals when favourable conditions are resumed.

1.5 Microalgal systematics

1.5.1 Principles of classification

Traditionally algae have been classified according to their colour and this characteristic continues to be of a certain importance. The current systems of classification of algae are based on the following main criteria: kinds of pigments, chemical nature of storage products and cell wall constituents. Additional criteria take into consideration the following cytological and morphological characters: occurrence of flagellate cells, structure of the flagella, scheme and path of nuclear and cell division, presence of an envelope of endoplasmic reticulum around the chloroplast, and possible connection between the endoplasmic reticulum and the nuclear membrane.

Lee (1989) was one of the first scientists to stress the phylogenetic importance of the additional membranes around the chloroplast envelope. He separated the algal divisions into four groups. The first group includes the prokaryotic algae: Cyanobacteria and Prochlorophyta. The other groups are classified with respect to the evolution of the chloroplast, and include the eukaryotic algae, which probably acquired the chloroplast along different evolutionary events. The second group, which includes Glaucophyta, Rhodophyta and Chlorophyta, has the chloroplast surrounded only by two chloroplast membranes. The third and fourth group have the chloroplast surrounded respectively by one (Dinophyta and Euglenophyta) or two additional

membranes of the endoplasmic reticulum (Cryptophyta, Chrysophyta, Prymnesiophyta, Bacillariophyta, Xanthophyta, Eustigmatophyta, Raphidophyta and Phaeophyta). The phylum Prochlorophyta contains chlorophylls *a* and *b* and, according to Castenholz (2001), the described genera (*Prochloron*, *Prochlorothrix* and *Prochlorococcus*) are included in the phylum Cyanobacteria.

The systematic position of the various algal group has changed many times over the years. The system of classification proposed by Lee (1989) has been largely adopted here to give a brief description of some of the divisions that this author considers.

1.5.2 General description of major Divisions and Classes

1.5.2.1 Prokaryotes

Cyanobacteria (*Cyanophyta* and *Prochlorophyta*)

The Cyanophyta and the Prochlorophyta are prokaryotic algae that contain chlorophyll *a*. The traditional name of blue-green algae for the Cyanophyceae is due to the presence of phycocyanin and phycoerythrin, which usually mask the chlorophyll pigmentation. The main storage product is glycogen (α-1,4-linked glucan). Cyanophycin is stored in large structured granules. Cell wall is composed of peptidoglycan with an external lipopolysaccharide layer, and it may also have a mucilagineous sheath. The cells may occur singly or in filaments, unbranched or branched, with uniseriate or multiseriate arrangment. The cells may aggregate to form colonies, which are surrounded by a firm or amorphous mucilage. Filaments may have cells differentiated into heterocysts and/or akinetes. Some planktonic forms can float owing to the presence of gas vacuoles, and most of the filamentous forms have gliding motility. The Cyanophyceae have a cosmopolitan distribution and inhabit marine and freshwater environments, moist soils and rocks, either as free-living or as symbiotic organisms.

The Prochlorophyta synthesize chlorophyll *b* in addition to chlorophyll *a*, and lack phycobiliproteins and phycobilisomes. Other major differences concern the morphology of thylakoids, which are paired rather than single, the synthesis of a starch-like polysaccharide as main storage compound, the absence of both cyanophycin and gas vacuoles, and the diffuse localization of DNA through the cytoplasm. They have unicellular or filamentous cell organization. They occur as free-living organisms in freshwater and marine habitats, and as symbionts in colonial ascidians.

Microcystis Kützing. *Microcystis* is a planktonic unicellular cyanobacterium that grows in freshwaters. Cells are spherical, 3–6 μm in diameter, and irregularly arranged in mucilaginous, spherical or irregularly lobed, colonies. The cells generally have a light refractile appearance due to the presence of gas vesicles. Cell division occurs by binary fission and reproduction by disintegration of colonies into single cells or group of cells. At the ultrastructural level, cells are characterized by thylakoids arranged in short bent or sinuous bundles running around the cell wall and occupying a large portion of the cytoplasm (Fig. 1.3). Gas vacuolated *Microcystis* species may give rise

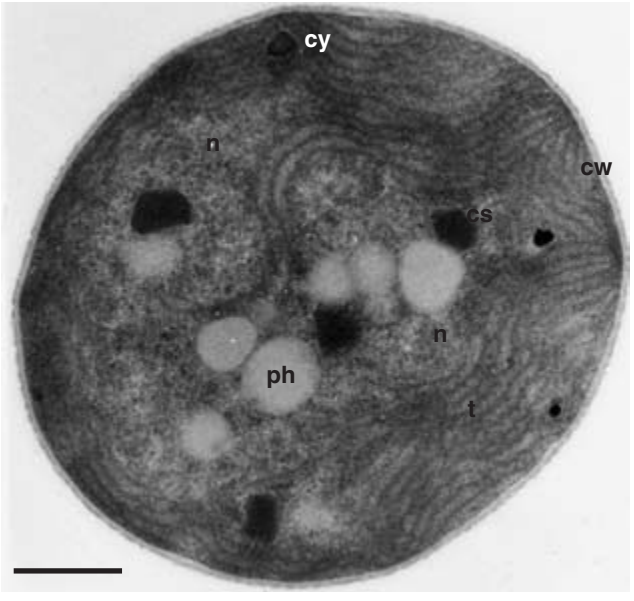

Fig. 1.3. Electron micrograph of a cell of *Microcystis* sp. in cross-section. Abbreviations: **cw** – cell wall, **t** – thylakoids, **n** – nucleoplasm, **cs** – carboxysomes, **cy** – cyanophycin granule and **ph** – poly-hydroxyalkanoate granules. Scale = 0.5 μm (Courtesy of M.R. Palandri).

to toxic blooms (Watanabe & Oishi, 1982). Like other cyanobacteria, they may produce toxins, such as microcystins (cyclic peptides) (Carmichael, 1996), which have become a concern for human health.

Arthrospira Stizenberger. *Arthrospira* is a planktonic filamentous cyanobacterium. It has multicellular left-handed helical trichomes, composed of shorter than broad cells (mean cell diameter 8 μm), and shows gliding motility by rotation along its axis (Fig. 1.4). Cells have visible cross walls, often masked by numerous gas vesicles (Fig. 1.5) (Tomaselli, 1997). Multiplication occurs by cell division in one plane and reproduction by hormogonia formation through trichome breakage at the sites of a lysing cell (necridium) (Tomaselli *et al.*, 1981). *Arthrospira* grows profusely in alkaline lakes of subtropical regions (Vonshak & Tomaselli, 2000). *Arthrospira*, the most cultivated phototrophic prokaryote as food supplement, is also used as a source of feed and fine chemicals (Richmond, 1986), and exploited as a therapeutic agent (Belay *et al.*, 1993). This genus is currently known under the name *Spirulina*, although *Arthrospira* and *Spirulina* have been recognized as separate genera (Castenholz & Waterbury, 1989; Tomaselli *et al.*, 1996).

1.5.2.2 Eukaryotes

Rhodophyta

The class Rhodophyceae, or red algae, includes multicellular and filamentous forms, whereas unicellular species are less represented. These algae have chlorophyll *a* and *d*, phycobiliproteins (phycoerythrin and phycocyanin), and floridean starch (α-1,4-linked glucan) as storage products accumulated

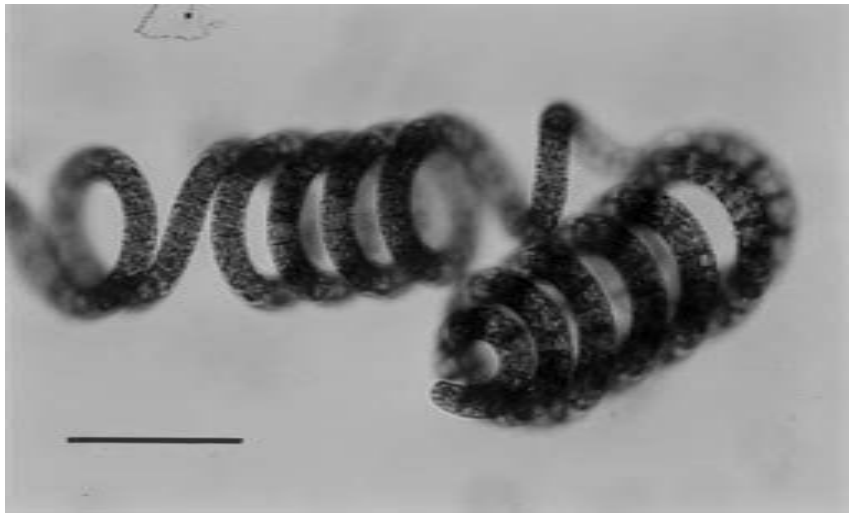

Fig. 1.4. Light micrograph of *Arthrospira maxima*. Scale = 50 μm (Courtesy of C. Sili).

in the cytoplasm outside the chloroplast. Rhodophyceae lack flagellate cells. Cell wall is composed of a microfibrillar layer of cellulose or xylan and amorphous polysaccharidic mucilages (agar or carrageenans). Chloroplasts contain the thylakoids with phycobilisomes, and pyrenoids.

Red algae represent the majority of seaweeds distributed mostly in temperate and tropical regions. Commercial utilization of red algae concerns the polysaccharidic mucilages of the cell wall, agar and carrageenan. The red microalga *Porphyridium* is an important source of sulphated polysaccharides (Arad *et al.*, 1985), and of polyunsaturated fatty acids, such as arachidonic acid (Ahern *et al.*, 1983).

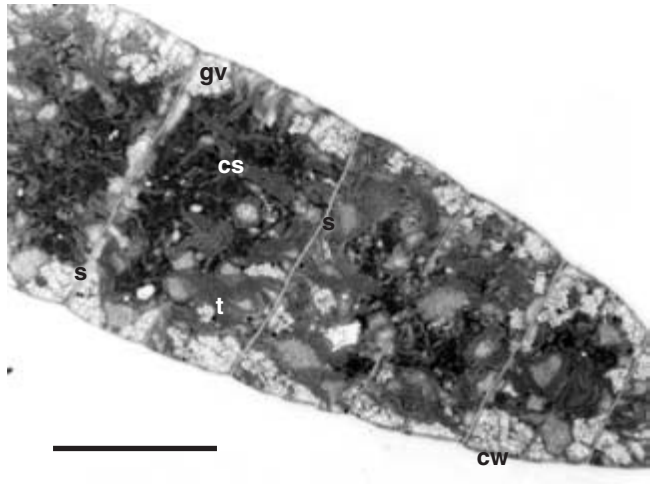

Fig. 1.5. Electron micrograph of a trichome of *Arthrospira maxima* in longitudinal section. Abbreviations: **cw** – cell wall, **s** – septa or cross walls, **t** – thylakoids, **gv** – gas vesicles, **cs** – carboxysome. Scale = 5 μm (Courtesy of M.R. Palandri).

Chlorophyta

The Chlorophyta, or green algae, embrace a large group of organisms with a great morphological variability, ranging from microscopic to macroscopic forms. They comprise four classes: Micromonadophyceae, Charophyceae, Ulvophyceae and Chlorophyceae. They have chlorophyll *a* and *b* and several carotenoids, that may be synthesized and accumulated outside the chloroplast under conditions of nitrogen deficiency and/or other stress, colouring the alga orange or red. The storage product is starch (α-1,4-linked glucan), composed of amylose and amylopectin. Unlike the other algae, it is formed within the chloroplast. Cell walls generally contain cellulose. Some species are naked. Chloroplasts may have an eyespot and pyrenoids. The group includes coccoid, unicellular or colonial flagellates, multicellular or multinucleate filaments. Most species have flagellate stages with the flagella apically inserted into the cell, the flagellar root system anchored with four sets of crucially arranged microtubules. The green algae are cosmopolitan. They are primarily freshwater, but a great number grow in marine, terrestrial and subaerial habitats. Some species occur in symbiotic associations, mostly with lichens.

Commercial exploitation of microscopic green algae comprises relatively few chlorophycean genera among which there are *Chlorella*, *Dunaliella* and *Haematococcus*. Moreover, *Botryococcus braunii* was proposed and cultivated as a renewable source of liquid fuel (Wolf *et al*., 1985), owing to the high hydrocarbon content (Knights *et al*., 1970). *Dunaliella* spp. from hypersaline environments have been extensively studied, cultivated and commercialized as a source of natural β-carotene (Ben-Amotz & Avron, 1983; Borowitzka *et al*., 1984; Richmond, 1990; Ben-Amotz, 1999).

Haematococcus Agardh. *Haematococcus* is a freshwater unicellular alga with ovoid cells actively motile by two smooth apical flagella. During its growth stages nonmotile cells, or cysts, also occur. The cell peculiarity is that the protoplast is separated from the cell wall, to which it is connected by radiating protoplasmic threads. Chloroplast is usually cup-like, parietal, and it contains pyrenoids and the eyespot. The cell content is usually green with a small part of the cell orange-red pigmented owing to the accumulation of the ketocarotenoid astaxanthin in globules outside the chloroplast (Lee & Ding, 1994). As the conditions become unfavourable, green and motile cells round off, then produce thicker walls, loose the flagella and finally cease to swim. In these cysts the protoplast fills the whole cell and a great increase in cell dimension occurs together with a change of pigmentation from green to orange-red, due to the increase of astaxanthin deposition. When the conditions become favourable for growth, the cysts germinate releasing a large number of new motile cells. The astaxanthin represents a high valuable product not only as a colouring agent for fishes and crustaceans in aquaculture but, since it is a potent natural antioxidant, it may also find important applications in medicine (Palozza & Krinsky, 1992). The process of astaxanthin production has been successfully developed in Israel (Boussiba & Vonshak, 1991).

Chlorella Beijerinck. *Chlorella* is a cosmopolitan genus with small, unicellular, ovoidal nonmotile cells; it does not produce zoospores. Cells have a thin cell wall, and cup-shaped chloroplast. Pyrenoid may be present. The accumulation of starch occurs within the chloroplast. *Chlorella* reproduces by forming daughter cells or autospores (4–8–16) of the same shape as the parent cell. It grows in autotrophic, heterotrophic and mixotrophic conditions. Besides autotrophic strains, heterotrophic strains are also cultivated. *Chlorella* is the most important species in the microalgal industry; it is cultivated and sold essentially as health food (Richmond, 1990).

Dinophyta

The class of Dinophyceae, formerly named Pyrrophyceae, includes a diverse assemblage of unicellular biflagellate planktonic algae of marine and freshwater habitats. They have chlorophylls *a* and c_2 and carotenoids. Starch is accumulated in the cytoplasm outside the chloroplast. The cell is composed of two parts, an epicone and a hypocone, and divided by the transverse girdle. Perpendicular to the latter there is a longitudinal groove. A layer of flat vesicles usually containing the cellulose plates surrounds the cell wall. In many species a polysaccharidic glycocalix surrounds the cell. Chloroplasts may have pyrenoids and a complex eyespot. The nucleus has the particularity of chromosomes always condensed. The Dinophyceae can produce large blooms, red or brown tides that colour the water and are highly toxic (Shilo, 1967). They are the main contributors to marine bioluminescence. The dinoflagellates are a potential source of ω-3 unsaturated fatty acids, eicosapentaenoic and docosaesaenoic acids (Pohl, 1982).

Chrysophyta

The Chrysophyta or golden-brown algae include two classes: the Chrysophyceae and the Synurophyceae. They mainly occur in freshwaters, especially in oligotrophic waters low in calcium. The chloroplasts contain chlorophylls *a* and c_1, c_2, fucoxanthin and β-carotene, which are responsible of the golden-brown colour. The storage product is chrysolaminarin (β-1,3-linked glucan), which is accumulated in a cytoplasmic vesicle. Most of the species are unicellular or colonial. Cells usually have two different apical flagella, one smooth, the other hairy, and contain two parietal chloroplasts with an eyespot. The cell wall is often lacking, or composed of cellulose. Silicified scales, polysaccharidic envelopes, or loricas and various cytoplasmic processes may occur external to the cell wall. Characteristic of the chrysophyta is the formation of special resting spores, statospores, enclosed in a silicified wall. Some species require vitamins and growth substances. *Ochromonas malhamensis*, which requires vitamin B12 to grow, has been used as an assay organism for this vitamin.

Prymnesiophyta

The Prymnesiophyta, also named Haptophyta for the presence of a thin filamentous appendage between two smooth flagella, called haptonema, include only the class Prymnesiophyceae. The cells are flagellate and have chlorophyll *a*, c_1 and c_2 and fucoxanthin as the major carotenoid. The storage

product is chrysolaminarin (β-1,3-linked glucan). Cells are usually covered with scales embedded in a mucilage and sometimes are calcified (coccoliths). These microalgae are widespread in marine environment, forming a major part of marine phytoplankton. Since many years, *Prymnesium parvum* is known for producing a potent exotoxin lethal for fish and molluscs (Shilo, 1967). Recently, toxic blooms of *Chrysochromulina polylepsis* have been reported (Nielsen *et al*., 1990). Strains of *Isochrysis* and *Pavlova* are investigated as a source of polyunsaturated fatty acids (PUFAs) (Lopez-Alonso *et al*., 1992).

Bacillariophyta

The class of Bacillariophyceae, or diatoms, includes a very conspicuous number of golden brown unicellular organisms. The diatoms live mostly singly or attached to one another in chains of cells or in colonial aggregations, in aquatic and terrestrial habitats. Their colour is due to the masking of the green colour of chlorophylls *a*, c_1 and c_2 by the brown and yellow pigmentation of the fucoxanthin and β-carotene. The storage product is chrysolaminarin (β-1,3-linked glucan). Lipids are also present. The cytoplasm is enclosed in a siliceous cell wall, the frustule, showing different structures and ornamentation, which are used as key features for diatom classification. The frustule consists of two overlapping halves joined by a girdle. The upper longer and wider half (epitheca) fits on the lower half (hypotheca) as the cover of a box. Cells contain two parietal chloroplasts; the nucleus is laterally or centrally placed and suspended by protoplasmic threads. Pyrenoids are sometimes present. Some diatoms may have a central raphe, or fissure. Diatoms with a raphe possess gliding motility. There are two major groups of diatoms: the pennate diatoms with bilateral symmetry and the central diatoms with radial symmetry. The latter are mostly widespread in marine environments, where they have a key role in food chains. Diatoms include photoautotrophic, auxotrophic and colourless heterotrophic species.

Deposits of fossil diatoms, known as diatomaceous earth, have many industrial uses (filtration and absorption processes), while commercial uses of living cells are mainly related to the aquaculture, since some diatoms contain significative amounts of PUFAs, especially eicosapentaenoic acid (Pohl, 1982).

Xanthophyta

The class of Xanthophyceae, also known as Tribophyceae, comprises freshwater and terrestrial species. Only few members are marine. The yellow-green colour is due to the presence of both chlorophyll *a* and carotenoids. Chlorophyll *c* may also be present, whereas fucoxanthin is absent. Most species are unicellular or colonial. Species with amoeboid, multicellular, filamentous or siphonaceous cell organization may also occur. A few unicellular species are motile and flagellate with two unequal flagella: a long hairy and a shorter smooth flagellum. Cells usually contain several parietal discoid chloroplasts, usually with pyrenoids and an eyespot. The cell wall, mainly formed by cellulose, is composed of two overlapping halves: a cap of constant size and a tubular basal portion, which elongates as the cell grows.

The storage product is chrysolaminarin (β-1,3-linked glucan). Mannitol and glucose are also accumulated in plastids. Some species, like *Monodus subterraneous*, contain appreciable amounts of arachidonic and eicosapentaenoic acids (Pohl, 1982).

Eustigmatophyta

The class of Eustigmatophyceae includes unicellular and coccoid organisms producing a small number of zoospores and living in freshwaters and soil. Previously these organisms were classified in the Xanthophyceae. The name was chosen because of the large size of the eyespot in the zoospore. The chloroplast have chlorophyll *a*; violaxanthin is the major light-harvesting carotenoid pigment. Vegetative cells usually have a polygonal pyrenoid, which is absent in the zoospores. The zoospores have one or two apical flagella. The cell wall is polysaccharidic. Unlike the Chrysophyta, the eyespot is not enclosed in the chloroplast. Cytoplasm and photosynthetic lamella lipids of many species are promising sources of eicosapentaenoic acid, particularly in *Monodus subterraneus* (Cohen, 1999).

Nannochloropsis. *Nannochloropsis* is a picoplanktonic genus of marine environment. The cells are small (2–4 μm in diameter), spherical to slightly ovoid, non-flagellate. They have one single chloroplast without pyrenoid and containing several bands of photosynthetic lamellae, each with three thylakoids per band. The chloroplast endoplasmic reticulum is continuous with the nuclear envelope. *Nannochloropsis* has polysaccharide cell walls. The cells do not accumulate starch. This alga has an important applied interest as a source of polyunsaturated fatty acids, since it accumulates significant amounts of eicosapentaenoic acid (Boussiba *et al*., 1987).

Rhaphidophyta

The class of Rhaphidophyceae, or chloromodas, includes a small group of unicellular flagellate containing chlorophyll *a*, c_1 and c_2, and some carotenoids, often fucoxanthin in marine species and heteroxanthin in freshwater species. The cells are naked and have two different apical or subapical flagella; there is no eyespot. The cytoplasm is subdivided into a peripheral part occupied by a layer of many discoid chloroplasts and vacuoles, and a central part containing the nucleus and the mitochondria. Species of marine genera (e.g. *Fibrocapsa japonica*) may give rise to massive blooms, red tides, that cause serious damages to fish-farming (van den Hoek *et al*., 1995).

Phaeophyta

The class of Phaeophyceae, or brown algae, includes a few microscopic filamentous forms and many giant forms, mostly seaweed. The colour results from the dominance of the fucoxanthin over the chlorophylls *a*, c_1 and c_2. The principal storage product is laminarin (β-1,3-linked glucan). The Phaeophyta occur almost exclusively in marine habitat, where they form rich underwater forests. Cell wall is composed of an inner cellulose microfibrillar layer and an outer amorphous slimy layer made up of alginates. Commercial uses of brown algae mainly concern the alginate industry.

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2 Photosynthesis in Microalgae

Jiří Masojídek, Michal Koblížek and Giuseppe Torzillo

It's not love or money that makes the world go round, it's photosynthesis.

2.1 The process of photosynthesis

Photosynthesis represents a unique process of sunlight energy conversion. In this process, inorganic compounds and light energy are converted to organic matter by photoautotrophs. Virtually, all forms of life on Earth depend directly or indirectly on photosynthesis as a source of energy for their metabolism and growth.

The earliest photoautotrophic organisms, anoxygenic photosynthetic bacteria, evolved 3.5 billion years ago. They use light energy to extract protons and electrons from a variety of donor molecules, such as H_2S, to reduce CO_2 to form organic molecules. In this treatise, we focus on oxygen-producing photosynthetic microorganisms – prokaryotic cyanobacteria and eukaryotic algae – which emerged later and created our oxygenous atmosphere on Earth.

Cyanobacteria (blue-green algae) are frequently unicellular, some species forming filaments or aggregates. The internal organisation of a cyanobacterial cell is prokaryotic, where a central region (nucleoplasm) is rich in DNA and a peripheral region (chromoplast) contains photosynthetic membranes. The sheets of the photosynthetic membranes are usually arranged in parallel, close to the cell surface.

Eukaryotic autotrophic microorganisms are usually divided according to their light-harvesting photosynthetic pigments: Rhodophyta (red algae), Chrysophyceae (golden algae), Phaeophyceae (brown algae) and Chlorophyta (green algae). Their photosynthetic apparatus is organised in special organelles, the chloroplasts, which contain alternating layers of lipoprotein membranes (thylakoids) and aqueous phases, the stroma (Staehelin, 1986).

Oxygenic photosynthesis can be expressed as a redox reaction driven by light energy (harvested by chlorophyll molecules), in which carbon dioxide and water are converted to carbohydrates and oxygen. The conversion is traditionally divided into two stages, the so-called *light reactions* and *dark reactions* (Fig. 2.1). In the light reactions, which are bound on photosynthetic membranes, the light energy is converted to chemical energy providing a *biochemical reductant* $NADPH_2$ and a *high energy* compound ATP. In the

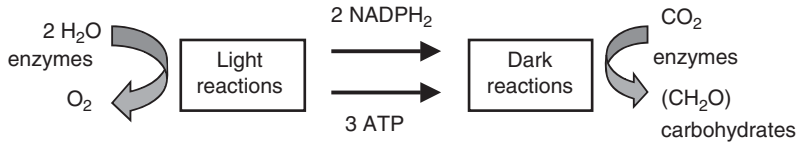

Fig. 2.1. Major products of the light and dark reactions of photosynthesis (adapted from Hall & Rao, 1999). The process of oxygenic photosynthesis is divided into two stages, the so-called *light reactions* and *dark reactions*. The light reactions include light absorption, transfer of excitons and electron and proton translocation resulting in the production of $NADPH_2$, ATP and O_2. The other phase, the dark reactions, which occur in the stroma, represent the reduction of carbon dioxide and the synthesis of carbohydrates using the $NADPH_2$ and ATP produced in the light reactions.

dark reactions, which take place in the stroma, $NADPH_2$ and ATP are utilised in the sequential biochemical reduction of carbon dioxide to carbohydrates.

The classical description of photosynthetic activity is based on measurements of oxygen evolution in proportion to light intensity, the so-called light-response (P/I) curve (Fig. 2.2). The initial slope $\alpha = P_{max}/I_k$, where I_k represents the saturation irradiance and P_{max} is the maximum rate of photosynthesis. In the dark, there is a net consumption of oxygen as a consequence of respiration (the negative part of the curve in Fig. 2.2). Thus, gross photosynthesis is considered as the sum of net photosynthesis (O_2 evolution) and respiration (O_2 uptake). At low irradiance (light-limited region), the rate of photosynthesis depends linearly on light intensity. With increasing light intensity, photosynthesis becomes less and less efficient. Finally, it reaches a plateau – the light-saturated value – where enzymatic reactions utilising fixed energy become rate limiting. Under prolonged supra-optimal irradiance, photosynthetic rates

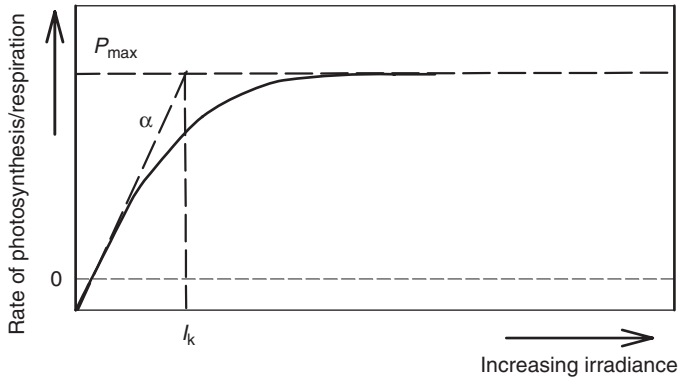

Fig. 2.2. A schematic representation of photosynthetic light-response curves, i.e. the dependency of photosynthesis vs irradiance. The initial slope of the curve (α) is the maximum light utilisation efficiency. The intersection between the maximum rate of photosynthesis P_{max} and α is the light saturation (optimum) irradiance. At supra-optimum irradiance, photosynthesis declines, which is commonly called down-regulation or photoinhibition.

usually decline from the light-saturated value. This phenomenon is commonly referred to as *photoinhibition* of photosynthesis.

2.2 The nature of light

The energy for photosynthesis is delivered in the form of light. Light is electromagnetic radiation and travels at the speed $c \sim 3 \times 10^8\,\mathrm{m\,s^{-1}}$. Based on the wavelength, electromagnetic radiation can be divided into several components (Fig. 2.3). As light is usually denoted radiation with wavelengths between 10^{-3} and 10^{-8} m. Gamma and X-rays have shorter wavelengths, while radio waves are above 10^{-3} m. The visible part of the spectrum ranges from the violet of about 380 nm to the far red at 750 nm. This range is usually expressed in nanometers ($1\,\mathrm{nm} = 10^{-9}$ m). The wavelengths of visible light also correspond to photosynthetically active radiation (PAR), i.e. radiation utilisable in photosynthesis.

According to the quantum theory, light energy is delivered in the form of separated packages called *photons*. The energy of a single light quantum, or *photon*, is the product of its frequency and Planck's constant, i.e. $h\nu$ ($h = 6.626 \times 10^{-34}$ J s). Since the energy is inversely related to wavelength, a photon of blue light (about 400 nm) is more energetic than that of red light (around 700 nm). Photosynthetic pigments absorb the energy of photons, and transfer it to the reaction centres where it is utilised for photochemistry. The photon should possess a critical energy sufficient to excite a single electron from one pigment molecule and initiate charge separation. According to Einstein's law, one mole of a compound must absorb the energy of N photons

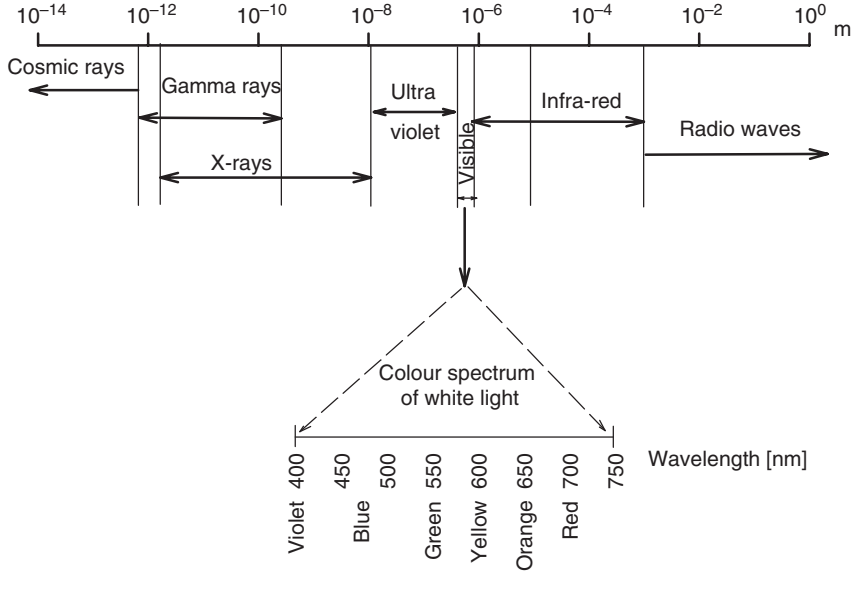

Fig. 2.3. Spectra of electromagnetic radiation and spectral pattern of visible light (adapted from Hall & Rao, 1999). Photosynthetically active radiation (PAR) ranges from 400 to 750 nm.

($N = 6.023 \times 10^{23}$, the Avogadro number) to start a reaction, i.e. N $h\nu$. This unit is called an Einstein ($E = 6.023 \times 10^{23}$ quanta).

Light flux is measured in *lumens* (lm); intensity of illumination is expressed in *lux* ($lm\,m^{-2}$) or historically in footcandles ($1\,lm\,ft^{-2}$, i.e. 1 ft candle equals 10.76 lux).

Photobiologists prefer to measure light energy incident on a surface, i.e. radiant flux energy or *irradiance*, in units of power per area ($W\,m^{-2}$ or $J\,m^{-2}\,s^{-1}$). Since photochemical reactions in photosynthesis depend on the number of photons incident on a surface, it is logical to express irradiance as number of quanta (photons) reaching unit surface in unit time, i.e. as the photosynthetic photon flux density measured in $\mu mol\,quanta\,m^{-2}\,s^{-1}$ or $\mu E\,m^{-2}\,s^{-1}$.

On a sunny day, average direct solar irradiance reaching the earth's surface is about $1000\,W\,m^{-2}$ (100 000 lux), of which photosynthetically active radiation (PAR, between 400 and 750 nm) represents about 40%, i.e. $400\,W\,m^{-2}$ or $1800\,\mu mol\,photon\,m^{-2}\,s^{-1}$. Thus, the approximate conversion factor for sunlight is $1\,W\,m^{-2}$ equals about $4.5\,\mu mol\,photon\,m^{-2}\,s^{-1}$. There exist different types of instruments to measure irradiance; most of them measure PAR in quanta $m^{-2}\,s^{-1}$ or in $W\,m^{-2}$.

2.3 Photosynthetic pigments

All photosynthetic organisms contain organic pigments for harvesting light energy. There are three major classes of pigments: chlorophylls, carotenoids and phycobilins. The chlorophylls (green pigments) and carotenoids (yellow or orange pigments) are lipophilic and associated in Chl–protein complexes, while phycobilins are hydrophilic.

Chlorophyll (Chl) molecules consist of a tetrapyrrole ring (polar *head*, chromophore) containing a central magnesium atom, and a long-chain terpenoid alcohol (except for Chl *c*) (Fig. 2.4A). These molecules are noncovalently bound to apoproteins. Structurally, the various types of Chl molecules designated *a*, *b*, *c* and *d* differ in their side-group substituents on the tetrapyrrole ring. All Chl have two major absorption bands: blue or blue-green (450–475 nm) and red (630–675 nm). Chl *a* is present in all oxygenic photoautotrophs as a part of the core and reaction centre pigment–protein complexes, and in light-harvesting antennae it is accompanied by Chl *b* or Chl *c*. The so-called accessory (antennae) pigments Chl *b*, *c* and *d* extend the range of light absorption.

Carotenoids represent a large group of biological chromophores with an absorption range between 400 and 550 nm. The basic structural elements of carotenoids are two hexacarbon rings joined by an 18-carbon, conjugated double-bond chain. They are usually either hydrocarbons (carotenes, e.g. α-carotene, β-carotene) or oxygenated hydrocarbons (xanthophylls, e.g. lutein, violaxanthin, zeaxanthin, fucoxanthin, peridinin) (Fig. 2.4C,D). Carotenoids have several roles in the photosynthetic apparatus, functioning as (i) accessory light-harvesting pigments transferring excitation to Chl *a*,

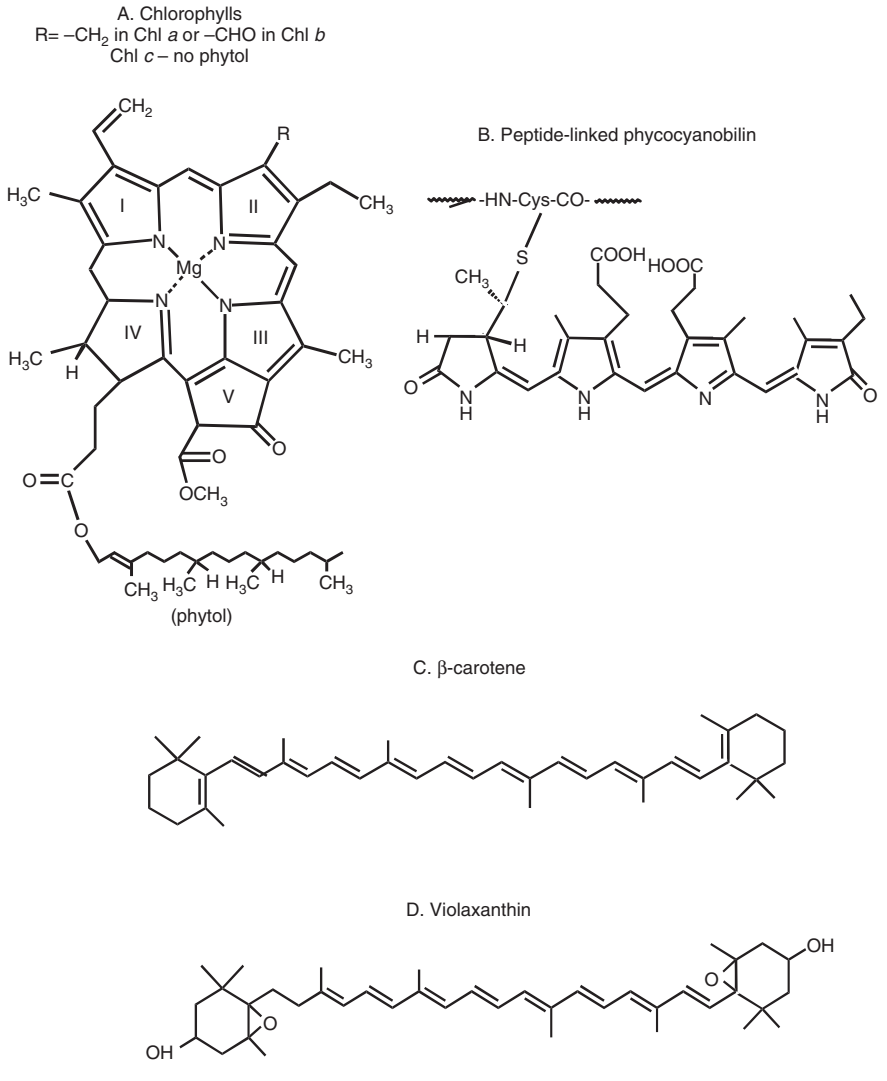

Fig. 2.4. The structures of the three principal groups of pigments in algae and cyanobacteria – chlorophylls (Chls), phycocyanobilin and carotenoids (β-carotene and violaxanthin). All Chls are tetrapyrroles, where nitrogen atoms are co-ordinated around a Mg atom. Chl *a* and *b* differ in the R group while Chl *c* does not contain a side chain of phytol. Phycobiliproteins are open tetrapyrroles, which are covalently linked to a protein. Carotenoids are conjugated isoprenes with cyclic 6-carbon side groups, whereas compared to carotenes, xanthophylls such as violaxanthin, are oxygenated.

(ii) structural entities within the light-harvesting and reaction centre pigment–protein complexes; and (iii) molecules required in the protection against excess irradiance, chlorophyll triplets and reactive oxygen species.

In cyanobacteria and red algae, the major antennae contain phycobilins (phycoerythrobilin, phycocyanobilin and phycourobilin), which are linear tetrapyrroles, nor are they associated with a magnesium atom. These accessory

pigments absorb blue-green, green, yellow, or orange light (500–650 nm). In contrast to the Chl-proteins and carotenoid-proteins, phycobiliproteins are water-soluble and the pigments are covalently bound to apoprotein (Fig. 2.4B).

Some pigments in algae do not transfer excitation energy. One group called secondary carotenoids, e.g. orange-red coloured xanthophylls, astaxanthin and canthaxanthin, are overproduced in some algal species (e.g. *Haematococcus pluvialis*) when grown under unfavourable conditions (i.e. combinations of nutrient deficiency, temperature extremes and high irradiance). These pigments are found in the cytoplasm and their metabolic role is unknown.

For quantification of Chls and carotenoids the pigments are extracted in organic solvents (methanol, ethanol, acetone, etc.). The absorbance of the extract is determined spectrophotometrically and the pigment content is calculated using mathematical formulae (e.g. Lichtenthaler & Wellburn, 1983). The separation and quantification of individual carotenoids can be achieved using high-performance liquid chromatography equipped with an absorption or fluorescence detector (e.g. by the method of Gilmore & Yamamoto, 1991).

2.4 The light reactions of photosynthesis

2.4.1 The photosynthetic membranes

The photosynthetic light reactions are located in the thylakoid membranes. These are composed of two major lipid components mono- and digalactosylglycerol arranged in a bilayer, in which proteins are embedded forming a liquid mosaic (Singer & Nicholson, 1972). They form closed, flat vesicles around the intrathylakoidal space, the lumen. Some protein–protein or pigment–protein complexes span the thylakoid membrane, whereas others only partially protrude with some functional groups facing the lumen or stroma.

In cyanobacteria (and also eukaryotic red algae), the photosynthetic lamellae occur singly, most likely as a result of the presence of hydrophilic phycobilisomes serving as outer (major) light-harvesting complexes. In the chloroplasts of higher plants, highly appressed regions of stacked thylakoids called *grana* are connected by single thylakoids called stromal lamellae. By contrast, in most algal strains, the thylakoids are organised in pairs or stacks of three.

The thylakoid membrane contains five major complexes: light-harvesting antennae, photosystem II (PS II) and photosystem I (PS I) (both containing a reaction centre), cytochrome b_6/f and ATP synthase, which maintain photosynthetic electron transport and photophosphorylation (Fig. 2.5).

2.4.2 Photosynthetic electron transport and phosphorylation

The main role of the light reactions is to provide the biochemical reductant ($NADPH_2$) and the chemical energy (ATP) for the assimilation of inorganic carbon. The light energy is trapped in two photoreactions carried out by two

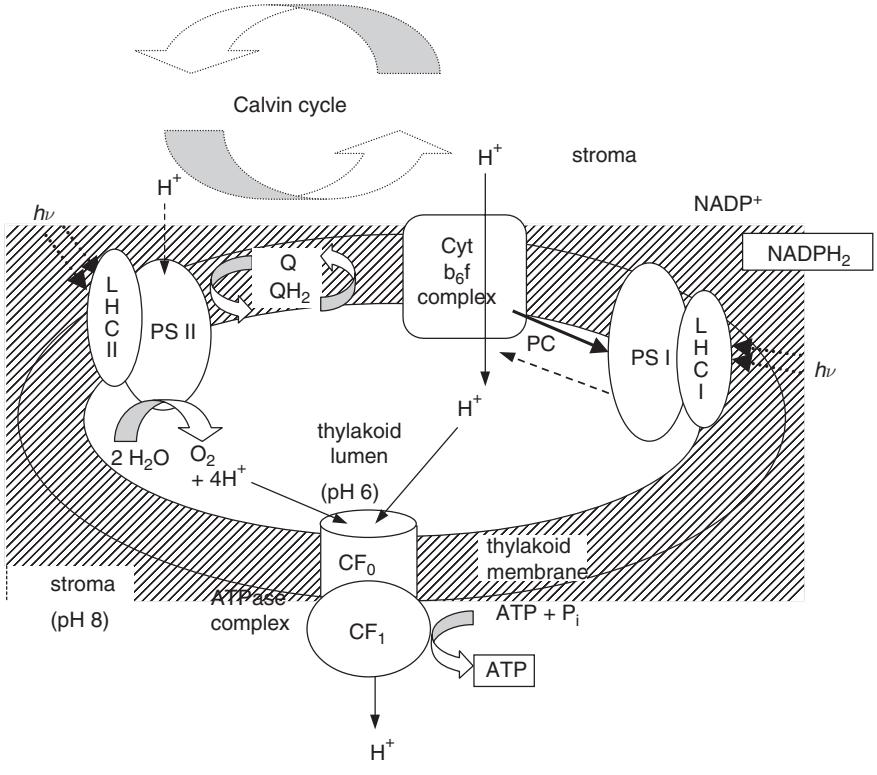

Fig. 2.5. Vectorial arrangement of photosystem I and II, the cytochrome b_6/f complex, and the ATP synthase within the thylakoid (adapted from Stryer, 1988). Electrons are removed from molecules of H_2O resulting in the evolution of O_2 as a by-product transported outside the thylakoid. Protons are translocated from an external space (stroma) into the intrathylakoid space during the light-induced electron transport. The flow of protons through the ATP synthase to the stroma leads to the generation of ATP from ADP and P_i in the stroma where the Calvin–Benson cycle reactions are carried out. $NADPH_2$ is also formed on the stromal side of the thylakoid.

pigment–protein complexes, PS I and PS II. The photosystems operate in series connected by a chain of electron carriers usually visualised in a so-called 'Z' scheme (Hill & Bendall, 1960). In this scheme, redox components are characterised by their equilibrium mid-point potentials and the electron transport reactions proceed energetically downhill, from a lower (more negative) to a higher (more positive) redox potential (Fig. 2.6).

Upon illumination, two electrons are extracted from water (O_2 is evolved) and transferred through a chain of electron carriers to produce one molecule of $NADPH_2$. Simultaneously, protons are transported from an external space (stroma) into the intrathylakoid space (lumen) forming a pH gradient. According to Mitchel's chemiosmotic hypothesis, the gradient drives ATP synthesis, which is catalysed by the protein complex called ATPase or ATP synthase (Fig. 2.5). This reaction is called photophosphorylation and can be expressed as:

$$2\ \mathrm{NADP} + 3\ \mathrm{H_2O} + 2\ \mathrm{ADP} + 2\ \mathrm{P_i} \xrightarrow[\text{chlorophyll}]{\text{light energy}} 2\ \mathrm{NADPH_2} + 3\ \mathrm{ATP} + \mathrm{O_2}$$

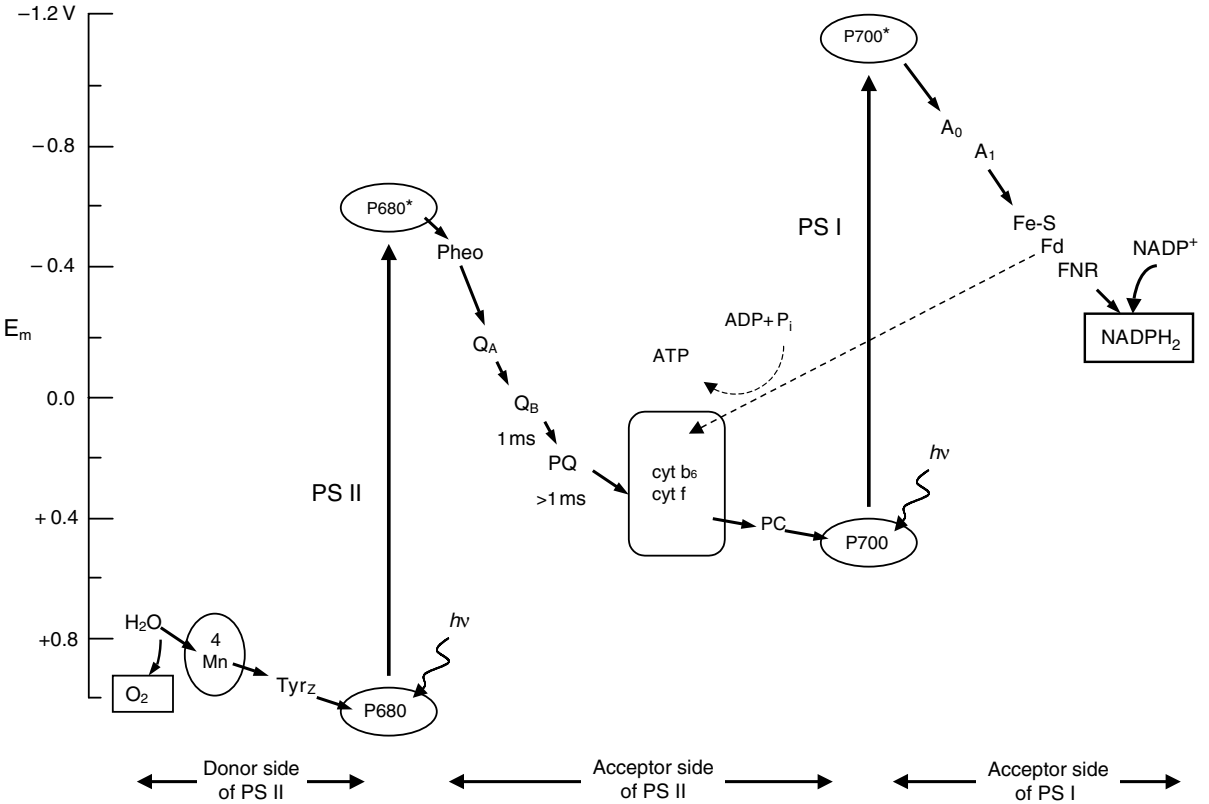

Fig. 2.6. The *Z* scheme for photosynthetic electron flow from water to $NADPH_2$ (Hill & Bendall, 1960). The electron transport carriers are placed in series on a scale of mid-point potentials. The oxidation of the primary electron donor P680 leads to a charge separation of about 1.2 V. The electron hole in $P680^+$ is filled by an electron from tyrosine Tyr Z, which obtains an electron from water via the four Mn ions. On the acceptor side of PS II, the pheophytin (Pheo) reduces the primary acceptor, Q_A, which is a plastoquinone molecule bound to a protein. Two electrons are sequentially transferred from Q_A to the secondary acceptor Q_B, the time constant of which is dependent on the level of reduction of Q_B. The reduced plastoquinone is oxidised by the cyt b_6/f complex. The re-oxidation of plastoquinol PQH_2 is the slowest reaction in the photosynthetic electron transport pathway. Plastocyanin (PC) carries one electron to the reaction centre of PSI, P700. On the acceptor side of PS I, the electron is passed through a series of carriers to ferredoxin, resulting finally in the reduction of NADP. The dotted straight arrow shows the pathway of cyclic photophosphorylation, where the electrons cycle in a *closed* system around PS I (from ferredoxin to the cyt b_6/f complex) and ATP is the only product.

2.4.3 The outer light-harvesting antennae

The primary function of the antenna systems is light-harvesting and energy transfer to the photosynthetic reaction centres (Fig. 2.7). The energy is funnelled to the reaction centres placed energetically *downhill*; some amount of heat is released during the transfer. All photosynthetically active pigments (chlorophylls, carotenoids and phycobilins) are associated with proteins, which are responsible for conferring a variety of specific functions in light-harvesting and electron transfer.

Two major classes of light-harvesting pigment–protein complexes can be identified: (i) hydrophilic phycobiliproteins, which are found in cyanobacteria and red algae, and (ii) hydrophobic pigment–protein complexes, such as LHC II and LHC I that contain Chl *a*, Chl *b* and carotenoids.

In cyanobacteria and red algae, the phycobiliproteins are assembled into multimeric particles called phycobilisomes, which are attached to the protoplasmic side of the thylakoid membrane. Phycobilisomes are assembled around an allophycocyanobilin-containing cores, which are coupled to the cores of PS II. The disks adjacent to the core of phycobiliproteins contain phycocyanobilin. The more distal disks consist of phycoerythrobilin or phycourobilin depending on the species. A special subdivision of cyanobacteria is Prochlorophyceae; unlike other cyanobacteria, they contain Chl *b* but no phycobiliproteins (Bryant, 1994).

In green algae (and higher plants), outer light-harvesting Chl *a*/*b*-protein complexes (called LHC II and LHC I) bind Chl *a* and *b* as well as xanthophylls (oxygenated carotenoids). A group of complexes LHC II serves PS II, and a genetically and biochemically different group called LHC I is associated with PS I.

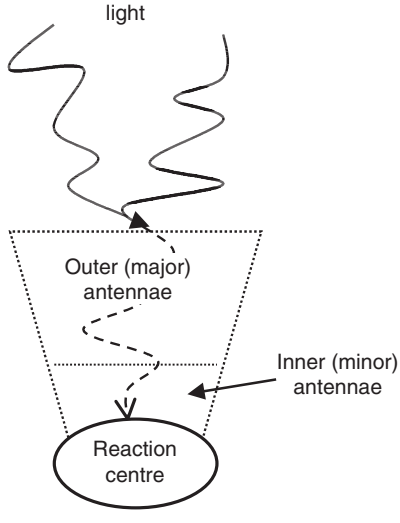

Fig. 2.7. The funnelling of excitation energy through the antenna array to the reaction centre (adapted from Ort, 1994). The light is captured by outer light-harvesting antennae, which are usually mobile. The excitation energy is transferred to the reaction centre via the inner antennae; it is the inner antennae and reaction centre that form the core of the photosystem.

In diatoms, the outer light-harvesting complexes contain Chl *a* and *c*, and fucoxanthin as the major carotenoid.

2.4.4 Photosystem II

Photosystem II represents a multimeric complex located in the thylakoid membrane, with more than 20 subunits and a relative molecular mass of about 300 kDa, composed of the reaction centre, the oxygen-evolving complex and the inner light-harvesting antennae. The PS II reaction centre contains the D1 and D2 proteins and the α and β subunits of cyt b_{559}. D1 and D2 proteins carry all essential prosthetic groups necessary for the charge separation and its stabilisation, tyrosine Z, the primary electron donor, P680, pheophytin and the primary and secondary quinone acceptors, Q_A and Q_B (Fig. 2.8). The inner core antennae are formed by the intrinsic Chl *a*-proteins CP43 and CP47, transferring excitation energy from the outer antennae to reaction centre (Fig. 2.7). As shown in Fig. 2.8, CP43 and CP47 are located on opposite sides of the D1–D2 reaction centre (Hankamer *et al.*, 2001). Recently, the X-ray crystal structure of PS II isolated from *Synechococcus elongatus* was resolved at 3.8 Å resolution (Zouni *et al.*, 2001).

2.4.5 Plastoquinone, the cytochrome b_6/f complex and plastocyanin

Electron transport between PS II and PS I is linked via the cytochrome b_6/f complex and assisted by two kinds of mobile carriers (Fig. 2.5). Plastoquinones (lipophilic benzoquinones with an isoprenoid chain) serve as

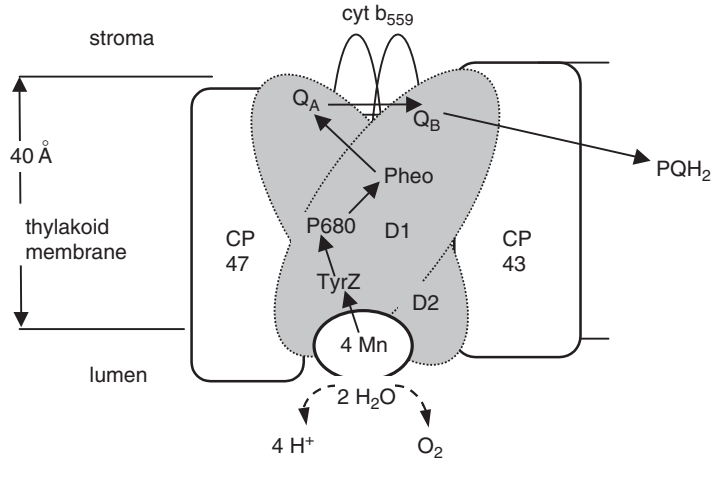

Fig. 2.8. Schematic diagram of molecular organisation of the PS II core. The major protein subunits CP43, CP47, cyt b_{559}, and the D1 and D2 proteins are labelled with bold letters. The two shaded protein subunits D1 and D2 are known to bind most of the electron carriers (a manganese cluster – 4 Mn, a tyrosine molecule Tyr Z, the special pair of chlorophyll *a* molecules P680, pheophytin Pheo, the plastoquinones Q_A and Q_B, and the plastoquinone pool PQH_2). The water-splitting complex represented by four manganese atoms is located in the thylakoid lumen. Arrows indicate principal electron transport pathways.

two-electron carriers between PS II and cytochrome b_6/f complexes. In parallel, the plastoquinone molecule translocates two protons from the stroma into the lumen. Plastocyanin (Cu-binding protein) operates in the thylakoid lumen, transferring electrons between the cytochrome b_6/f complex and PS I (for review, see Gross, 1996).

2.4.6 Photosystem I

Photosystem I is a multi-subunit intermembrane complex composed of about ten proteins, 100 chlorophylls and a molecular mass of about 360 kDa. PS I performs the photochemical reactions that generate the low redox potential (about -1 V) necessary for reducing ferredoxin and subsequently producing $NADPH_2$. The two large *PsaA* and *PsaB* proteins are located at the centre of the monomer which bears the major prosthetic cofactors of the reaction centre. Embedded within the complex are the Chl dimer P700 (where primary charge separation is initiated) and electron carriers A_0 (Chl *a*), A_1 (phylloquinone) and F_X (4Fe–4S). Generated electrons are further transported to the 4Fe–4S electron acceptors F_A and F_B of the *PsaC* subunit and to the terminal mobile electron acceptor, which is ferredoxin (Fig. 2.6). Recently, the 2.5 Å X-ray structure of cyanobacterial PS I was resolved (Jordan *et al.*, 2001).

2.4.7 ATP synthase/ATPase

ATP synthase is a membrane-bound enzyme that is composed of two oligomeric subunits, CF_0 and CF_1 (with relative molecular masses of 110–160 kDa and about 400 kDa, respectively). The complex powered by the pH gradient catalyses the synthesis of ATP from ADP and P_i (Fig. 2.5). The hydrophobic CF_0 spans the thylakoid membrane, whereas the hydrophilic CF_1 is attached to CF_0 on the stromal side of the membrane. The subunits CF_0 act as a proton channel and the flux of protons drives the subunits CF_1, which form a ring structure with catalytic sites for ATP synthesis. A passage of about four protons is required for the synthesis of one ATP molecule (Kramer *et al.*, 1999).

2.5 The dark reactions of photosynthesis

2.5.1 Carbon assimilation

The fixation of carbon dioxide happens in the dark reaction using the $NADPH_2$ and ATP produced in the light reaction of photosynthesis. The reaction can be expressed as:

$$CO_2 + 4\ H^+ + 4\ e^- \xrightarrow[\text{enzymes}]{2\,NADPH_2,\ 3\,ATP} \underset{\text{carbohydrates}}{(CH_2O)} + H_2O$$

In order to fix one molecule of CO_2, two molecules of $NADPH_2$ and three molecules of ATP are required (representing an energy of 5.2×10^4 J, about

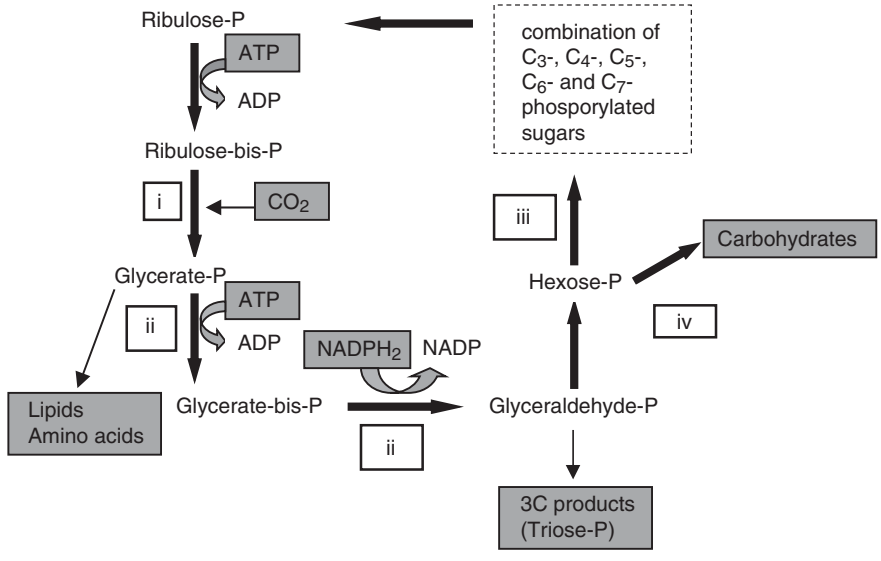

Fig. 2.9. The photosynthetic carbon fixation pathways – the Calvin–Benson cycle. The fixation of CO_2 to the level of sugar can be considered to occur in four distinct phases: (i) *Carboxylation phase* – a reaction whereby CO_2 is added to the 5-carbon sugar, ribulose bisphosphate (Ribulose-bis-P), to form two molecules of phosphoglycerate (Glycerate-P). This reaction is catalysed by the enzyme ribulose bisphospate carboxylase/oxygenase (Rubisco); (ii) *Reduction phase* – to convert Glycerate-P to 3-carbon products (Triose-P), the energy must be added in the form of ATP and $NADPH_2$ in two steps, the phosphorylation of Glycerate-P to form diphosphoglycerate (Glycerate-bis-P), and the reduction of Glycerate-bis-P to phosphoglyceraldehyde (Glyceraldehyde-P) by $NADPH_2$; (iii) *Regeneration phase* – Ribulose-P is regenerated for further CO_2 fixation in a complex series of reactions combining 3-, 4-, 5-, 6- and 7-carbon sugar phosphates, which are not explicitly shown in the diagram; (iv) *Production phase* – primary end-products of photosynthesis are considered to be carbohydrates, but fatty acids, amino acids and organic acids are also synthesised in photosynthetic CO_2 fixation.

13 kcal). As concern the quantum efficiency of CO_2 fixation, it was found that at minimum ten quanta of absorbed light are required for each molecule of CO_2 fixed or O_2 evolved.

The reaction mechanism of carbon fixation was worked out by Calvin and Benson in the 1940s and early 1950s using ^{14}C radiolabelling technique (Nobel Prize, 1961). The conversion of CO_2 to sugar (or other compounds) occurs in four distinct phases (Fig. 2.9) forming the so-called Calvin–Benson cycle:

1. *Carboxylation phase* The reaction whereby CO_2 is added to the 5-carbon sugar, ribulose bisphosphate (Ribulose-bis-P), to form two molecules of phosphoglycerate (Glycerate-P). This reaction is catalysed by the enzyme ribulose bisphospate carboxylase/oxygenase (Rubisco).
2. *Reduction phase* In order to convert phosphoglycerate to 3-carbon products (Triose-P) the energy must be added in the form of ATP and $NADPH_2$ in two steps: phosphorylation of phosphoglycerate to form diphosphoglycerate and ADP, and secondly, reduction of diphosphoglycerate (Glycerate-bis-P) to phosphoglyceraldehyde (Glyceraldehyde-P) by $NADPH_2$.

3. *Regeneration phase* Ribulose phosphate (Ribulose-P) is regenerated for further CO_2 fixation in a complex series of reactions combining 3-, 4-, 5-, 6- and 7-carbon sugar phosphates. The task of generating 5-carbon sugars from 6-carbon and 3-carbon sugars is accomplished by the action of the transketolase and aldolase enzymes.
4. *Production phase* Primary end-products of photosynthesis are considered to be carbohydrates, but fatty acids, amino acids and organic acids are also synthesised in photosynthetic CO_2 fixation. Various end-products can be formed under different conditions of light intensity, CO_2 and O_2 concentrations, and nutrition.

2.5.2 Photorespiration

Photorespiration represents a competing process to carboxylation, where the organic carbon is converted into CO_2 without any metabolic gain. In this process, Rubisco functions as an oxygenase, catalysing the reaction of O_2 with ribulose bisphosphate to form phosphoglycolate. After dephosphorylation, glycolate is converted, in several steps, to serine, ammonia and CO_2.

Photorespiration depends on the relative concentrations of oxygen and CO_2 where a high O_2/CO_2 ratio (i.e. high concentration of O_2 and low concentration of CO_2) stimulates this process, whereas a low O_2/CO_2 ratio favours carboxylation. Rubisco has low affinity to CO_2, its K_m (half-saturation) being roughly equal to the level of CO_2 in air. Thus, under high irradiance, high oxygen level and reduced CO_2, the reaction equilibrium is shifted towards photorespiration. Photosynthetic organisms differ significantly in their rates of photorespiration: in some species it may be as high as 50% of net photosynthesis.

For optimal yields in microalgal mass cultures, it is necessary to minimise the effects of photorespiration. This might be achieved by an effective stripping of oxygen and by CO_2 enrichment. For this reason, microalgal mass cultures are typically grown at a much higher CO_2/O_2 ratio than that found in air.

2.6 Light adaptation (Falkowski & Raven, 1997)

In the natural environment, photosynthetic organisms can face frequent changes in irradiance – in the range of one to two orders of magnitude. To cope with such changes plants have developed several acclimation mechanisms. The aim of acclimation processes is to balance the light and dark photosynthetic reactions. Since the levels of Rubisco seem to be relatively constant (Sukenik *et al.*, 1987), the major regulation occurs on the light reactions' side, mainly in PS II. The regulation of the PS II output can be performed in two ways – by modulation of its light-harvesting capacity, or by changes in the number of PS II reaction centres.

In light-limiting conditions, the organism increases pigmentation, i.e. increases the number of photosynthetic units, the size of light-harvesting complexes. Under supra-optimal irradiance the pigmentation is reduced. The changes of pigmentation occur at a timescale of days; so, to respond

to fast changes in irradiance, other mechanisms have to be employed. In many species, the build-up of the pH gradient results in enhanced thermal dissipation (quenching) of harvested quanta, reducing the amount of energy utilised in photochemistry (Briantais *et al.*, 1979). Though, in cyanobacteria, the ΔpH-regulated dissipation does not seem to exist. In higher plants and green algae, the pH gradient build-up is accompanied by a reversible conversion of violaxanthin into zeaxanthin. In higher plants, it was demonstrated that zeaxanthin content correlates well with the extent of thermal dissipation (Demmig *et al.*, 1987). However, in green algae, the zeaxanthin-dependent dissipation seems to play only a minor role (Casper-Lindley & Björkman, 1998; Masojídek *et al.*, 1999). An analogous cycle (monoepoxide diadinoxanthin $\leftrightarrow$ diatoxanthin) has been found in Chrysophyceae and Phaeophyceae. As in the case of zeaxanthin in green plants, the presence of diatoxanthin results in enhanced thermal dissipation of light energy (Arsalane *et al.*, 1994).

The light inactivation of the PS II function (PS II photoinactivation) can be viewed as an emergency acclimation process reducing the number of redundant PS II units. As it happens, light energy causes an inevitable modification of the PS II reaction centres, which, if not repaired by continuous D1 replacement, leads to the inactivation of the PS II function (Prasil *et al.*, 1992). The photoinactivation is manifested as an exponential (single-order) decline of variable fluorescence F_V (F_0 remains constant), paralleled by a decline of the Hill reaction (Šetlik *et al.*, 1990). The rate of this decline is directly proportional to light intensity (Šetlik *et al.*, 1987; Tyystjärvi & Aro, 1996) suggesting that the damage represents a single-photon process with a very low quantum yield. The inactivation of a part of the units caused by excess irradiance does not necessarily reduce the overall rates of electron transfer. At saturating light intensities, the rate of photosynthesis usually depends on the CO_2 fixation rate (Sukenik *et al.*, 1987), and a moderate reduction in the number of active PS II units might not have any effect (Behrenfeld *et al.*, 1998).

2.7 Selected monitoring techniques used in microalgal biotechnology

2.7.1 Measurement of photosynthetic oxygen evolution (Walker, 1993)

Routine measurements of photosynthetic oxygen production in algal cultures are usually carried out with an oxygen electrode. It is a special form of electrochemical cell, in which the generated current is proportional to the activity of oxygen present in a solution, capable of detecting changes of the order of 10 μM.

A Clark-type oxygen electrode, which is the most widely used, consists of a platinum cathode (but gold or other metals can also be used) and a silver/silver chloride anode. When the voltage (-0.71 V) is applied across the electrodes, the oxygen undergoes electrolytic reduction ($O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O$). The electrodes are placed in an electrolyte (saturated KCl) separated from the suspension by a thin,

gas-permeable membrane (Teflon, polypropylene). The electrode consumes oxygen, and therefore the suspension has to be mixed. Oxygen production is usually expressed in μmol or mg O_2 per mg^{-1} (Chl) h^{-1}, or per cell h^{-1}.

Recently, optical oxygen sensors have been developed that are based on the fluorescence and phosphorescence quenching of certain luminophores in the presence of oxygen (e.g. PreSens, Precision Sensing GmbH, Germany; Optod Ltd, Moscow, Russia). Although not widely used, these sensors have sensitivity comparable to Clark-type electrodes, and yet show a few advantages, namely: no consumption of oxygen, stability against electrical and thermal disturbances, and high storage and mechanical stability.

2.7.2 Measurement of photosynthetic carbon fixation

Since photosynthetic carbon fixation in cell suspension cannot be easily followed by infrared gas analysis, special electrodes are used to measure the partial pressure of carbon dioxide (p_{CO_2}) in solutions. The principle is based on the relationship between pH, and the concentration of CO_2 and bicarbonate in the solution ($K_s = [HCO_3^-] \times [H^+]/[CO_2]$). The p_{CO_2} electrode is constructed as a combined glass and Ag/AgCl electrode.

The method of ^{14}C radiolabelling has been widely used to study photosynthetic carbon metabolism, but it also provides a measure of the photosynthetic assimilation rate. The population (or culture) of microalgae is exposed to ^{14}C for a fixed period of time. The reaction is then stopped by the addition of concentrated HCl and the amount of ^{14}C incorporated is determined by a scintillation counter. This technique is widely employed in phytoplankton studies, but can also be exceptionally used in mass cultures in photobioreactors.

Biomass production might be roughly estimated as optical density (OD) at 750 nm, or measured as dry weight per volume of sample. Exact determination of carbon (and nitrogen) content in the biomass can be done by a CHN analyser.

2.7.3 Chlorophyll fluorescence

Chlorophyll fluorescence has become one of the most common and useful techniques in photosynthesis research. Its non-invasiveness, sensitivity, as well as the wide availability of reliable commercial instruments, also make it a convenient and suitable technique in algal biotechnology.

Chlorophyll fluorescence directly reflects the performance of photochemical processes in PS II; the contribution of PS I emission in the total signal at ambient temperature is rather small and for practical purposes is often neglected. However, in cyanobacteria, the fluorescence of numerous PS I complexes and phycobilisomes contributes significantly to the total signal, which affects the correct determination of certain parameters (e.g. F_V/F_M). Upon illumination, the PS II chlorophyll molecules are excited to a singlet excited state (Chl a^*). The energy of the excited state is transferred to the reaction centre to be used for photochemical charge separation. Alter-

natively, the excitation energy can be dissipated as heat, or re-emitted as fluorescence (Fig. 2.10). The sum of energy entering these three competing processes is equal to the absorbed light energy. Any change of photochemistry or dissipation results in a change of fluorescence, providing a direct insight into the energetics of PS II.

In the dark, all the reaction centres are in the so-called *open* state and photochemistry is at a maximum. The fluorescence yield in this state is low, designated as F_0. When PS II is exposed to a strong pulse of light, the reaction centres undergo charge separation and the electron is moved to the first electron acceptor Q_A. When Q_A is reduced, the reaction centres are in the *closed* state and photochemistry is transiently blocked. Since the yield of photochemistry is zero, the dissipation and fluorescence yields rise proportionally. The high fluorescence yield of the closed centres is described as F_M. Since the fluorescence yield rises proportionally to the level of the PS II closure, the open reaction centre acts as a fluorescence quencher. This phenomenon is called photochemical quenching qP and can be calculated as $(F_M - F')/(F_M - F_0)$, where F' is a steady-state yield of fluorescence. The values of qP range from 0 to 1 reflecting the relative level of Q_A oxidation (Fig. 2.11).

The difference between the maximum fluorescence F_M (all Q_A reduced) and minimum fluorescence F_0 (all Q_A oxidised) is denoted as the variable fluorescence F_V. The ratio between the variable fluorescence and maximum fluorescence (F_V/F_M) ranges from 0.65 to 0.80 in dark-adapted green algae. This ratio is frequently used as a convenient estimate of the photochemical yield of PS II. The yield varies significantly, depending on the irradiance regime and physiological treatment.

When the photosynthetic apparatus is exposed to light, a decrease in F_M is usually observed. The lowered fluorescence yield is described as F'_M. This phenomenon is called non-photochemical quenching and indicates an

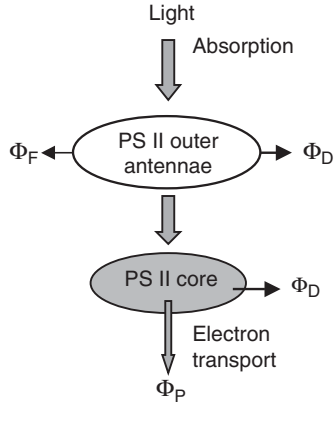

Fig. 2.10. A schematic representation of absorbed light energy distribution in the PSII complex between photochemistry Φ_P, fluorescence Φ_F and non-radiative dissipation Φ_D; the latter (Φ_D) can occur in the antennae as well as in the reaction centre. Φ_P, Φ_F and Φ_D represent the yield of photochemistry, fluorescence and non-radiative dissipation, respectively.

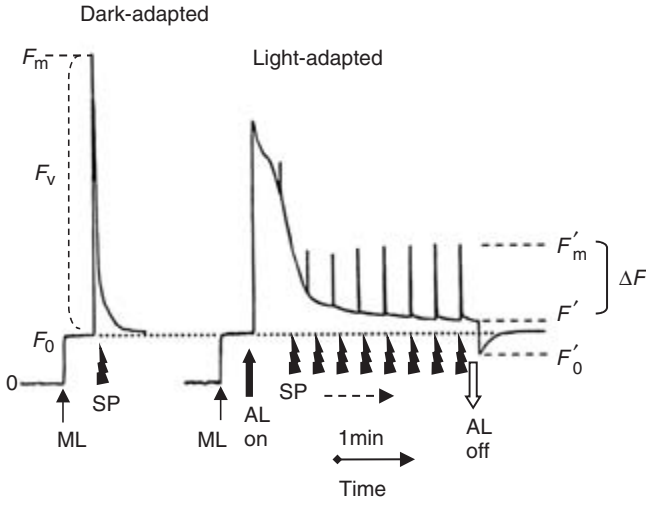

Fig. 2.11. A schematic representation of the saturation pulse method (adapted from Schreiber *et al.*, 2000). The minimum and maximum fluorescence levels F_0 and F_m are measured in the dark-adapted sample, using modulated measuring light (ML) and a saturating light pulse (SP). Next, the sample is illuminated with actinic light (AL) and a series of saturating pulses in order to reach the steady state F' and F'_m level. Finally, the actinic light and saturating pulses are switched off to measure the F'_0 level.

increased heat dissipation of excitation (Fig. 2.10). In principle, non-photochemical quenching is inversely related to photochemistry, and is considered a safety valve protecting PS II reaction centres from damage by excess irradiance. Selected parameters calculated from chlorophyll fluorescence measurements are listed in Table 2.1.

Table 2.1. Selected parameters calculated from chlorophyll fluorescence measurements (Fig. 2.11). F_0, F_V, F_M – minimum, variable and maximum fluorescence in dark-adapted state; F'_0, F', F'_V, F'_M – minimum, steady-state, variable and maximum fluorescence in light-adapted state; a_{PSII} – optical absorption cross-section of PS II; PPFD – photosynthetic photon flux density.

Parameter	Symbol	Formula
Maximum photochemical yield of PS II	F_V/F_M	$F_V/F_M = (F_M - F_0)/F_M$
Effective PS II photochemical yield	Φ_{PSII} or $\Delta F/F'_M$	$\Phi_{PSII} = (F'_M - F')/F'_M$
Relative electron transport rate through PS II (rate of photochemistry)	rETR	$rETR = \Phi_{PSII} \times PPFD$
Actual electron transport rate through PS II (correlated with primary productivity)	ETR	$ETR = \Phi_{PSII} \times PPFD \times a_{PSII}$
Non-photochemical quenching	qN	$qN = 1 - (F'_M - F'_0)/(F_M - F_0)$
Photochemical quenching qN	qP	$qP = (F'_M - F')/(F'_M - F'_0)$
Stern–Volmer coefficient of non-photochemical quenching	NPQ	$NPQ = (F_M - F'_M)/F'_M$

2.8 Theoretical limits of algal productivity

An understanding of photosynthesis is fundamental for microalgal biotechnology. Mass cultures of unicellular microalgae (cyanobacteria and algae) grown in the laboratory and outdoors represent a special environment, where rather dense suspensions of cells, colonies, coenobia or filaments are usually cultivated under conditions of low irradiance per cell, high concentration of dissolved oxygen and limited supplies of inorganic carbon (carbon dioxide or bicarbonate). Therefore, the growth critically depends on the interplay of several parameters: average irradiance per cell, mixing, gas exchange and temperature.

Ideally, the theoretical maximum rate of growth of an algal culture should be equal to the maximum rate of photosynthesis. In a fast-growing culture adapted to high irradiance, the turnover of electron transport can reach 2 ms, which probably corresponds to the turnover of the PS II complex. At this rate, up to 50 atoms of carbon can be fixed per individual RC per second, if we consider that ten electrons are transferred per C atom or per molecule of O_2 fixed. Assuming 300 chlorophyll molecules per PS II unit, then the rate of photosynthesis can be about 660 μmol C (or O_2) mg^{-1} (Chl) $h^{-1} = 7.9$ g C (or 21.1 O_2) g^{-1} (Chl) h^{-1}. This rate, considering a carbon per Chl ratio of 30 (w/w), results in a growth rate of $\mu \sim 0.2\,h^{-1}$. (See, however, the distinction made between productivity and growth rate in Chapter 7.)

In all cultivation facilities, the growth of algae is spatially confined by the dimensions of the cultivation vessel. This confinement, together with a given solar input, leads to a finite amount of light energy that can be delivered to such a system. Therefore, cultivation facilities have to be designed such that the light conversion efficiency is maximised, which means the use of dense cultures fully absorbing the delivered light. Unfortunately, the steep light gradient formed in these cultures results in an overexposure of the upper layers of the suspension and leads to a low efficiency of light conversion. To avoid this situation, the cultures have to be rapidly mixed to prevent prolonged light saturation (Nedbal *et al.*, 1996). Special designs of photobioreactors might improve the light distribution in a suspension, but proposed solutions are frequently difficult to scale up to the industrial level (Carlozzi & Torzillo, 1996; Tredici & Chini Zittelli, 1997). The third approach to the problem is to modify the optical properties of the cells in order to assure better light utilisation in the suspension (Melis *et al.*, 1999). The genetic modification of reducing the antenna size could reduce the excitation pressure of the photosynthetic units under high irradiance and maintain a high efficiency of light conversion.

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