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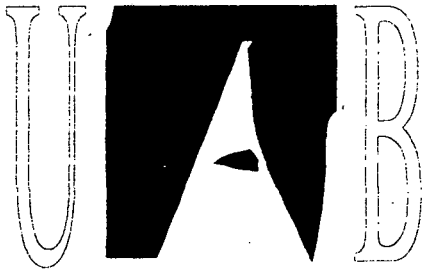

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LIGHT-LIMITED GROWTH OF *Chromatium vinosum*

OLGA SÀNCHEZ MARTÍNEZ

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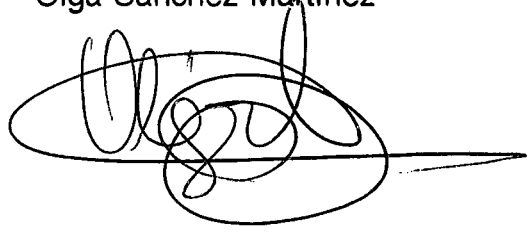
DEPARTAMENT DE GENÈTICA I MICROBIOLOGIA

LIGHT-LIMITED GROWTH OF *Chromatium vinosum*

Memòria redactada per optar al Grau de Doctor en Ciències, Secció de Biològiques, per la Universitat Autònoma de Barcelona,
per
Olga Sànchez Martínez



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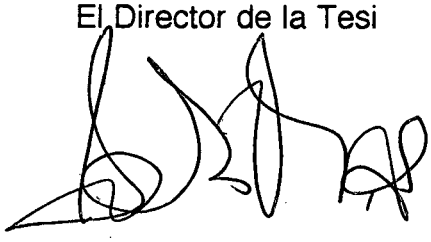

Dr. Jordi Mas Gordi

Bellaterra, novembre de 1996

Al Pere i l'Helena

SUMMARY

This thesis addresses the study of the mechanisms through which the purple sulfur bacterium *Chromatium vinosum* adapts to light limitation. *C. vinosum* was chosen as a model organism representative of purple sulfur bacteria, a group of phototrophic microorganisms often found in anoxic, sulfide–containing illuminated environments. The study attempted to analyze two major aspects: first, the capacity of *C. vinosum* to adapt the photosynthetic response when grown at low irradiances, and second, the ability to change the allocation of reducing power to different cell fractions therefore modifying the relative content of several storage compounds.

The experiments had to be carried out in a culture system which allowed to change the light supply while maintaining constant biomass, sulfide and nutrient concentrations. In conventional chemostats, variations of the light supply result in changes in these variables, and as a consequence, it is virtually impossible to discriminate between the effect of light limitation and the effect of changes in the variables mentioned above. Thus, a system was developed (Chapter 4), in which the dilution rate was controlled through sulfide-dependent redox changes monitored through a redox controller. The system allowed to maintain a constant level of hydrogen sulfide (50 μM) while undergoing a series of shifts in irradiance (868 to 113, 113 to 23 and 23 to 7 $\mu E.m^{-2}.s^{-1}$).

It was also necessary to define an appropriate variable to describe the amount of light available for growth. Previous literature referred activity data to light irradiance at the surface of the culture, ignoring that the light climate experienced by the cells depends to a large extent on the biomass of the culture and the size of the culture vessel. In the procedure developed in this thesis (Chapter 2), the rate of light absorption was measured as the difference between the integrated light output from the surface of a culture vessel containing an active culture, and the integrated output of a vessel containing only culture medium. Division of the rate at which light was absorbed by the culture by the biomass contained in the culture, yielded the specific rate of light uptake (q_e), which has been subsequently used to describe the energy available to the organisms. A study on the factors likely to affect q_e (Chapter 3) indicated that increases in the biomass of the culture and in the size of the culture vessel maximize light absorption but decrease the amount of light available per cell. The presence of refractile sulfur inclusions also reduces the specific light absorption in dense cultures. However, it has the opposite effect in diluted suspensions, actually increasing the amount of light absorbed per cell.

In order to study the mechanisms involved in the adaptation to light-limiting conditions, four steady states were tested at different light supplies (103.0, 30.9, 16.8 and 7.1 $\mu E.mg^{-1}$ protein.h^{-1}). The results (Chapter 5) show that *C. vinosum* increases the specific pigment content (from 24 to 85 μg Bchl a. mg^{-1} protein) when irradiance decreases, and as a consequence, the protein cross-section (a^*_{prot}) increases (from 0.00019 to 0.00038 $m^2.mg^{-1}$ protein). The quantum yield for photosynthesis also increases with light limitation (0.048 to 0.140 μmol $C.\mu E^{-1}$) indicating an enhanced efficiency of the photosynthetic apparatus. The photosynthetic capacity of the organism (P_{max}) remained constant under all the conditions tested, suggesting that the adaptation of the photosynthetic apparatus does not involve changes in the number of reaction centres. The maintenance coefficient of the cells (m) suffered a fivefold increase under conditions of light limitation, reaching a maximum value of 3.4 $\mu E.mg^{-1}$ protein.h^{-1}). Analysis of the main storage components (Chapter 6) indicates that glycogen was stored in large amounts (31.6% of the total stored reducing power) under light-saturating conditions, while sulfur was accumulated preferentially (95.7% of the stored reducing power) under light limitation. Conversion of the different cell fractions into reducing equivalents allowed to calculate the electron budget. The results show that, under light limitation, a considerable amount of reducing power (41%), initially present as sulfide, was not recovered in any of the cell fractions analyzed. Analysis of dissolved organic carbon confirms the existence of an important organic fraction in supernatants of the culture, thus supporting the idea that a considerable part of the electrons derived from hydrogen sulfide was conducted to the synthesis of excretion products.

RESUM

Aquesta tesi tracta l'estudi dels mecanismes mitjançant els quals el bacteri vermell del sofre *Chromatium vinosum* s'adapta a la limitació per la llum. *C. vinosum* va ésser escollit com a organisme model representatiu dels bacteris vermells del sofre, un grup de microorganismes fototròfics que sovint es troba en ambients il.luminats anòxics i en presència de sulfhídric. L'estudi intenta analitzar dos aspectes principals: primer, la capacitat de *C. vinosum* d'adaptar la resposta fotosintètica quan creix a irradiàncies baixes i, segon, la possibilitat d'assignar el poder reductor a diferents fraccions cel.lulars modificant així el contingut relatiu de diversos composts de reserva.

Els experiments es van haver de dur a terme en un sistema de cultiu que permetés el canvi de les condicions d'il.luminació mentre es mantenien constants la biomassa i les concentracions de sulfhídric i de nutrients. En els quimiostats convencionals, aquestes variables es veuen afectades per canvis en el subministrament de llum i, com a conseqüència, és virtualment impossible discriminar entre l'efecte de la limitació per la llum i l'efecte de canvis en les variables abans esmentades. Per tant, es va desenvolupar un sistema (Capítol 4) en el qual, el potencial redox, depenent de la concentració de sulfhídric, regulava la taxa de dilució mitjançant un controlador de redox. El sistema permetia mantenir un nivell contant de sulfhídric (50 μM) al variar la irradiància (868 a 113, 113 a 23 i 23 a 7 $\mu E.m^{-2}.s^{-1}$).

També va ésser necessari definir una variable apropiada que permetés descriure la quantitat de llum disponible pel creixement. En treballs previs, les dades d'activitat es relacionaven amb la irradiància incident en la superfície del cultiu sense tenir en compte que el camp de llum experimentat per les cèl.lules depèn sobretot de la biomassa del cultiu i de la mida del recipient de cultiu. El procediment desenvolupat en aquesta tesi (Capítol 2) determina la taxa d'absorció de llum com la diferència entre la quantitat de llum que surt de la superfície d'un recipient de cultiu que conté un cultiu actiu i la quantitat de llum que surt d'aquest recipient quan només conté medi de cultiu. El quocient entre la taxa d'absorció de llum del cultiu i la biomassa continguda en el cultiu proporciona la taxa específica d'absorció de llum (q_e), la qual va ésser utilitzada subseqüentment per descriure l'energia disponible pels organismes. Un estudi sobre els possibles factors que podien afectar q_e (Capítol 3) va indicar que increments en la biomassa del cultiu i en la mida del recipient de cultiu maximitzen l'absorció de llum però disminueixen la quantitat de llum disponible per cèl.lula. La presència d'inclusions refringents de sofre també redueix la taxa específica d'absorció de llum en cultius densos. Malgrat això, té l'efecte oposat en suspensions diluïdes, incrementant de fet la quantitat de llum absorbida per cèl.lula.

Per tal d'estudiar els mecanismes involucrats en l'adaptació a condicions de limitació per la llum, es van dur a terme quatre estats estacionaris a diferents nivells d'il.luminació (103.0, 30.9, 16.8 i 7.1 $\mu E.mg^{-1}$ proteïna.h^{-1}). Els resultats (Capítol 5) mostren que *C. vinosum* incrementa el contingut específic de pigments (des de 24 a 85 μg Bchl a.mg^{-1} proteïna) quan la irradiància disminueix i, com a conseqüència, el coeficient d'absorció de les proteïnes (a^*_{prot}) incrementa (des de 0.00019 fins a 0.00038 $m^2.mg^{-1}$ proteïna). El rendiment quàntic de la fotosíntesi també augmenta amb la limitació per la llum (de 0.048 a 0.140 μmol C.μE^{-1}) indicant un increment en l'eficiència de l'aparell fotosintètic. La capacitat fotosintètica de l'organisme (P_{max}) es va mantenir constant en totes les condicions d'il.luminació, la qual cosa suggereix que l'adaptació de l'aparell fotosintètic no involucra canvis en el nombre de centres de reacció. El coeficient de manteniment (m) va experimentar un increment de cinc vegades en condicions de limitació per la llum, assolint un valor màxim de 3.4 $\mu E.mg^{-1}$ proteïna.h^{-1}. Les anàlisis dels compostos de reserva (Capítol 6) indiquen que el glicogen s'acumulava en gran quantitats (31.6% del total de poder reductor magatzemat) en condicions de saturació per la llum, mentre que el sofre s'acumulava preferentment (95.7% del poder reductor magatzemat) quan la llum era limitant. La conversió de les diferents fraccions cel.lulars en equivalents de poder reductor va permetre el càlcul del balanç d'electrons. Els resultats mostren que, en condicions de limitació per la llum, una quantitat considerable de poder reductor (41%), present inicialment com a sulfhídric, no va ésser recuperat en cap de les fraccions analitzades. Les anàlisis de carboni orgànic dissolt confirmen l'existència de una fracció orgànica important en sobrenadants del cultiu, recolzant la idea que una part considerable dels electrons derivats del sulfhídric va ésser destinada a la síntesi de productes d'excreció.

RESUMEN

Esta tesis trata el estudio de los mecanismos mediante los cuales la bacteria roja del azufre *Chromatium vinosum* se adapta a la limitación por la luz. *C. vinosum* fue elegido como organismo modelo representativo de las bacterias rojas del azufre, un grupo de microorganismos fototróficos que a menudo se encuentra en ambientes iluminados anóxicos y en presencia de sulfhídrico. El estudio intenta analizar dos aspectos principales: primero, la capacidad de *C. vinosum* de adaptar la respuesta fotosintética cuando crece a irradiancias bajas y, segundo, la posibilidad de asignar el poder reductor a diferentes fracciones celulares modificando así el contenido relativo de diversos compuestos de reserva.

Los experimentos se tuvieron que llevar a cabo en un sistema de cultivo que permitiera el cambio de las condiciones de iluminación mientras se mantenían constantes la biomasa y las concentraciones de sulfhídrico y de nutrientes. En los quimiostatos convencionales, estas variables se encuentran afectadas por cambios en el suministro de luz y, como consecuencia, es virtualmente imposible discriminar entre el efectode la limitación por la luz y el efecto de cambios en las variables citadas anteriormente. Por tanto, se desarrolló un sistema (Capitulo 4) en el cual el potencial redox, dependiente de la concentración de sulfhídrico, regulaba la tasa de dilución mediante un controlador de redox. El sistema permitía mantener un nivel constante de sulfhídrico (50 μM) al variar la irradiancia (868 a 113, 113 a 23 i 23 a 7 $\mu E.m^{-2}.s^{-1}$).

También fue necesario definir una variable apropiada que permitiera describir la cantidad de luz disponible para el crecimiento. En trabajos previos, los datos de actividad se relacionaban con la irradiancia incidente en la superficie del cultivo sin tener en cuenta que en campo de luz experimentado por las células dependía sobre todo de la biomasa del cultivo y de la medida del recipiente de cultivo. El procedimiento desarrollado en esta tesis (Capítulo 2) determina la tasa de absorción de luz como la diferencia entre la cantidad de luz que sale de la superficie de un recipiente de cultivo que contiene un cultivo activo y la cantidad de luz que sale de este recipiente cuando sólo contiene medio de cultivo. El cociente entre la tasa de absorción de luz del cultivo y la biomasa contenida en el cultivo proporciona la tasa específica de absorción de luz (q_e), la cual fue utilizada subsiguientemente para describir la energía disponible para los organismos. Un estudio sobre los posibles factores que podían afectar q_e (Capítulo 3) indicó que incrementos en la biomasa del cultivo y en la medida del recipiente de cultivo maximizan la absorción de luz pero disminuyen la cantidad de luz disponible por célula. La presencia de inclusiones refringentes de azufre también reduce la tasa específica de absorción de luz en

cultivos densos. Sin embargo, tiene el efecto opuesto en suspensiones diluidas, incrementando de hecho la cantidad de luz absorbida por célula.

Con el fin de estudiar los mecanismos implicados en la adaptación a condiciones de limitación por la luz, se llevaron a cabo cuatro estados estacionarios a diferentes niveles de iluminación (103.0, 30.9, 16.8 i 7.1 μE.mg^{-1} proteína.h^{-1}). Los resultados (Capítulo 5) muestran que *C. vinosum* incrementa el contenido específico de pigmentos (desde 24 a 85 μg Bchl a.mg^{-1} proteína) cuando la irradiancia disminuye y, como consecuencia, el coeficiente de absorción de las proteínas (a^*_{prot}) incrementa (desde 0.00019 hasta 0.00038 m^2.mg^{-1} proteína). El rendimiento cuántico de la fotosíntesis también aumenta con la limitación por la luz (de 0.048 a 0.140 μmol C.μE^{-1}) indicando un incremento en la eficiencia del aparato fotosintético. La capacidad fotosintética del organismo (P_{max}) se mantuvo constante en todas las condiciones de iluminación, lo cual sugiere que la adaptación del aparato fotosintético no involucra cambios en el número de centros de reacción. El coeficiente de mantenimiento (m) experimentó un incremento de cinco veces en condiciones de limitación por la luz, alcanzando un valor máximo de 3.4 μE.mg^{-1} proteína.h^{-1}. Los análisis de los compuestos de reserva (Capítulo 6) indican que el glicógeno se acumulaba en grandes cantidades (31.6% del total del poder reductor almacenado) en condiciones de saturación por la luz, mientras que el azufre se acumulaba preferentemente (95.7% del poder reductor almacenado) cuando la luz era limitante. La conversión de las diferentes fracciones celulares en equivalentes de poder reductor permitió el cálculo del balance de electrones. Los resultados muestran que, en condiciones de limitación por la luz, una cantidad considerable de poder reductor (41%), presente inicialmente como sulfhídrico, no fue recuperado en ninguna de las fracciones analizadas. Los análisis de carbono orgánico disuelto confirman la existencia de una fracción orgánica importante en sobrenadantes del cultivo, apoyando la idea de que una parte considerable de los electrones derivados del sulhídrico fue destinada a la síntesis de productos de excreción.

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CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Phototrophic microorganisms, in particular purple and green sulfur bacteria, generally develop in aquatic ecosystems exposed to light. Their growth is controlled by a number of environmental factors, such as irradiance (Takahashi and Ichimura 1970, Parkin and Brock 1980a, Pedrós-Alió et al 1983), the spectral composition of incident light (Parkin and Brock 1980b, Montesinos et al 1983), oxygen tension, temperature, pH, nutrient concentration and the presence of reduced sulfur compounds (van Gemerden and Mas 1995). Among these parameters, light availability seems to play a crucial role in the development of these organisms, as shown by studies which proof the existence of light limitation in natural environments (Bergstein et al 1979, Parkin and Brock 1980a, Guerrero et al 1985, Mas et al 1990).

This thesis attempts to analyze how a model organism, the purple sulfur bacterium *Chromatium vinosum* adapts to light limiting conditions. The results of the study will hopefully contribute to improve our knowledge of the mechanisms of adaptation of this group of organisms to light-limited environments.

This introduction describes some theoretical aspects which are necessary to understand the principles of light measurement in aquatic systems and in culture vessels, as well as the physiological response of phototrophic organisms to changes in the spectral composition and the quantity of light they are exposed to.

1.1. LIGHT MEASUREMENT

1.1.1. Photometry and radiometry

The electromagnetic spectrum extends over 19 orders of magnitude. From these, light occupies only a small part (less than one order of magnitude, extending between 200 and 1000 nm) (Fig. 1.1). Since the human eye can only sense radiation in the segment of the electromagnetic spectrum between 400 and 700 nm, physicists have defined the primary standard of light in terms of the visual response of the human eye (Fig. 1.2). Thus, photometry

refers to the measurement of radiation between 400 and 700 nm and, as a consequence, does not describe the total amount of energy received, but rather the energy received that can effectively be transduced by the human eye with a sensor which has a spectral response curve equal to that of the human eye. Thus, while photometric measurements are suitable to describe the light conditions in studies related to human light perception, such as in the illumination of work areas, television screens, etc., their utilization in studies in which the subject has a transducer with a spectral response different from that of the human eye (e.g., photosynthetic apparatus), results in appreciable errors which in occasions can render the data worthless.

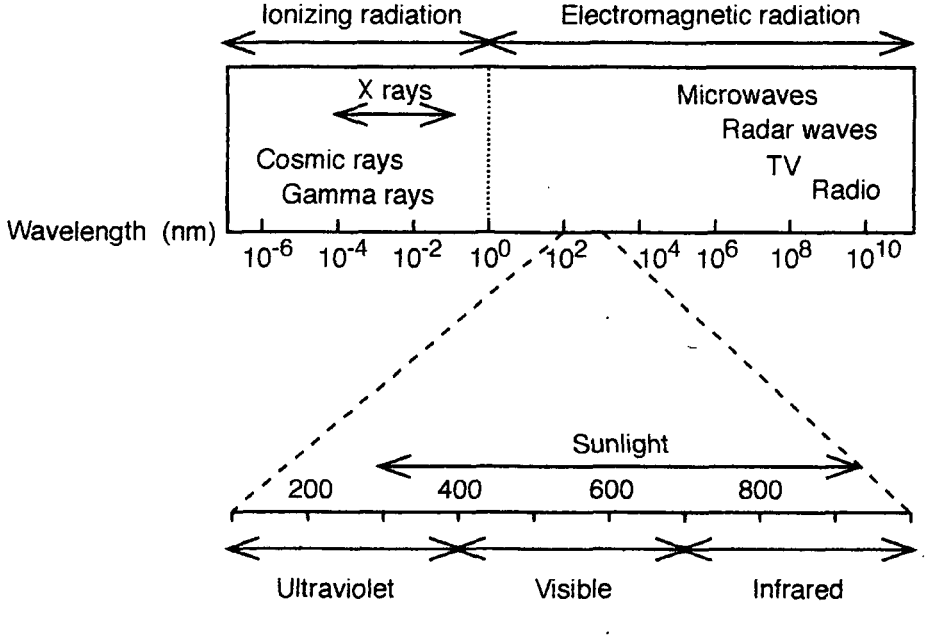

Fig. 1.1. Electromagnetic spectrum.

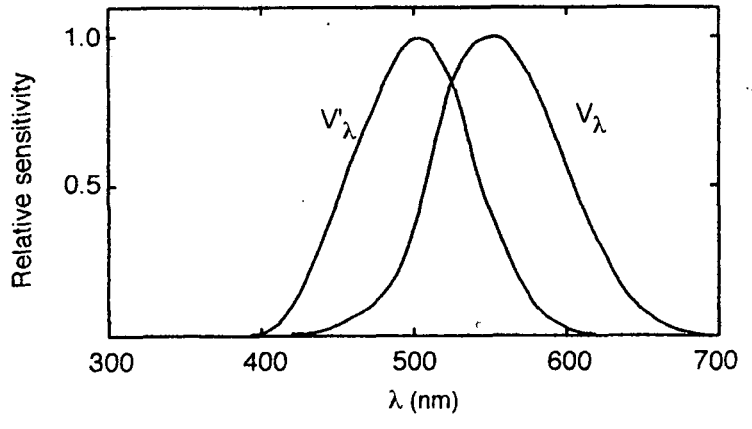

Fig. 1.2. Photopic (V_λ) (light-adapted) and scotopic (V'_λ) (dark-adapted) spectral sensitivity curves for the human eye (reproduced from Arnold 1975).

To avoid these problems, an ideal light meter should provide the same level of sensitivity all over the spectrum. Sensors with this type of response are referred to as radiometers, and their integrated output gives the actual amount of energy emitted in a certain part of the spectrum.

Photometric and radiometric measurements can be used to define four different quantities, which are described in Fig. 1.3 and defined below:

Photometric		Radiometric
Luminous flux	Total energy emitted by a light source per unit time	*Radiant power*
Luminous intensity	Energy flux emitted per unit solid angle	*Radiant intensity*
Illuminance	Energy flux per surface unit	*Irradiance*
Luminance	Amount of energy emitted from a surface	*Radiance*

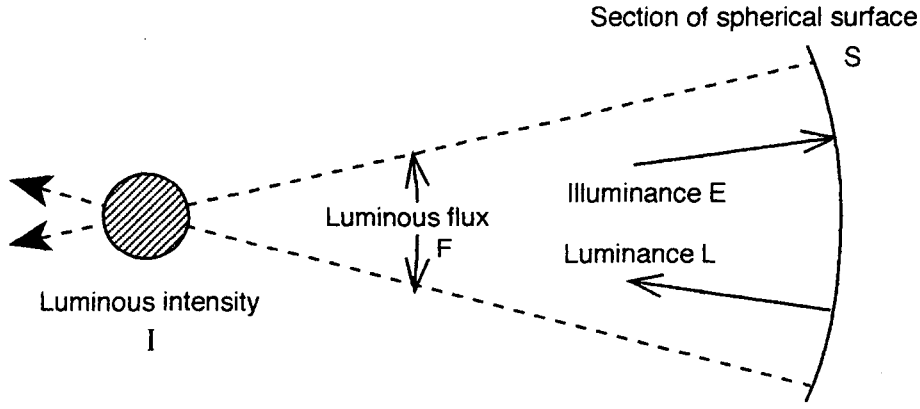

Fig. 1.3. The four basic photometric quantities.

As a consequence of the way in which most light meters are designed (as a flat surface receiving a certain amount of radiation per unit time and per unit surface), the quantities most frequently measured are illuminance and irradiance.

The symbols and units corresponding to each of the quantities defined above are listed in Table 1.1.

Table 1.1. Equivalent photometric and radiometric units.

Photometric quantity	Symbol	Photometric unit	Abbreviation	Radiometric quantity	Radiometric unit
Luminous flux	F, Ø	Lumen	lm (cd.sr)	Radiant power	W
Luminous intensity	I	Candela	cd ($lm.sr^{-1}$)	Radiant intensity	$W.sr^{-1}$
Illuminance	E	Lux	lx ($lm.m^{-2}$)	Irradiance	$W.m^{-2}$
Luminance	L	Nit	nt (cd. m^{-2})	Radiance	W.sr-1.m-2

1.1.2. Implications of quantum theory for light measurement

Light occurs as indivisible units referred to as quanta or photons. However, despite its particulate nature, it also behaves as a wave, with every photon having a certain wavelength λ. The content of energy ε of a photon varies inversely with the wavelength according to the following equation:

$$\varepsilon = h \cdot \frac{c}{\lambda} \qquad (1)$$

in which h is Planck's constant (6.63×10^{-34} J.s.) and c is the speed of light (3×10^{8} $m.s^{-1}$)

Even though two photons with different wavelengths have different energy contents, in the context of photosynthesis and due to the characteristics of the light absorption process, they bring about the same photosynthetic response. As a consequence, photosynthetic activity is more closely related to the flux of quanta than to the flux of energy. Thus, a light meter should be designed to respond to the amount of quanta rather than to the amount of radiant energy. An irradiance meter with these characteristics is commonly referred to as a quanta meter, and unlike a photoelectric cell, is equally sensitive to all quanta in the range between 400 and 700 nm (Fig. 1.4).

A monochromatic energy flux expressed in $J.s^{-1}$ (watts) can readily be converted to $quanta.s^{-1}$, and vice versa, a radiation flux ϕ expressed in $quanta.s^{-1}$, can be converted to W using the following relationship, in which λ is expressed in nm:

$$quanta.s^{-1} = 5.03 \,.\, \phi \,.\, \lambda \,.\, 10^{15} \qquad (2)$$

In the case of radiation covering a broad spectral band, a simple conversion cannot be carried out accurately since the value of λ varies across the spectral band. If the distribution of quanta or energy across the spectrum is known, this conversion can be carried out for a series of relatively narrow bands and then the results can be summed for the whole waveband. Alternatively, an approximate conversion factor from W to $quanta.s^{-1}$ of 2.77×10^{18} $quanta.s^{-1}.W^{-1}$ can be used (Morel and Smith 1974).

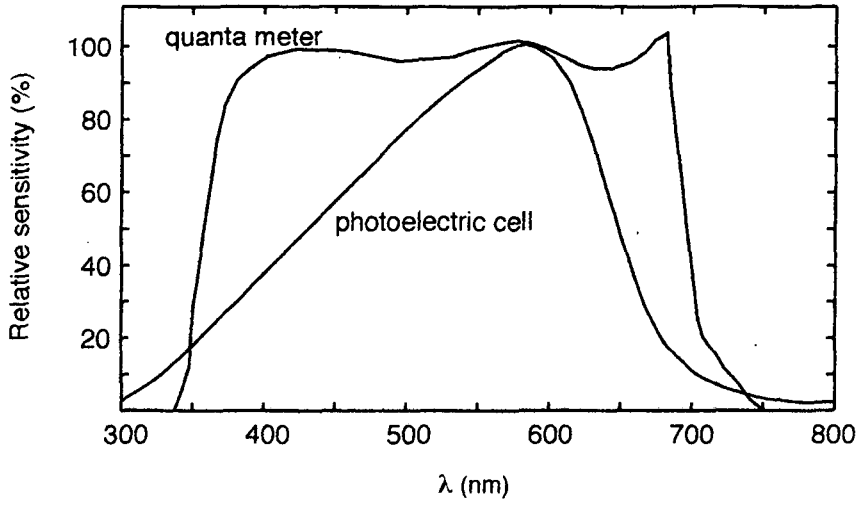

Fig. 1.4. Sensitivity curves of the photoelectric cell and the quanta meter.

Although quanta meters measure the quantity of light available for photosynthesis, they are not sensitive out of the 400-700 nm range, and besides, they do not give information about the amount of light available for every wavelength. In order to describe radiation completely it is therefore necessary to make spectroradiometric measurements, which give the power distribution as a function of the wavelength. An instrument that measures irradiance across the spectrum is known as a spectroradiometer. A spectroradiometer is an irradiance meter in which a variable monochromator is interposed between the collector and the photodetector. In its simplest form the monochromator can consist of an array of colour filters which can be sequentially moved in front of the photodetector. The values of energy can then be converted to units of quanta.

1.2. FACTORS AFFECTING IRRADIANCE IN AQUATIC SYSTEMS

The factors affecting the quantity and the spectral composition of light in aquatic systems fall within either of the following categories (Gibson and Jewson 1984):

1. *External factors,* which affect the irradiance reaching the surface of the aquatic medium as well as the proportion of reflected light, and therefore the total photon flux entering the water body.

2. *Optical properties of the water body*, which determine the extinction of light with depth and the spectral changes caused by the differential absorption of certain wavelengths.

1.2.1. External factors

When solar radiation crosses the atmosphere, the energy of certain wavelengths is preferentially absorbed and dispersed. Ultraviolet energy is absorbed by ozone and oxygen, and infrared energy by water vapour, ozone and carbon dioxide. As a consequence, radiation reaching the Earth's surface is considerably enriched between 400 and 700 nm. However, a sizable amount of energy above 700 nm (i.e. not visible) is also transmitted and can be used by organisms with pigments adequate to absorb at those wavelengths. On the other hand, the amount of solar energy reaching the Earth's surface (Fig. 1.5) varies markedly with latitude, season (solar elevation) (Fig. 1.6), diurnal variation, altitude and atmospheric conditions (Kirk 1983).

When light reaches the surface of an aquatic medium, it does not penetrate totally in the water. A fraction is reflected back and lost, unless it returns to the water after being redispersed from the atmosphere or the surrounding topography. The degree of reflection varies with the incidence angle and the characteristics of the water surface.

1.2.2. Optical properties of the water body

Light entering the water body is altered due to dispersion and absorption. Essentially absorption of light by natural waters can be attributed to four components of the aquatic ecosystem: water itself, dissolved and colloidal coloured organic compounds (gelbstoff or humic substances), detrital and inorganic particulate material (tripton) and phototrophic microorganisms (including phytoplankton and bacteria).

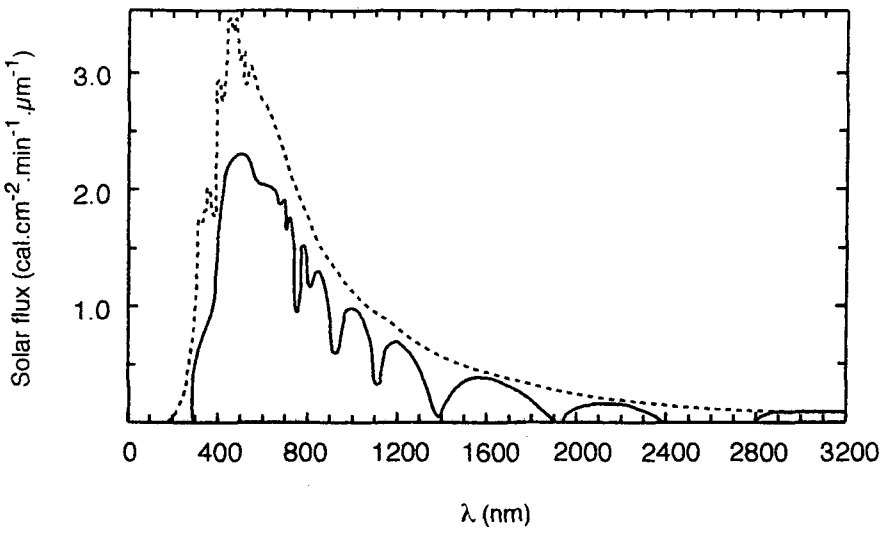

Fig. 1.5. Solar flux outside the atmosphere (dotted line) and solar flux on the earth surface (solid line) (modified from Wetzel 1983).

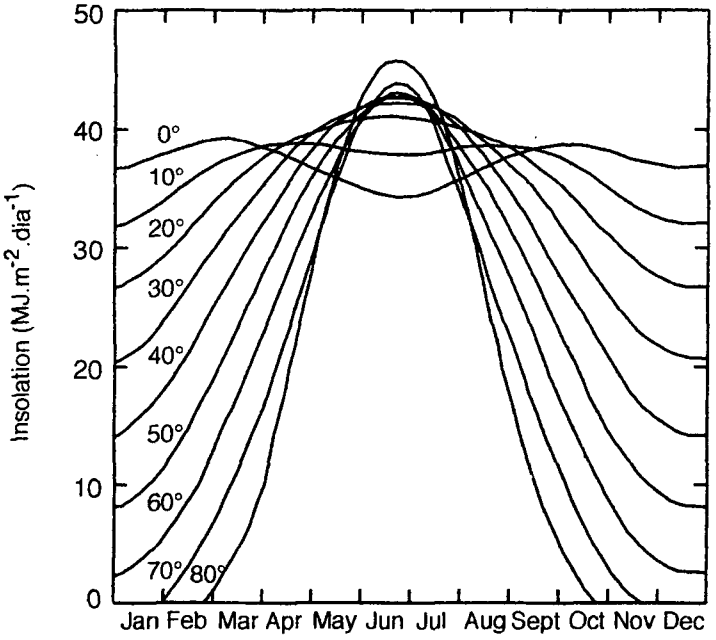

Fig. 1.6. Change in calculated daily insolation (ignoring the influence of the atmosphere) throughout the year at different latitudes in the northern hemisphere. The latitude is indicated above each curve (plotted from data of Kondratyev 1954).

Water absorbs weakly in the blue and green regions of the spectrum, but has a considerable absorption in the red and infrared parts of the spectrum above 550 nm (Fig. 1.7).

Humic substances, an ill defined group of polymeric compounds originated during the decomposition of plant tissues, absorb strongly in the blue part of the spectrum (Fig. 1.7). The extent of their contribution to light extinction varies greatly depending on the type of environment, being rather abundant in lakes with a significant output of detrital plant materials.

The inanimate particulate matter, or tripton, of natural waters contributes little to light extinction in the way of absorption (Fig. 1.7). Its main contribution resides in its scattering properties. The attenuation due to tripton is variable between systems and depends to some extent on the degree of turbulence, since resuspension of bottom material or continual mixing can maintain very high concentrations of inorganic material throughout the year.

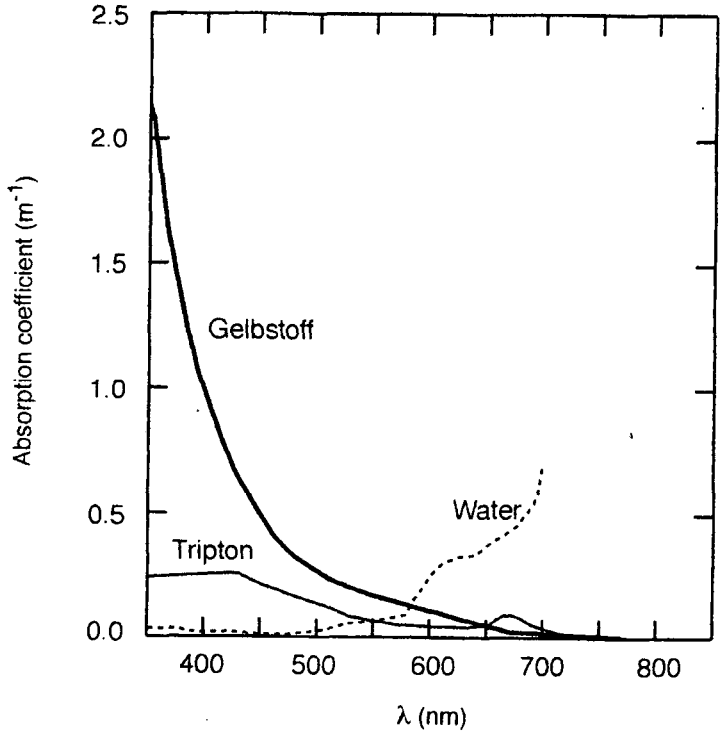

Fig. 1.7. Comparison of the spectral absorption properties of the different fractions in an estuarine water from southeast Australia (Lake King, Victoria). Phytoplankton were present at a level corresponding to 3.6 mg Chl $\underline{a}.m^{-3}$ (from Kirk 1983).

Attenuation of light by phototrophic microorganisms, both algae and bacteria, involves absorption and scattering. In some cases, microorganisms can be responsible for the interception of 80% of the incident light. The amount of light intercepted by this fraction depends not only on the total amounts of photosynthetic pigments present, but also on the size and shape of the cells in which pigments are located.

1.3. ATTENUATION OF IRRADIANCE IN AQUATIC SYSTEMS

In general, as a result of absorption and scattering of the solar flux, the downward irradiance of the light field decreases exponentially with depth according to the following expression:

$$E(z) = E(o) \cdot e^{-k_d \cdot z} \tag{3}$$

or

$$\ln E(z) = -k_d \cdot z + \ln E(o) \tag{4}$$

where $E(z)$ and $E(o)$ are the values of downward irradiance at z m and just below the surface, respectively, and k_d is the vertical attenuation coefficient for downward irradiance.

However, the spectral composition of the light field is also modified depending of the absorption spectrum of the aquatic medium. In non-productive oceanic waters generally, where water itself is the main absorber, blue and green light penetrate deeper, while red light is attenuated much more rapidly (Fig. 1.8A). In productive oceanic waters, in which blue light is preferentially absorbed by phytoplankton pigments, and red light is absorbed by water, green light penetrates deeper. In coastal waters, which contain more yellow substances and phytoplankton, green is again the most penetrating waveband (Fig. 1.8B).

In lakes, the infrared and ultraviolet parts of the spectrum are also rapidly attenuated, while in sediments infrared radiation penetrates deeper. Fig. 1.9 illustrates the modification in the spectral composition of the light field in lake Kinneret (Fig. 1.9A) and in sediments of San Francisco Bay (Fig. 1.9B). In lake Kinneret light is enriched in a narrow band around 550 nm. On the contrary, in the San Francisco Bay sediments, at a depth of 1 mm, most of the light belongs to wavelengths above 850 nm due to scattering of the sediment particles and due to absorption by phototrophic oxygenic microorganisms (Jørgensen and Des Marais 1986, 1988).

Phototrophic organisms capture light by means of their photosynthetic pigments. Their absorption spectrum (Fig. 1.10) can offer information about which pigments are present and allows us to know the extent to what an organism is able to harvest light in a certain environment.

Therefore, in aquatic environments, where purple and green anoxygenic bacteria can develop, and where only green and blue-green light arrives, carotenes, and not bacteriochlorophylls, play a crucial role in the capture of light. In sediments, on the contrary, where the infrared part of the spectrum penetrates, bacteriochlorophylls play a more important role.

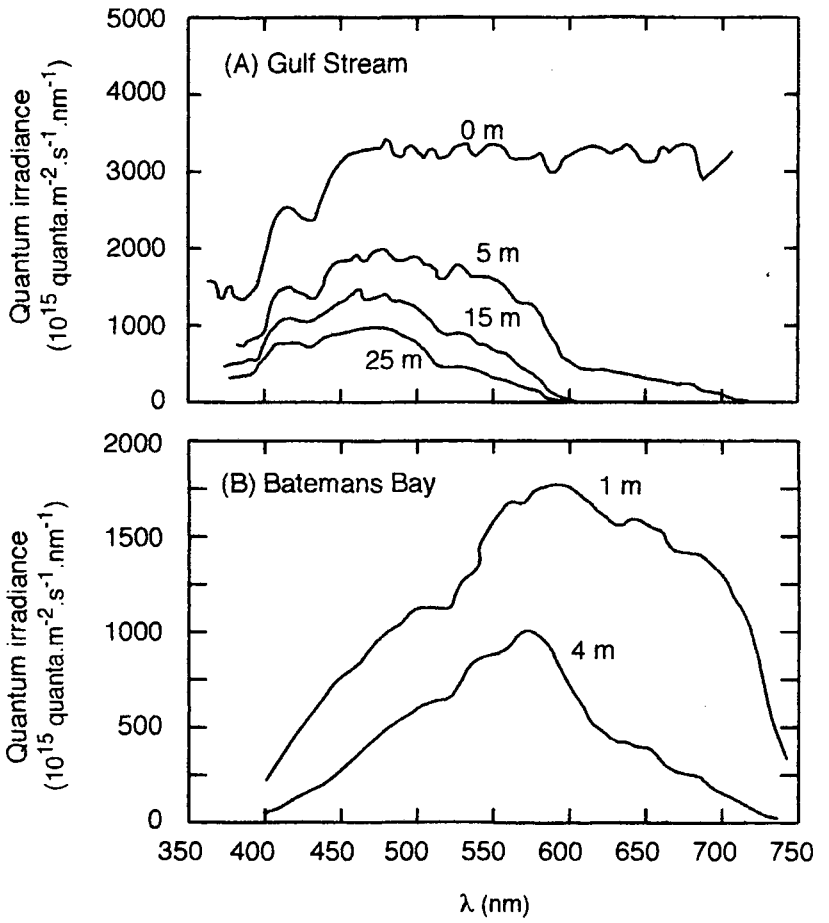

Fig. 1.8. Spectral distribution of downward irradiance in the Gulf Stream (Atlantic Ocean) off the Bahamas Islands, a non-productive oceanic water (A), and in Batemans Bay, Australia, a coastal/estuarine oceanic water (B) (modified from Kirk 1983).

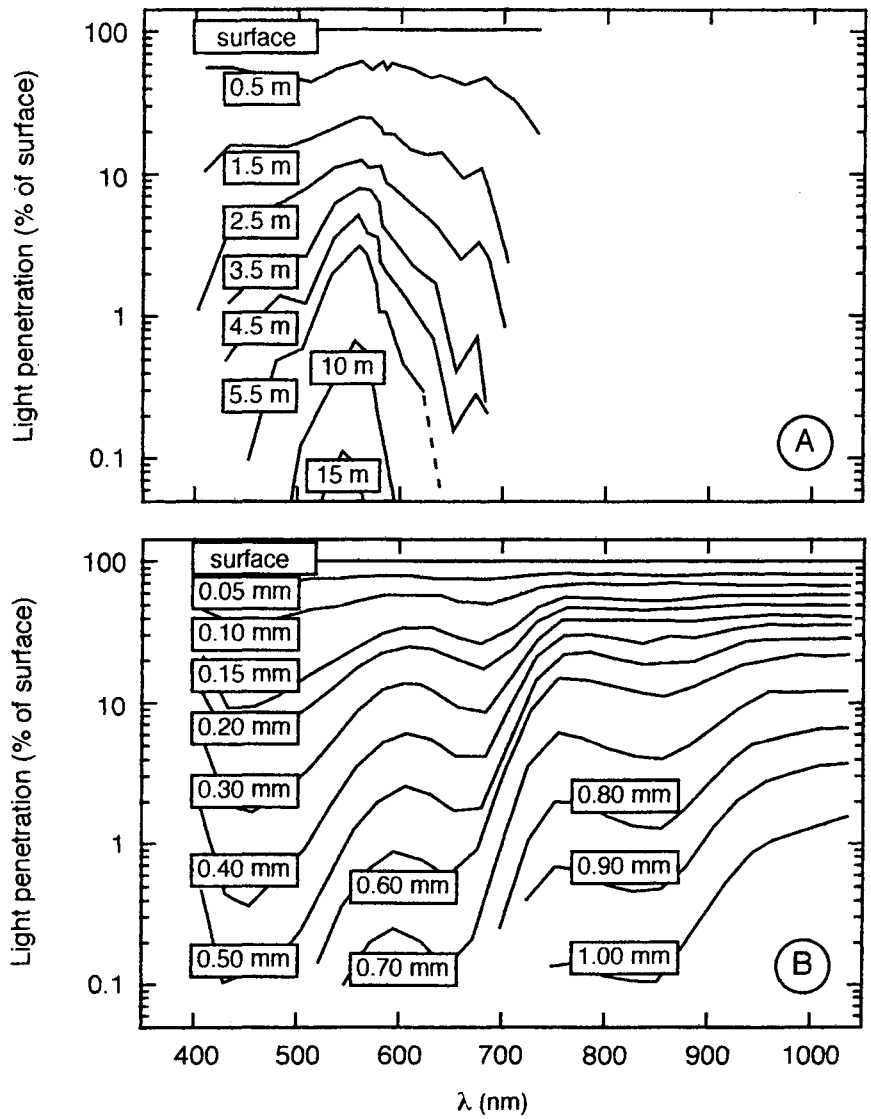

Fig. 1.9. Light penetration at different wavelengths in a lake and a sediment. Fig. A shows the spectral composition of light at different depths in Lake Kinneret (modified from Dubinsky and Berman 1979). Fig. B indicates light penetration in sediments of San Francisco Bay (Jørgensen and Des Marais 1986).

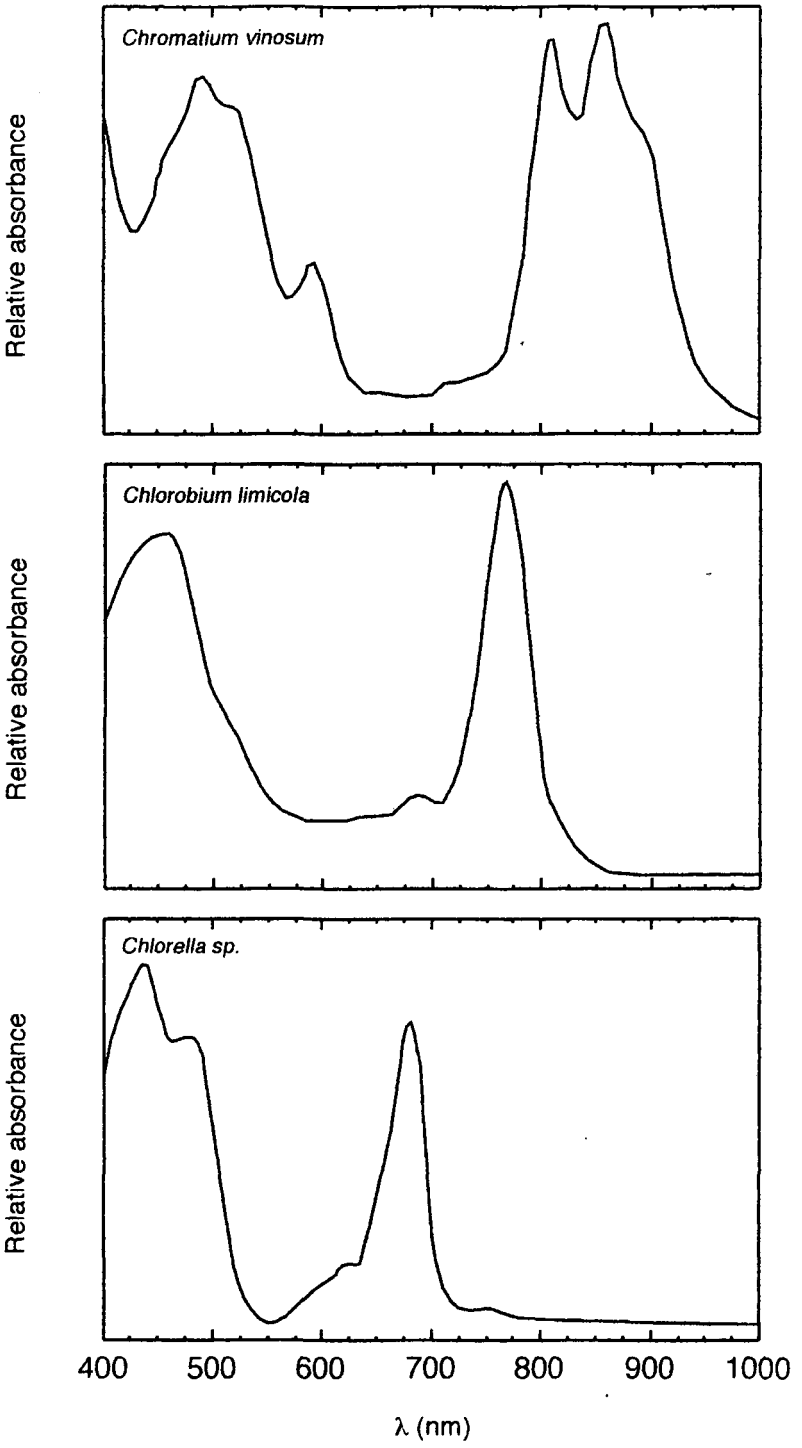

Fig. 1.10. *In vivo* absorption spectra of a purple sulfur photosynthetic bacteria (*Chromatium vinosum*), a green sulfur photosynthetic bacteria (*Chlorobium limicola*), and an algae (*Chlorella*).

1.4. ABSORPTION OF LIGHT BY CULTURES OF PHOTOTROPHIC MICROORGANISMS

The light climate inside a culture vessel varies widely due to absorption and scattering by the cell suspension. Therefore, irradiance is not equally distributed and the light field inside the culture is heterogeneous. As a consequence, laboratory experiments which use incident irradiance as a measurement of the amount of light available for growth do not reflect the actual amount of energy received by the cells. Several authors, in an attempt to circumvent this problem, introduced the concept of mean irradiance (Rabe and Benoit 1962, Göbel 1978, Van Liere et al 1978), which represents the average irradiance cells are exposed to when traveling within the stirred culture. Some of the studies measure light irradiance at the front and back of the culture vessel (Van Liere and Walsby 1982), while others average the intensity of the light field at different positions inside the culture vessel (Van Liere et al 1978). The first of these two approaches may result inaccurate, because it does not take into account scattering and light may leave the culture vessel in directions other than opposite to the light source. The second approach, although accurate, requires special equipment for the determination of the light field at different positions within the culture vessel and may interfere with the culture operation.

Van Liere and Mur (1979) introduced a new concept, the specific light uptake rate (q_e), which results from the measurement of the light absorbed by the culture divided by the total biomass, and constitutes a better indicator of the level of light supply. This variable provides an indication of the average rate of light absorbed per cell, and it can be directly related to the biological activity through a yield coefficient (Van Liere and Mur 1978, Pirt et al 1980, Evers 1991). In order to measure the energy absorbed by the culture, several procedures have been reported. Some authors (Göbel 1978, Gons and Mur 1980) measured light absorption by subtracting the average output irradiance from the average incident irradiance. Others (Evers 1991) utilized a theoretical model, which determined the light output as a function of the incident irradiance, the biomass of the culture, the geometry of the vessel, and the extinction coefficient of the organisms, but did not take into account the role of scattering. A new procedure has been developed by Sánchez and Mas (1996, Chapter 2), in which light absorption is determined as the difference between the integrated light output of a blank vessel containing only growth medium and the light output of the same vessel containing the suspension of microorganims. This procedure characterizes the outcoming light field, takes into consideration losses due to scattering, and allows to determine the optical properties of the suspension (Chapter 3).

1.5. PHYSIOLOGICAL RESPONSES TO CHANGES IN LIGHT QUALITY AND LIGHT QUANTITY

The quantity and the spectral distribution of the light field change markedly with depth and with the optical properties of the water body, as we have seen above. Furthermore, light availability varies with time, both within the day and with the seasons during the course of the year. Phototrophic organisms must be able to cope with these variations and, in fact, several strategies have evolved which allow their adaptation to a changing light climate.

1.5.1. Responses to light quality

Since the last century, it has been a common belief that the most important factor determining the zonation of phototrophic organisms was the variation of the light field with depth. This theory has taken two forms. According to the *chromatic adaptation* theory of Engelmann (1883) it was the varying spectral distribution of the light with depth which determined this distribution; those organisms with absorption bands corresponding to the spectral distribution of light would photosynthesize more effectively and therefore would be more abundant. On the other hand, Berthold (1882) and Oltmanns (1892) proposed that it was the variation of irradiance with depth which determined the distribution of the different types of organisms. Harder (1923) suggested that both theories were involved.

There are some evidences in favour of the chromatic adaptation theory. Some cyanobacteria are able to change their phycoerythrin or phycocyanin content in response to spectral changes of light (Bogorad 1975, Tandeau de Marsac 1977). Chromatic adaptation is also found in benthic marine algae (Levring 1966). However, the adaptive significance of such changes is not clear, and suggested advantages have not been clearly demonstrated in nature. What appears to be chromatic adaptation may be a response to light quantity (Dring 1981). Furthermore, pigment shifts in natural populations may be due to factors other than the spectral composition of light, such as nutrient starvation (Jewson 1976).

1.5.2. Responses to light quantity

Changes in photosynthetic characteristics

The photosynthetic response of an organism can be examined taking as a basis the photosynthesis-irradiance relationship, which adjusts to a saturation curve whose mathematical expression has originated a number of models (Smith 1936, Rabinowitch 1951, Van Oorschot

1955, Steele 1962, Vollenweider 1965, Bannister 1974, Jassby and Platt 1976, Iwakuma and Yasuno 1983). These models define some parameters like the photosynthetic capacity (P_{max}) which gives the maximum rate of photosynthesis, and the photosynthetic efficiency (α) which is the initial slope of the photosynthesis-irradiance plot. Talling (1957) also defined the parameter I_k as the irradiance at which an extension of the initial slope α intersects P_{max}, and which gives an approximate idea of the irradiance above which photosynthesis saturates.

The photosynthetic efficiency α depends on the specific content of pigments (Kirk 1983, Dubinsky et al 1995). When irradiance falls below the saturating value for photosynthesis, cell pigment usually increases and so does α. This increase in pigment content can reflect either an increase in the total number of reaction centres (PSU or photosynthetic units) and their associated antennal pigments or an increase in the antennal pigment associated with a constant number of reaction centres. An increase in the number of PSU would result in a higher photosynthetic capacity. This strategy is characteristic of some species of marine phytoplankton, like *Scenedesmus* (Senger and Fleischhacker 1978, Falkowski and Owens 1980), and can also be found in organisms like *Chlorella* and *Dunaliella*, which grow in high-light environments. On the other hand, changes in the size of PSU would be reflected in variations in α. Variations of α with a constant P_{max} could be important in low-light environments. This type of response has been found in the cyanobacterium *Oscillatoria* (Foy and Gibson 1982, Post et al 1985), which predominates in waters with a high content of phytoplankton and a low irradiance. Other organisms, like *Microcystis* (Zevenboom and Mur 1984), experiment changes in α and P_{max}.

Changes in pigment content

As mentioned above, when photosynthesis is light-limited, the specific pigment content can increase as an attempt to improve the light harvesting capacity of the organism. This phenomenon has been observed in algae (Myers 1946, Cook 1963, Beale and Appleman 1971, Gons and Mur 1975, Yoder 1979), in cyanobacteria (Myers and Kratz 1955, van Liere and Mur 1978) and in other photosynthetic bacteria (Sistrom 1962, Holt and Mar 1965, Uffen 1973, Trentini and Starr 1967, Broch-Due et al 1978, van Gemerden 1980).

Some organisms respond to light limitation by changing their chlorophyll-carotenoids ratio (Halldal 1970). This phenomenon has been observed in the marine dinoflagelate *Glenodinium* (Prézelin 1976, 1981), which increases the pyridine/Chl a ratio, and in some cyanobacteria (increase of the phycocianin/Chl a ratio) (Foy and Gibson 1982, Post et al 1985). In the anoxygenic photosynthetic bacterium *Chromatium minus* the carotenoid/Bchl a ratio has been observed to increase in relation with the specific content of Bchl a (Montesinos 1982). Although the adaptive meaning of these physiological changes has not been well established, they are

probably related to the fact that in aquatic environments a lower irradiance is usually related to a modified spectrum (Fig. 1.9). Since accessory pigments absorb at wavebands different from those of chlorophylls, these changes can actually be considered as a mechanism to improve light harvesting in low-light environments.

1.6. OUTLINE OF THE THESIS

The present study was aimed to characterize the physiological response of the purple sulfur bacterium *Chromatium vinosum* to light limitation. In order to provide a constant light climate and controlled environmental conditions, the experiments had to be carried out using a continuous culture. In particular, two aspects were addressed. First, the capacity of the organism to adapt its photosynthetic response when challenged with limiting irradiances (Chapter 5). Second, the possible existence of changes in the relative content of storage inclusions which could contribute to an increased survival under light limitation (Chapter 6).

However, to cover these two points, a number of technical questions had to be previously solved. First, current measurements of incident irradiance at the surface of the culture vessel were not suitable to describe the amount of energy available for photosynthesis. Light available to individual organisms depends not only on the external light supply but also in the amount of biomass sharing this supply. Therefore, throughout the work, light availability has been described as the specific rate of light uptake (q_e), expressed in $\mu E.mg^{-1}protein.h^{-1}$, and measured as the amount of light absorbed by the culture, divided by the biomass present within the culture. The methodology for measuring q_e is described in Chapter 2. A detailed study of how q_e is affected by variables such as the biomass of the culture, the size of the culture vessel, and the presence of refractile structures, is presented in Chapter 3.

Chapter 4 describes the continuous culture utilized in these experiments, which offers a number of advantages for the growth of sulfide-oxidizing phototrophs. Its design allows the establishment of steady states in which the dilution rate depends exclusively on the conditions of illumination imposed. At the same time, the culture is able to maintain a constant concentration of hydrogen sulfide inside the culture vessel. Thus, the physiological events observed are not a consequence of collateral effects due to the excess or limitation of substrate.

Finally, Chapter 7 presents an overview of the results together with a general discussion of the main aspects of the work.

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CHAPTER 2

MEASUREMENT OF LIGHT ABSORPTION AND DETERMINATION OF THE SPECIFIC RATE OF LIGHT UPTAKE (q_e) IN CULTURES OF PHOTOTROPHIC MICROORGANISMS

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CHAPTER 2

MEASUREMENT OF LIGHT ABSORPTION AND DETERMINATION OF THE SPECIFIC RATE OF LIGHT UPTAKE (q_e) IN CULTURES OF PHOTOTROPHIC MICROORGANISMS

This article describes a novel method for the measurement of light absorption by cultures of phototrophic microorganisms. The rate of light absorption is calculated as the difference between the rate of light output from a culture containing cells and the rate of light output from a culture containing only growth medium. The specific rate of light uptake is calculated by dividing the rate of light absorption by the total biomass present in the culture. Application of the method to several case studies shows that light output from a culture varies widely depending on the absorption and scattering characteristics of the suspension.

INTRODUCTION

Development of phototrophic microorganisms in laboratory cultures is strongly conditioned by their light supply. As an indication of the amount of energy available for growth, incident irradiance at the surface of the culture has been often used, overlooking the fact that light available to individual organisms is determined not only by the external light supply but also by the amount of biomass sharing this light supply. In early attempts to find an appropriate variable to describe light availability, several authors (Rabe and Benoit 1962, Göbel 1978, Van Liere et al 1978) introduced the concept of mean irradiance, which referred to the average irradiance the cell might be experiencing when circulating within a well-mixed culture. This concept was further used and modified by Van Liere and Mur (1979), who suggested a new variable, the specific light uptake rate (q_e). This was calculated as the total amount of energy absorbed by the culture divided by the total biomass present in the culture. Determination of the energy absorbed by the culture was at the time, and still is, far from straightforward, and several procedures have been proposed for its measurement. In some of them (Göbel 1978, Gons and Mur 1980), light output from the culture is measured and subtracted from the average irradiance entering the culture. In others (Evers 1991), the irradiance output from the culture is estimated from the average incident irradiance, the biomass of the culture, the size of the culture vessel, and the extinction coefficient of the organisms. This approach uses a theoretical model which takes into account the effect of self shading but does not consider the role of scattering. As shown later in

this paper, this constitutes an important shortcoming, which in some cases cannot be disregarded without incurring serious mistakes. Although a theoretical model predicting the optical behaviour of cultures of microalgae or phototrophic bacteria seems quite feasible, we felt compelled to develop a method to measure the amount of light absorbed by a culture and to determine the specific rate of light uptake.

The procedure we have developed originated from the ideas put forward by Van Liere and Mur (1979), who used the difference between the average light output and the average incident irradiance at the surface of the culture vessel as a measure of the energy absorbed. In our procedure, light absorption is estimated as the difference between the integrated light output from the surface of a culture vessel when it contains an active culture and the light output from a blank consisting of the same vessel filled exclusively with culture medium. Because the same experimental setup is used in both cases, differences in the integrated light output can be attributed only to absorption by the cells. The procedure does not interfere with regular culture operation; also, because output is measured at several locations (as many as 52) at the surface of the vessel, losses due to scattering are also taken into account. Furthemore, the discrete measurements needed to calculate the light output provide valuable information about the exiting light field, which can be related to the reflecting and scattering properties of the suspension. This procedure has been tested in three different situations: (i) with only growth medium, (ii) with a dense culture of a photosynthetic microorganism, and (iii) with a dense culture of the same organism but loaded with refractile inclusions of elemental sulfur. The reproducibility of the procedure was checked by a series of determinations carried out on separate days on a steady-state continuous culture of *Chromatium vinosum*. The results indicate how this method can be used to estimate the rate at which light is absorbed by a culture of microorganisms and to provide some information about the optical properties of the suspension.

MATERIALS AND METHODS

Organisms and growth conditions. Experiments were performed with the purple sulfur bacterium *C. vinosum* DSM 185. The organism was grown at 25°C in an all-glass cylindrical culture vessel (1.4 liters) in a complex mineral medium which contained carbonate as the only carbon source and hydrogen sulfide as electron donor (Mas and Van Gemerden 1987). The culture, placed in a dark room, was illuminated from one side using two 60-W incandescent light bulbs. Incident irradiance at the surface of the culture vessel was set at 220 $\mu E.m^{-2}.s^{-1}$. Irradiance was measured with a quanta meter (no. Li-185B; LiCor Inc.). Experiments requiring growth in continuous culture were carried out in a glass vessel with no headspace, using the

medium cited above, with a 2 mM concentration of sulfide in the reservoir. In these experiments dilution rate was set at 0.12 h^{-1} and the pH was kept at 7.2 by means of a pH controller. As in the previous case, the culture was placed in a dark room and illumination was provided from one side using an incident irradiance of 114 $\mu E.m^{-2}.s^{-1}$.

Sampling and analyses. For the analysis of cell components (sulfur and proteins), 10-ml samples were centrifuged (4400 x g 10 min), supernatant liquids were discarded and the resulting pellets were frozen and kept at -20°C until analyzed. Sulfur was estimated spectrophotometrically at 770 and 265 nm in methanol extracts of whole cells. Concentrations were calculated with the extinction coefficients provided by Stal et al. (1984). Protein was determined with the Folin-Phenol reagent (Lowry et al 1951) after treatment of the pellets with methanol (to remove sulfur, which interferes strongly with the assay) and extraction in 1 N NaOH at 100 °C (Herbert et al 1971). Bovine serum albumin (BSA) was used as the standard.

Determination of light output and calculation of the specific rate of light uptake. Light output was determined by measuring the photon flux ($\mu E.m^{-2}.s^{-1}$) exiting the surface of the culture vessel at different angles (0, 45, 90, 112.5, 135, 157, 180, 202.5, 225, 247.5, 270, 315, 360°) relative to the light source. Measurements were carried out with the light meter positioned perpendicularly to the surface, and each set of measurements was performed at three levels which corresponded to the top, central and bottom parts of the vessel (Fig. 2.1). Since differences between levels were small, the values were averaged and the results were plotted both, as polar plots and using Cartesian x-y layouts. The resulting curves were integrated between 0 and 360° and divided by 360°, thus providing the average photon flux exiting the culture vessel through the sides. Similar determinations at the lid and bottom of the vessel (an average of eight measurements distributed all over each of the surfaces) were also performed to calculate the average light flux exiting through the top and bottom of the culture. Both values (lateral and top plus bottom) were then multiplied by their respective areas (lateral surface and top plus bottom surfaces) yielding the overall light output. The overall light output from the culture was subtracted from the output determined in a blank containing exclusively medium without cells. This difference was then divided by the total biomass present in the culture (expressed as protein), yielding the specific rate of light uptake in $\mu E.mg^{-1}$ protein.h^{-1}.

The entire procedure can be summarized and expressed as the following equation:

$$q_e = \frac{\left(\overline{E}_{L_{blank}} - \overline{E}_{L_{culture}}\right) \cdot A_L + \left(\overline{E}_{T+B_{blank}} - \overline{E}_{T+B_{culture}}\right) \cdot A_{T+B}}{V \cdot C}$$

where $\overline{E}_{L_{blank}}$ and $\overline{E}_{T+B_{blank}}$ are the average lateral (L) and top-plus-bottom light outputs ($T + B$) (in $\mu E.m^{-2}.s^{-1}$) from a vessel containing only culture medium, $\overline{E}_{L_{culture}}$ and $\overline{E}_{T+B_{culture}}$ are the average lateral (L) and top-plus-bottom ($T + B$) light outputs (in $\mu E.m^{-2}.s^{-1}$) from a vessel containing both bacteria and medium (in $\mu E.m^{-2}.s^{-1}$), A_L and A_{T+B} are the areas of the lateral and the top-plus-bottom surfaces (in m^2), V is the volume of the culture (in L), and C is the biomass concentration (in mg protein.L^{-1}).

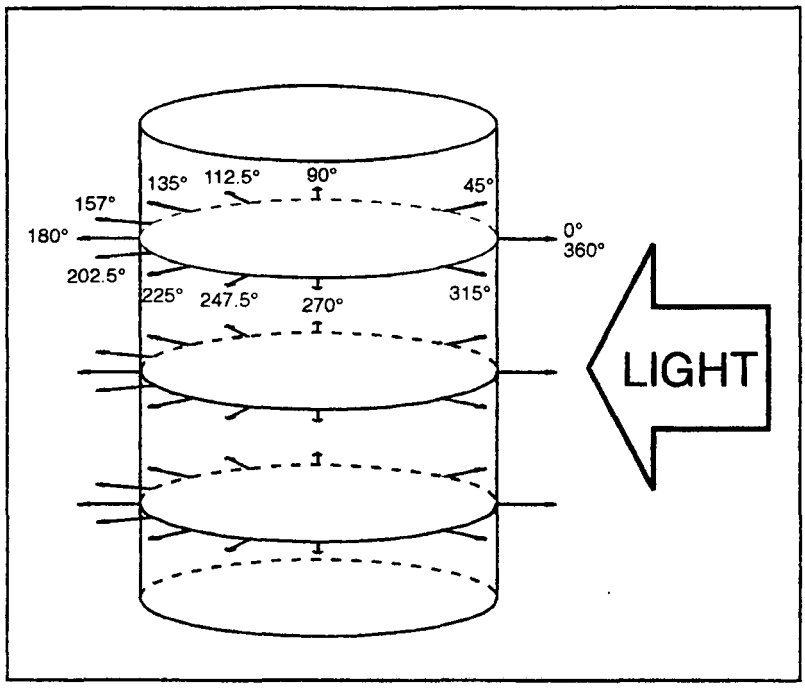

Fig. 2.1. Diagram summarizing the procedure followed to determine light output from the culture vessel. Measurements were taken at several angles relative to the light source (0, 45, 90, 112.5, 135, 157, 180, 202.5, 225, 247.5, 270, 315, 360°). Each set of measurements was repeated at three levels which corresponded to the top, central and bottom parts of the culture vessel. Vertical light output (not shown) was also determined at eight different positions at the lid and the bottom of the vessel.

RESULTS AND DISCUSSION

Light output from the culture was measured at different angles as described above. The results are plotted in Fig. 2.2 as a function of the output angle, for three different situations. The first, represented in Fig. 2.2A, corresponds to a blank in which the vessel contained only growth medium. In this case, there were no particles in suspension and therefore scattering was absent and absorption was minimal. Light exited the vessel at between 135 and 225°, with a maximum output at 180°. Integration of the light output (area underneath the curve) divided by 360° provides the average irradiance leaving the culture. This value, 17.2 $\mu E.m^{-2}.s^{-1}$, corresponds to the maximum possible output from the culture under the current conditions of illumination and thus provides a reference against which all other measurements will be compared. In the second case (Fig. 2.2B) the culture contained a dense suspension (116 mg protein.L^{-1}) of sulfur-depleted *C. vinosum*. The average light output from this culture was 4.6 $\mu E.m^{-2}.s^{-1}$, much lower than the value determined above, as a consequence of absorption by the cells. The angular distribution of the light output differs remarkably from the previous situation, since outward irradiance is much higher at 135 and at 225° than at 180°, probably a consequence of scattering by the cell suspension.

Analysis of a third situation (Fig. 2.2C) yielded still different results. In this case, the suspension of *C. vinosum* (at the same concentration as above) had been fed hydrogen sulfide (final concentration, 1.8 mM) and incubated for 3 h in the light until the sulfide was depleted. As a consequence, the cells had accumulated up to 17 μmol of elemental sulfur (S°) per mg protein (approx. 20% of the cellular dry weight). The distribution of the light output, as can be seen in Fig. 2.2C, was rather different from the distribution found when cells did not contain sulfur. Light was predominantly reflected, and output was maximum between 270 and 90°. A similar phenomenon has been qualitatively described in suspensions of cyanobacteria containing gas vesicles (Van Liere and Walsby 1982). The average output irradiance calculated from the data in Fig. 2.2C was again much lower than the average output irradiance from the blank in Fig. 2.2A (6.12 $\mu E.m^{-2}.s^{-1}$). The difference, as before, can only be attributed to absorption.

The data shown in Fig. 2.2 correspond only to the light output through the sides of the culture vessel. The procedure described in Materials and Methods, however, also requires the determination of light outputs through the top and the bottom of the culture. In our experiment, this could be readily accomplished because the lid and bottom of the culture were made entirely of glass. In many cases, though, culture reactors are fitted with metal lids, which do not allow measurements of light losses through the top of the culture. In these cases, application of the procedure described above might prove difficult. To estimate the magnitude of the error incurred in disregarding vertical outputs, we carried out a series of determinations which

compare the rates of light absorption and the specific rates of light uptake in cultures with different characteristics depending on whether vertical outputs were included.

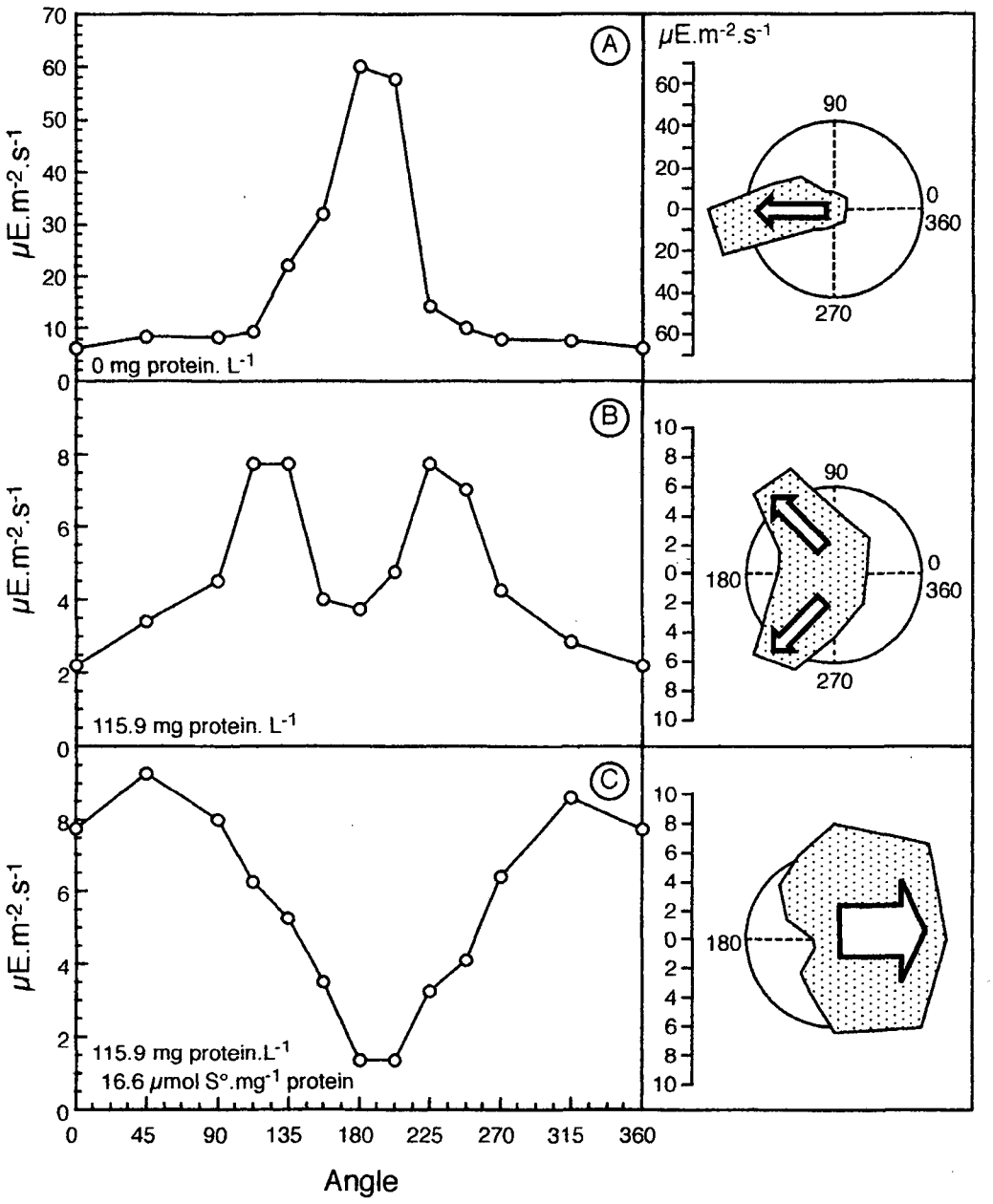

Fig. 2.2. Light output from the culture vessel under different conditions. The left side of the figure shows x-y plots of the irradiance as a function of the angle of output. (A) Light output from a culture vessel filled with medium (blank). (B) Vessel filled with a dense sulfur-depleted culture of *C. vinosum*. (C) Culture of *C. vinosum* containing refractile intracellular sulfur globules. The right side of the figure shows polar plots of the same data, in which the distance of the points on the polygon from the center of the circle represents the magnitude of the light output. The thick arrows in these plots emphasize the preferred directions at which light exits the culture.

These measurements were carried out with dense and diluted cultures of *C. vinosum* with different sulfur contents. The results are summarized in Table 2.1. When the culture vessel contained only medium, as much as a 92.6% of the output exited through the sides and only 7.4% escaped through the top and bottom. The situation was different when cells were present. In general, light output through the sides decreased and a larger amount left through the top and bottom; however, the magnitude of these changes seemed to depend on the biomass. Thus, in a diluted culture containing 6.8 mg protein.L^{-1} the variation was small (lateral output, 92.6%; top-plus-bottom, 7.4%), while in a dense culture containing 89 mg protein.L^{-1}, 78.5 % of the light output occurred through the sides and 21.5% occurred through the top and bottom. The presence of refractile structures such as sulfur inclusions also increased light losses through the top and bottom. Therefore, in a diluted culture (6.8 mg protein.L^{-1}) containing 14 μmol S°.mg $^{-1}$ protein, light output through the top and bottom increased up to 21.9%, while in a dense culture (89 mg protein.L^{-1}) containing an equivalent amount of sulfur, this value increased to 27.3%.

Table 2.1. Percentage of light output through the sides and through the top and bottom boundaries of a culture of *C. vinosum* as a function of the biomass and sulfur contents[a].

Area of vessel	% of light output in the presence of:				
	Growth medium alone	6.8 mg protein.L^{-1}		89 mg protein.L^{-1}	
		-S°	+S°	-S°	+S°
Sides	92.6	92.6	81.4	78.5	72.7
Top and Bottom	7.4	7.4	18.6	21.5	27.3

[a]-S°, 0 μmol S°.mg^{-1} protein; +S°, 14 μmol S°.mg^{-1} protein

The error due to unaccounted losses through top and bottom of vessels decreases somewhat due to partial reflection of the light. The importance of this reflection depends on the type of metal. For stainless steel, and taking into account published reflection coefficients (Weast 1986-1987), 55% of the light impinging on the internal side of the lid and bottom would be reflected back to the culture vessel. Therefore, light losses would be approximately half of the values estimated above.

While these results show that an important fraction of the exiting light is not taken into account when light outputs through the top and the bottom of the vessel are not considered, differences are less evident when q_e is calculated. The values of the specific rates of light uptake for the cultures mentioned above are in Table 2.2. The error caused by calculating q_e without taking into account top and bottom losses is very small (approx. 2%) in dense cultures, no matter whether the culture contains sulfur. Larger effects are observed in diluted cultures. There, the effect of omitting top and bottom outputs is relatively small (7%) when cultures do not contain sulfur, but becomes much more important in the sulfur-containing cultures (43%). In the presence of metal surfaces, as pointed out above, light would be partially reflected into the culture, thus decreasing the magnitude of these errors. These results indicate that the measurement of q_e could actually be simplified to some extent by measuring exclusively lateral outputs; however, this simplification should preferably be applied in dense cultures, especially in cases in which no refractile structures such as sulfur inclusions are present.

Table 2.2. Specific rates of light uptake in cultures of *C. vinosum* determined considering only lateral light output, or considering both lateral and vertical outputs[a]

Output direction	Rate of light uptake ($\mu E.mg^{-1}$ protein.h^{-1}) in the presence of[b]:			
	6.8 mg protein.L^{-1}		89 mg protein.L^{-1}	
	-S°	+S°	-S°	+S°
Lateral	90.96	201.7	41.9	40.5
Lateral + vertical	98.26	140.4	42.9	39.6

[a] Determinations have been made in cultures differing in both biomass and sulfur content.
[b] -S°, 0 μmol S°.mg^{-1} protein; +S°, 14 μmol S°.mg^{-1} protein.

Testing this method for reproducibility required carrying out independent determinations with different cultures of identical characteristics. Since the outcome of the measurements depended to a large extent on the biomass and sulfur content of the cultures, it was critical to ensure that the concentration, composition, and physiological state of the cells did not change between measurements. To do that, *C. vinosum* was inoculated into a continuous culture and allowed to reach steady state under anoxic conditions. Once the culture was in steady state, biomass and cell composition were constant for as long as the dilution rate remained

unchanged. During this period, light output was determined at three different times (on separate days) and the data obtained were used to calculate the specific rates of light uptake.

The results obtained during these measurements have been presented in Fig. 2.3, and show a remarkable agreement, with virtually identical light distributions for the three cases. Both the rates of light output (0.61, 0.65 and 0.64 $\mu E.s^{-1}$) and the specific rates of light uptake calculated from these data (33.9, 28.8 and 30.6 $\mu E.mg^{-1}.protein.h^{-1}$) were very similar on all the occasions, when they were measured, thus indicating a good reproducibility. Although these data do not allow for a detailed analysis of precision and accuracy, they indicate the degree of variability which can be expected from the method.

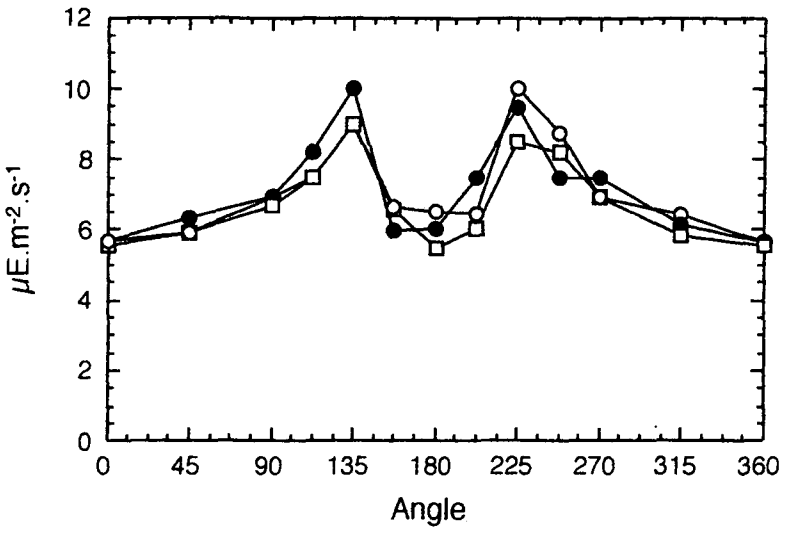

Fig. 2.3. Serial measurements of light output from a steady-state continuous culture of *C. vinosum*. Measurements were carried out at 24-h intervals. Differences between the curves illustrate the variability of the technique.

Determination of the specific rate of light uptake by this procedure relies on the assumption that light absorbed by the organisms falls within the range of sensitivity of the light meter used. However, this is not always the case. In the determinations described above, the light meter measured photon fluxes between 400 and 700 nm whereas the emission spectrum of the incandescent light source used had a strong infrared component. For algae, which absorb light only below 700 nm, these differences would not affect the determinations. For anoxygenic phototrophs, however, the results would be biased by the fact that these organisms are able to absorb light around 800 to 900 nm. Thus, utilization of a quanta meter would allow for determination of the amount of light absorbed between 400 and 700 nm but light absorbed in the infrared would not be taken into account. The result would be an underestimation of the

actual amount of energy absorbed by the organisms. Although this discrepancy does not invalidate the method described as a procedure to standardize light conditions in the laboratory, it is extremely important when trying to compare the experimental results with field observations from aquatic environments in which irradiance falls mainly in the 400 to 700 nm interval. The problem can be avoided either by using a light source that mimics the spectrum found in aquatic environments, or using a spectroradiometer to carry out light measurements in the laboratory. Utilization of a spectroradiometer would constitute the most accurate solution by far, although the amount of data generated would make routine calculation rather cumbersome.

Determination of the specific rate of light uptake requires an accurate characterization of the exiting light field. If light output in growing cultures occurred following the same distribution observed in a blank containing only culture medium, a simple measurement of the light output at 180° (opposite to the light source) would simplify the calculation of q_e. The results presented above, however, show rather clearly that this is not the case and that measurement of the light output only at 180° would be inappropriate, specially in situations such as the one shown in Fig. 2.2C, in which a large fraction of the light output occurs through the front part of the culture vessel (approximately a 70% of the light output). In this case, the light output at 180° constitutes a minor portion of the total exiting light. Further studies on the factors affecting both absorption and scattering of light by cultures of phototrophic microorganisms will no doubt contribute to our understanding and will enable us to predict the behaviour of illuminated cultures.

ACKNOWLEDGMENTS

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CHAPTER 3

ABSORPTION OF LIGHT BY SUSPENSIONS OF PHOTOTROPHIC MICROORGANISMS: EFFECTS OF SCATTERING, CELL CONCENTRATION AND SIZE OF THE CULTURE VESSEL

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CHAPTER 3

ABSORPTION OF LIGHT BY SUSPENSIONS OF PHOTOTROPHIC MICROORGANISMS: EFFECTS OF SCATTERING, CELL CONCENTRATION AND SIZE OF THE CULTURE VESSEL.

This paper analyzes how absorption of light by suspensions of phototrophic microorganisms is modulated by changes in the biomass of the culture, the size of the culture vessel and the presence of refractile structures within the cells. Increases in biomass and culture size result in higher rates of light absorption but, on the other hand, decrease the amount of energy available per cell. The presence of refractile structures has different consequences depending on the biomass concentration. In dense cultures, accumulation of refractile structures increases reflection of light, therefore reducing specific light absorption. In diluted cultures, however, the effect is the opposite, and rather than increasing reflection, refractile structures seem to increase light absorption. A theoretical model developed to interpret the experimental observations, has allowed us to estimate several parameters related to the optical properties of the organisms.

INTRODUCTION

The physiological state of phototrophic microorganisms is strongly conditioned by their light supply. Variations in this supply induce changes in cellular composition, in the degree of development of the photosynthetic apparatus as well as in several cellular activities. Thus, laboratory experiments intending to analyze the physiology of phototrophic bacteria or algae must pay special attention to the light climate cells are exposed to. In general, experiments are set in such a way that a light source provides controlled and reproducible illumination to the culture vessel, however, it is seldom realized that light conditions inside a culture vessel are significantly influenced by factors other than the incident irradiance. The occurrence of self shading, and variations in factors affecting its magnitude, such as population density or the size and shape of the culture vessel, are often overlooked.

In an attempt to circumvent the problems arising from the existence of a heterogeneous light field inside the culture, several authors have suggested solutions based in the assessment of the mean light intensity (Rabe and Benoit 1962) either through the measurement of light

irradiance at the front and back of the culture vessel (Van Liere and Walsby 1982), or after averaging the intensity of the light field at different positions inside the culture vessel (Van Liere et al 1978). The first approach may result in inaccuracies in cultures with a high scattering, in which light might exit the vessel at directions other than opposite to the light source. The second approach, although accurate, is somewhat cumbersome since it requires specialized built equipment for the analysis of the light field and, also, interferes with the regular operation of the culture. In general, mean light intensity represents an average value for the irradiance experienced by the cells when traveling within the stirred culture. Further improvements in this area have been introduced with the determination of the specific light energy uptake rate (Van Liere and Mur 1978, Pirt et al 1980, Evers 1981). To calculate this variable the total amount of light absorbed by the culture is divided by the total biomass present in the culture, thus providing an indication of the average instantaneous specific rate at which light is absorbed by the organisms. The resulting value can be directly related to biological activity through a yield coefficient (Van Liere and Mur 1978, Pirt et al 1980, Evers 1981) and constitutes a much better predictor of the level of energy supply. A direct procedure for the determination of light absorption and the specific light uptake rate in cultures of phototrophic microorganisms, has been recently developed (Sánchez and Mas 1996, Chapter 2). Using this procedure, we have carried out a study which analyzes the contribution of several factors to both, the characteristics of the light field exiting the vessel, and the magnitude of the absorption. The work described in this paper does not attempt to constitute an exhaustive study of all the factors determining the inherent optical properties of a culture but rather, tries to provide some insight into the phenomenon of light absorption by suspensions of photosynthetic microorganisms. Thus, important elements such as cell size and shape, specific content of photosynthetic pigments, formation of multicellular filaments or aggregates have not been considered. Actually, the observations focus on three factors: biomass concentration, dimensions of the culture vessel and presence of intracellular refractile inclusions likely to affect scattering. As a model organism we have chosen the purple sulfur bacterium *Chromatium vinosum*. This organism can be grown at high cell densities and does not form aggregates which might interfere with the measurements. Besides, it can accumulate large amounts of sulfur (as much as 40% of its dry weight) deposited intracellularly as highly refractile inclusions. To interpret the observations we have elaborated a mathematical model which explains variations in the rate of light absorption (Q_e) and in the specific rate of light uptake (q_e) as a function of cell concentration and the size of the culture vessel.

MATERIALS AND METHODS

Organisms and growth conditions. Experiments were carried out with *Chromatium vinosum* DSM 185 grown at 25 °C on the mineral medium described by Mas and Van Gemerden (1987). Illumination was provided by incandescent light bulbs.

Sampling and analyses. Protein, bacteriochlorophyll a (BChl a) and elemental sulfur were analyzed in 10-ml aliquots from the culture. The samples were centrifuged (4400 x g, 10 min) and after discarding the supernatants, the pellets were frozen and kept at -20 °C. BChl a and elemental sulfur were measured in methanol extracts of the samples using the procedure described by Stal et al. (1984). The protein content of the pellets remaining from the previous extraction was determined according to Lowry et al. (1951) after extraction at 100 °C with 1N NaOH. Sulfide was measured using the method of Pachmayr (1960) as described by Trüper and Schlegel (1964) in samples taken directly from the culture.

Light absorption and specific rate of light uptake. The rate at which light was absorbed by the culture ($\mu E.h^{-1}$) was determined according to the procedure described by Sánchez and Mas (1996, Chapter 2) as the difference between the rate of light output from a culture containing cells, and the rate of light output from a culture containing only medium. Outputs were measured taking into account the photon fluxes exiting the culture vessel at different angles, an approach which also allowed the characterization of the light field. Calculation of the specific rate of light uptake (q_e) was performed dividing the rate of light absorption (Q_e), by the total biomass in the culture (Sánchez and Mas 1996, Chapter 2).

Light distribution. In order to analyze how variations in the different factors studied affected the spatial distribution of the exiting light field, we defined three different quantities which somehow summarize this distribution. The first quantity is the fraction of transmitted light, calculated as the integrated light output between 135° and 225°. This value provides an indication of the fraction of light neither absorbed nor scattered and exhibits a maximum value when the culture contains only medium. The second quantity is the amount of forward scattered light, which is calculated as the integrated light output between 90° and 135° plus the light output between 225° and 270°. It gives an indication of the light which, although crossing the vessel, does not leave exactly opposite to the light source. The third quantity is the amount of backward scattered light, which provides a measure of the light reflected by the culture. It is determined as the integrated light output between 270° and 90°.

THEORETICAL ASPECTS

In order to interpret the experimental observations collected during this work, we have developed a theoretical model which allows prediction of both the rate of light absorption (Q_e) and the specific rate of light uptake (q_e) as a function of the size of the culture vessel, and the concentration of biomass.

The model, in its simplified form, assumes a prismatic vessel with parallel walls, containing a non scattering medium which absorbs light according to Beer-Lambert's Law, and which is illuminated from one side by a collimated light source perpendicular to the vessel. In such an ideal system, the rate of light absorption (Q_e) is defined by the following equation:

$$Q_e = (E_0 - E_x) \cdot A \qquad (1)$$

in which E_0 is the incident irradiance at the front of the vessel, E_x is the irradiance exiting the vessel and A is the cross-sectional area of the vessel.

Taking into account the integrated form of Beer-Lambert's equation, E_x can be expressed as:

$$E_x = E_0 \cdot e^{-a^* \cdot c \cdot x} \qquad (2)$$

in which c is the concentration of absorbing material, a* is the absorption coefficient and x the length of the light path.

After substituting equation 2 for E_x in equation 1, we obtain:

$$Q_e = A \cdot E_0 \cdot \left(1 - e^{-a^* \cdot c \cdot x}\right) \qquad (3)$$

The specific rate of light uptake (q_e) can be derived from equation 3 after dividing by the total biomass in the culture. Total biomass equals biomass concentration times the volume of the culture. The volume of the culture vessel can be defined as the product of the culture cross section (A) by the length of the optical path (x). Therefore q_e can be expressed as:

$$q_e = E_0 \cdot \left(1 - e^{-a^* \cdot c \cdot x}\right) \cdot \frac{1}{c \cdot x} \qquad (4)$$

Equations 3 and 4, although conceptually sound, are seriously flawed in practice by the fact that several of the premises on which the model is based, are not true. First, equations 3 and 4 only hold when the light source is collimated. When this is not the case, and due to the inverse square law of illumination (Arnold 1975), irradiance decreases with distance even when light travels through a non-absorbing medium therefore inducing serious errors in the calculations. Second, the assumption of a prismatic vessel with parallel walls is not consistent with the systematic use in the laboratory of cylindrical culture vessels which ensure a better stirring and mixing. Finally, scattering by suspensions of microorganisms can be considerable. Utilization of equations 3 and 4 to describe our experimental data, required therefore some modifications in order to correct for these shortcomings. The modifications introduced are described below.

Correction for distance-dependent attenuation. When a non-collimated light source is utilized, the light beam diverges while crossing the culture vessel, thus resulting in a decrease in irradiance which, in fact, is not related to light absorption. In this case, the light output $E_{x'}$ can be predicted from the inverse square law taking into account the incident irradiance (E_0), the distance between the culture vessel and the light source (L_0) and the length of the optical light path in the vessel (x):

$$E_{x'} = \frac{L_0^2}{(L_0 + x)^2} \cdot E_0 \qquad (5)$$

Thus, $E_{x'}$ represents exclusively the light output if there were no absorption by the medium.

In an absorbing medium, light decreases as a consequence of the combined effect of absorption and attenuation due to the distance. In this case, light output (E_x) is given by the following equation:

$$E_x = \frac{L_0^2}{(L_0 + x)^2} \cdot E_0 \cdot e^{-a^* \cdot c x} \qquad (6)$$

In equation 1, the rate of light absorption is proportional to the difference between light input and light output (E_0-E_x). Since E_x decreases not only due to absorption, but also to attenuation by distance, (E_0-E_x) overestimates the amount of energy absorbed by the culture. In order to compensate for this, a correction factor (E_0-$E_{x'}$) must be included. After adding this correction, equation 1 becomes:

$$Q_e = \left[(E_0 - E_x) - (E_0 - E_{x'})\right] \cdot A \qquad (7)$$

Substituting equations 5 and 6 in equation 7, we obtain:

$$Q_e = \left[\left(E_0 - \frac{L_0^2}{(L_0 + x)^2} \cdot E_0 \cdot e^{-a^* \cdot c \cdot x}\right) - \left(E_0 - \frac{L_0^2}{(L_0 + x)^2} \cdot E_0\right)\right] \cdot A \qquad (8)$$

Which after simplifying becomes:

$$Q_e = \frac{L_0^2}{(L_0 + x)^2} \cdot E_0 \cdot \left(1 - e^{-a^* \cdot c \cdot x}\right) \cdot A \qquad (9)$$

Correction for shape and scattering. Due to the fact that culture vessels are not square but cylindrical, and due to the existence of scattering, light does not exit the vessel opposite to the light source. Actually, what happens is that light exits in all directions but with a preferential orientation which depends on the concentration and scattering characteristics of the cell suspension. As a consequence, Beer-Lambert's Law is not applicable because irradiance at the end of the theoretical light path (this is at the far end of the culture vessel) is determined not only by losses through absorption, but also by emission due to scattering in directions at an angle to the theoretical light path.

To overcome this problem light output has been measured using a procedure based on the integration of the total light output around the culture vessel (see Materials and Methods), which corrects for losses due to scattering. Since measurements carried out with this procedure correspond to the total light output of the culture, the predictive capacity of Beer-Lambert's law can still be applied and equations 3 and 4 should still be valid. However, a new problem arises related to the actual length of the optical path. In the case of a cylindrical vessel, the length of the optical path should be a function of the diameter of the culture, but also of the trajectory followed by light inside the vessel. We felt that inclusion of a detailed treatment of these factors in the present model would exceed the theoretical aspects of this paper and thus we decided to combine them in an expression in which the length of the light path (x) is defined as:

$$x = \varnothing \cdot f \qquad (10)$$

where Ø is the diameter of the culture vessel, and f is a correction factor which modifies the diameter and summarizes the combined effects of the cylindrical geometry of the vessel, and the possible increase of the light path due to repeated scattering by the cell suspension.

Substituting equation 10 for x in equation 9 and considering that the cross sectional area equals the diameter times the height of the culture (h), the following expression arises:

$$Q_e = \frac{L_0^2}{(L_0 + \varnothing \cdot f)^2} \cdot E_0 \cdot \left(1 - e^{-a^* \cdot c \cdot \varnothing \cdot f}\right) \cdot \varnothing \cdot h \qquad (11)$$

From this expression, the specific rate of light uptake q_e can be calculated dividing by the total biomass contained in the culture. In the case of a cylindrical vessel, total biomass equals cell concentration (c) times the volume of the vessel (V). If the volume is expressed as $\pi.(\varnothing/2)^2.h$, the following equation results:

$$q_e = \frac{L_0^2}{(L_0 + \varnothing \cdot f)^2} \cdot E_0 \cdot \left(1 - e^{-a^* \cdot c \cdot \varnothing \cdot f}\right) \cdot \frac{4}{c \cdot \pi \cdot \varnothing} \qquad (12)$$

Equations 11 and 12 can be fitted to experimental data with q_e and Q_e as dependent variables and either the diameter of the culture (Ø) or the concentration of biomass (c) as independent variables. Since E_0 and L_0 are known, these regressions will allow estimation of a* and f. Fitting of the model to experimental data was carried out by non-linear regression using least-squares as a loss function.

RESULTS

Effect of sulfur accumulation on light absorption. In order to assess how the presence of highly scattering structures such as sulfur inclusions affects light absorption, an experiment was designed in which a culture of *C. vinosum* was progressively forced to accumulate sulfur. The organism was fed neutralized hydrogen sulfide at the beginning of the experiment up to a concentration of 1.25 mM. When sulfide was depleted a second addition was performed (1.5 mM). Utilization of H_2S during the experiment and the concomitant accumulation of elemental sulfur have been represented in Fig. 3.1A. Sulfur, which initially was present at a very low concentration (0.03 mM) increased during the experiment, attaining a maximum value of 2.4 mM approximately four hours later. The concentration of protein remained virtually constant during the same period (around 116 $mg.L^{-1}$). The concentration of BChl a (not shown) did not change during the experiment, thus resulting in a constant specific content of 49.7 μg BChl a.mg^{-1} protein. The specific content of sulfur, on the contrary, increased steadily until reaching 21.4 μmol S°.mg^{-1} protein, approximately 25.5% of the dry weight.

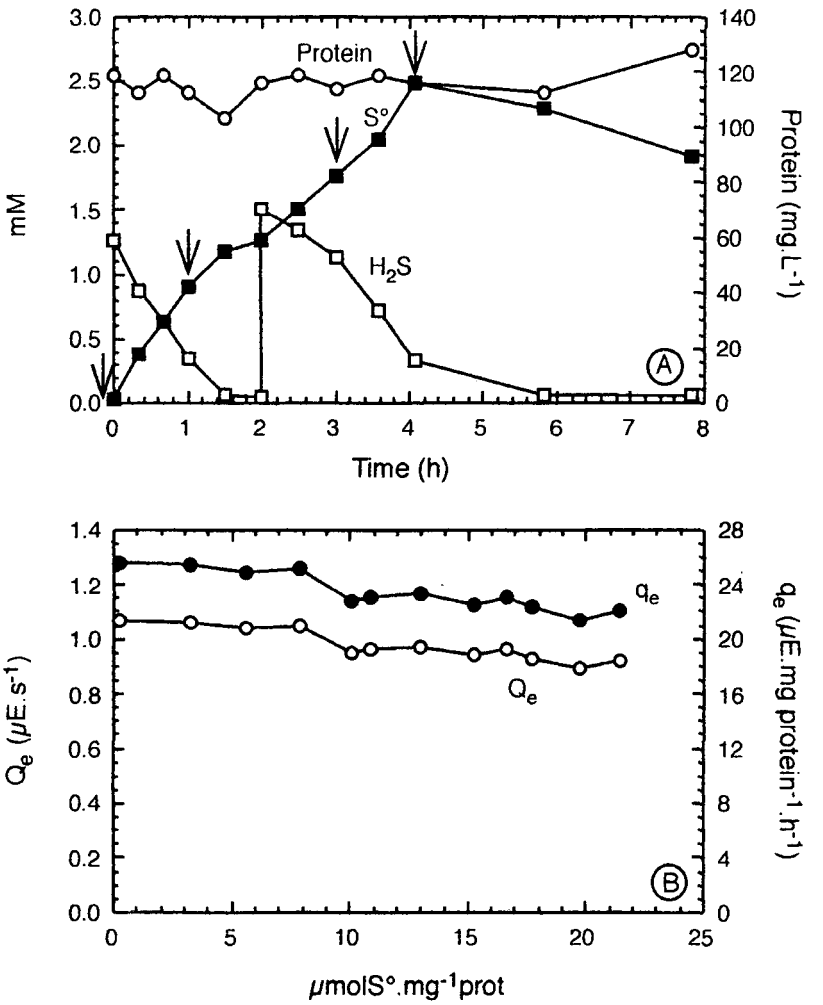

Fig. 3.1. (A) Variations along time of the concentrations of sulfide, sulfur and protein in a culture of *C. vinosum* DSM 185 after initial addition of sulfide. Two hours after the beginning of the experiment, a second addition of sulfide was made, resulting in further accumulation of sulfur. Arrows indicate the time at which the measurements of light output (represented in Fig. 2) were carried out. (B) Rate of light absorption (Q_e) and specific rate of light uptake (q_e) as a function of the specific content of sulfur. The experiment was carried out in a culture vessel 20 cm high and 10.4 cm in diameter. Incident irradiance at the surface of the vessel was 220 μE.m^{-2}.s^{-1}.

Accumulation of sulfur gave rise to a gradual change in the optical characteristics of the culture. The color, which initially was a deep shade of red progressively acquired a milky appearance indicating a higher reflection of the incident light. Measurements along time of the light output at different angles confirmed this observation. Some of the results (corresponding to sulfur contents of 0.2, 7.9, 16.6 and 21.4 μmol S°.mg^{-1} protein) have been represented in Fig. 3.2 as both, x-y graphs and polar plots.

At the beginning of the experiment, when cells were virtually depleted of sulfur (Fig. 3.2A), light escaped from the culture through the back at 135° and 225°, probably as a consequence of forward scattering. As sulfur accumulated, back scattering increased and light output shifted progressively from the back to the front of the culture vessel (Figs. 3.2B, C and D). Overall, the

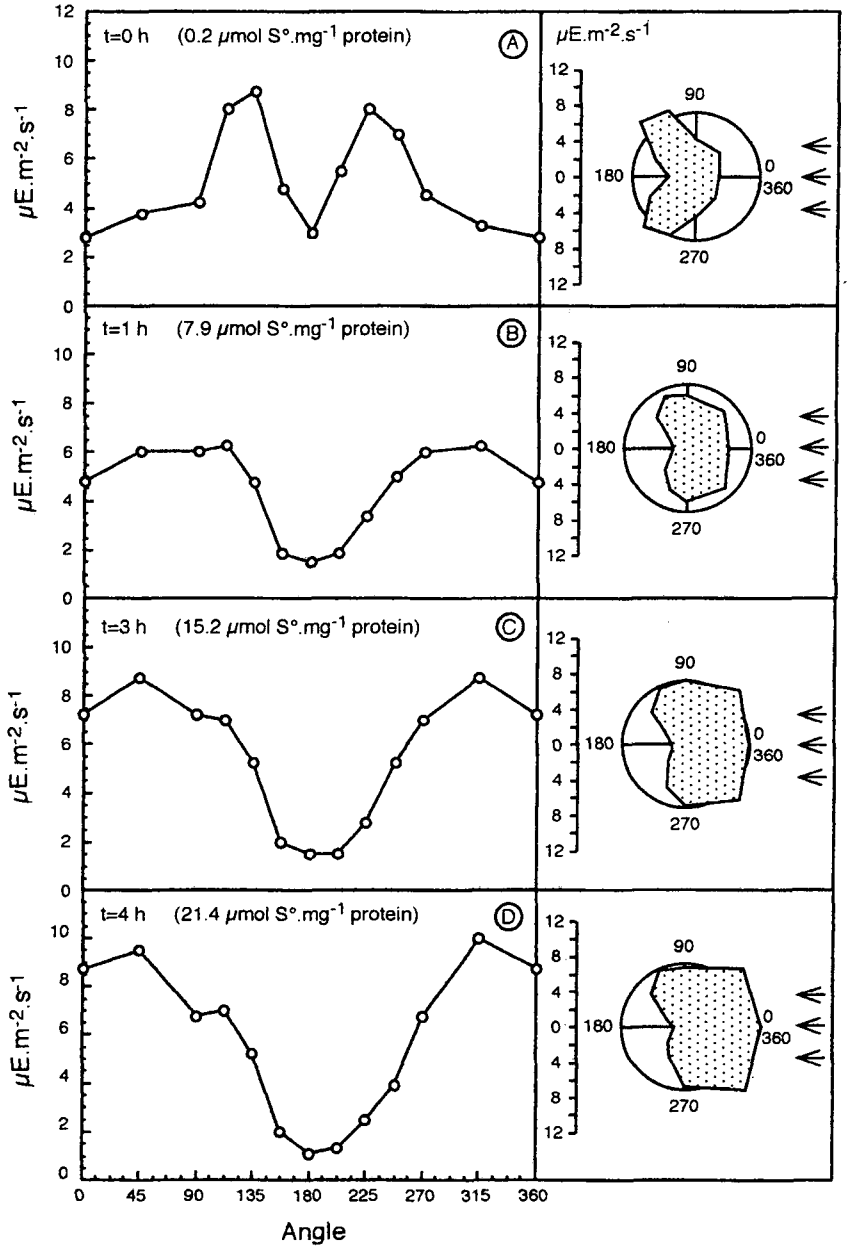

Fig. 3.2. Light output as a function of the angle in cultures of *C. vinosum* with different sulfur contents. The results have been represented as x-y graphs and polar plots. In these plots the distance of the points on the polygon from the center of the circle represents the magnitude of the light output. Arrows indicate the position of the light source.

total amount of light exiting the culture (dotted area in the polar plots) changed very little, increasing slightly as sulfur accumulated. The consequence of this increase in light output was a small decline in light absorption (Fig. 3.1B) which resulted in a parallel reduction in the specific rate of light uptake (q_e).

The results described above correspond to a dense culture with a high biomass. In dense cultures the presence of refractile structures, which increase scattering, seems to have little effect on the rate at which light is harvested by the organisms, however, it has a dramatic effect on the distribution of light exiting the culture. This observation, as will be shown later on in this paper, does not hold for diluted cultures in which sulfur accumulation appears to intensify light uptake by the organisms.

Effect of culture size on light absorption. The effect of culture size on light absorption was studied using culture vessels with different diameters. In order to standardize the results, the height of the vessel was 20 cm in all the experiments. For each case we determined the spatial distribution of the light output from a blank containing medium, and from a vessel containing a dense culture (74.4 mg protein.L^{-1}) of *C. vinosum* depleted of sulfur. In both cases, the vessels were illuminated by incandescent light bulbs placed in one side, supplying an incident irradiance of 92 $\mu E.m^{-2}.s^{-1}$. A total of eight different diameters were analyzed (3.7, 4.4, 5.2, 6.7, 7.9, 10.1, 13.8 and 18.5 cm). Light output as a function of the angle has been represented for all of them in Fig. 3.3A.

Most of the light exited the vessel at 180°, opposite to the light source. Total light output (area underneath the curves) decreased steadily as diameter increased, due to a higher absorption by the culture.

The rate of light absorption (Q_e) and the specific rate of light uptake (q_e) have been represented in Fig. 3.3B together with the theoretical curves predicted by the model. The rate of light absorption increases with diameter. At high diameters, the rate at which light is absorbed by the culture flattens reaching a maximum value which, as will be discussed below (see Discussion), is partly determined by the distance between the light source and the culture vessel. Although light absorption increases with diameter, the specific rate of light uptake decreases dramatically when increasing the size of the culture vessel. Thus, an increase of approximately five times in the diameter gives rise to a tenfold decrease in q_e. Since photosynthetic activity is proportional to q_e, it is anticipated that the size of the culture vessel will have a substantial effect on the behaviour of phototrophic cultures.

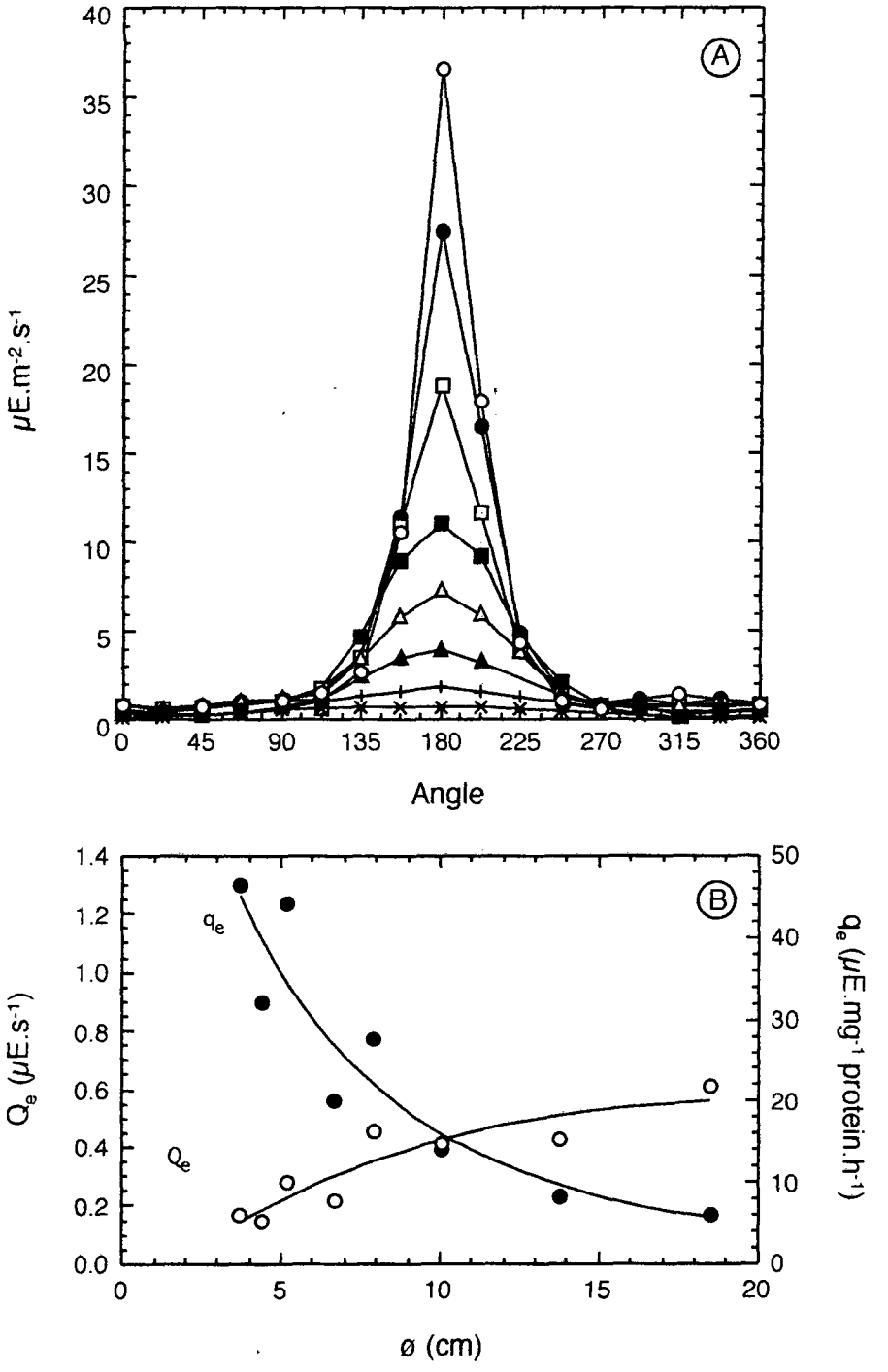

Fig. 3.3. (A) Irradiance as a function of the output angle for vessels of different sizes containing a sulfur-depleted culture (0.4 μmol S°.mg^{-1} protein) of *C. vinosum* of 74.37 mg protein.L^{-1}. Incident irradiance was 92 μE.m^{-2}.s^{-1} (○ 3.7cm, ● 4.4 cm, □ 5.2 cm, ■ 6.7 cm, △ 7.9 cm, ▲ 10.1 cm, + 13.8 cm, × 18.5 cm). (B) Rate of light absorption (Q_e) and specific rate of light uptake (q_e) as a function of the diameter of the vessel in cultures of *C. vinosum*. The curves have been drawn after fitting the model described in Materials and Methods.

Effect of biomass on light absorption. Light absorption by cultures of phototrophic organisms was also expected to change as a function of biomass. To study the effects of this variable, a dense culture of *C. vinosum* (116 mg protein.L^{-1}) was diluted several fold using fresh culture medium. Each of the dilutions was placed in a culture vessel and light output was measured at different angles. Measurements were carried out using a culture vessel 10.4 cm in diameter and 20 cm high. Parallel determinations were also carried out in dilutions of a culture with the same initial biomass but containing 16.6 μmol S° per mg protein. In both cases, the vessels were illuminated by incandescent light bulbs placed in one side, providing an incident irradiance of 220 $\mu E.m^{-2}.s^{-1}$. Light output as a function of the angle has been represented in Fig. 3.4 for some of the dilutions from the sulfur-depleted culture. Light output from a blank has also been included in the same graph for comparison (Fig. 3.4A).

At low biomass (6.7 mg protein.L^{-1}) light distribution resembles very much the light distribution of the blank. As biomass increases light output decreases and the distribution changes. While at low biomass, light exits the culture vessel preferentially at 180°, high biomass results in a decrease in the output at 180° and a relative increase of the outputs at 135° and 225°, as a consequence of forward scattering within the culture.

The specific rate of light uptake (q_e) has been represented in Fig. 3.5 as a function of protein concentration together with the rate of light absorption (Q_e). Q_e increases with protein attaining a maximum value at high concentrations when all light has been absorbed by the culture. On the contrary, q_e decreases approaching zero. Both variables change following the same pattern observed in Fig. 3.3B indicating that biomass and culture size have nearly equivalent effects on Q_e and q_e.

Changes in q_e can also be observed in cultures which have accumulated sulfur. Fig. 3.6 shows the relationship between q_e and biomass (expressed as protein) in cultures of *C. vinosum* depleted of sulfur and in cultures containing 16.6 μmol S°.mg^{-1} protein. Differences in q_e were more pronounced in cultures with low cell density. In diluted cultures, the presence of refractile structures causes scattering, increasing the light path inside the culture vessel and therefore, affecting the rate at which light is harvested by the organisms. As a consequence, the overall light output decreases and q_e shows a substantial increase when sulfur is present. This conclusion does not hold for dense cultures, in which structures apt to increase scattering seem to have little effect on the specific rate of light uptake.

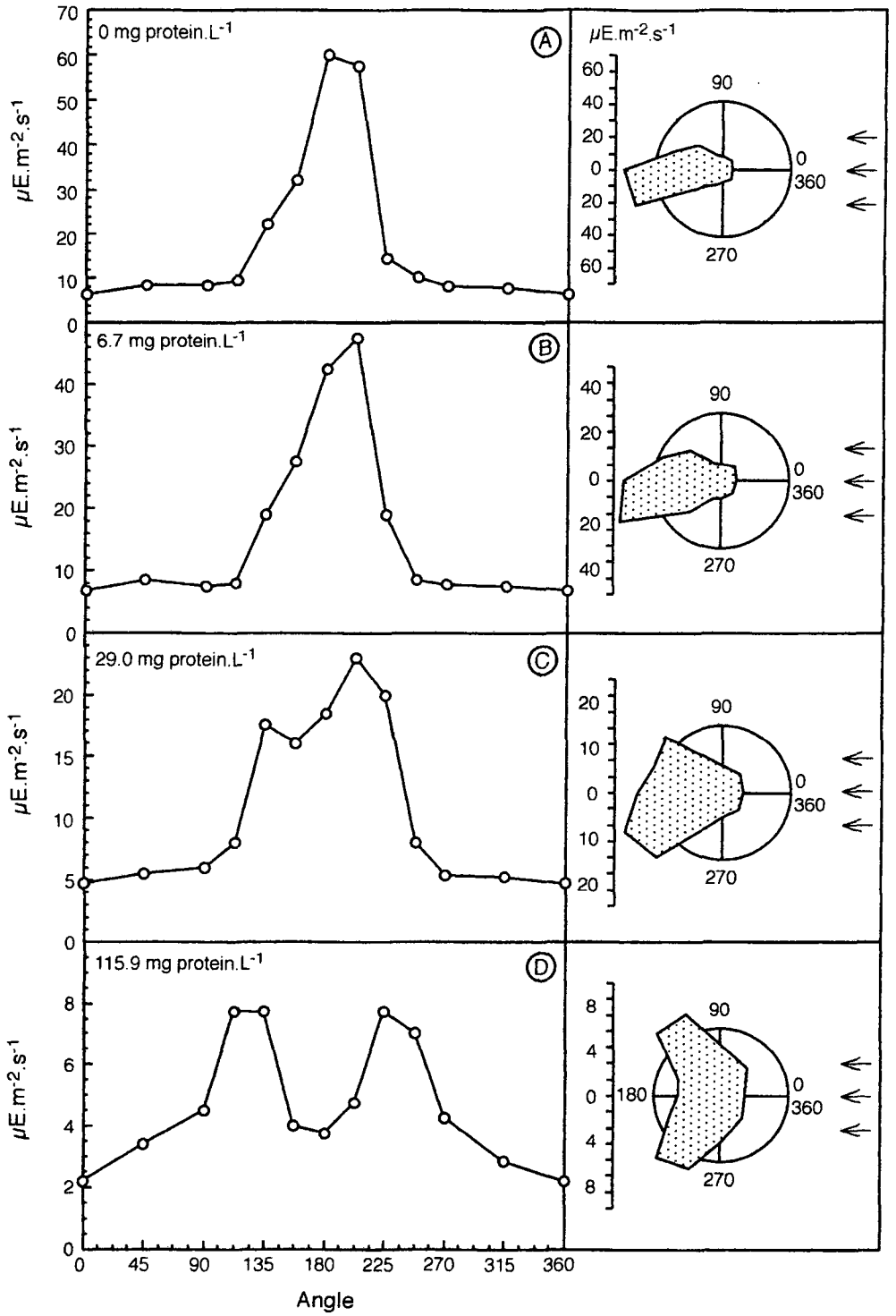

Fig. 3.4. Irradiance as a function of the output angle in cultures of *C. vinosum* at different biomass concentrations. The results have been represented as x-y graphs and polar plots. Arrows indicate the position of the light source.

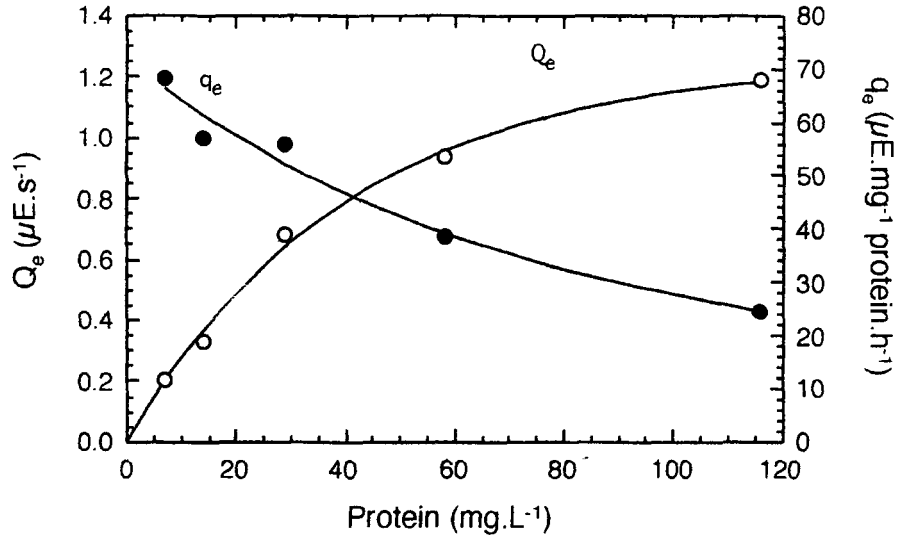

Fig. 3.5. Rate of light absorption (Q_e) and specific rate of light uptake (q_e) as a function of biomass in cultures of *C. vinosum*. The curves have been drawn after fitting the model described in Materials and Methods.

This effect is more apparent when looking at the distribution of the light output for the two extreme situations in Fig 3.6A. These situations have been represented as polar plots in Figures 3.6B and 3.6C. In Fig. 3.6B, the distribution of the light output corresponds to two diluted cultures of *C. vinosum* with the same biomass (6.7 mg protein.L^{-1}), one depleted of sulfur and the other containing 16.6 μmol S°.mg^{-1} protein. The presence of sulfur does not increase back scattering, but rather decreases transmitted light and results in a reduction of the overall output, therefore suggesting an increase in light absorption. A different situation can be observed in Fig. 3.6C which represents the light output of two dense cultures containing the same biomass (115.9 mg protein.L^{-1}) and different sulfur contents (0.2 and 16.6 μmol S°.mg^{-1} protein). In this case, the presence of refractile inclusions increases dramatically the amount of back scattered light. This results in a light distribution radically different from the distribution found in the sulfur-depleted culture, in which light is scattered forward and exits the culture at 135° and 225°.

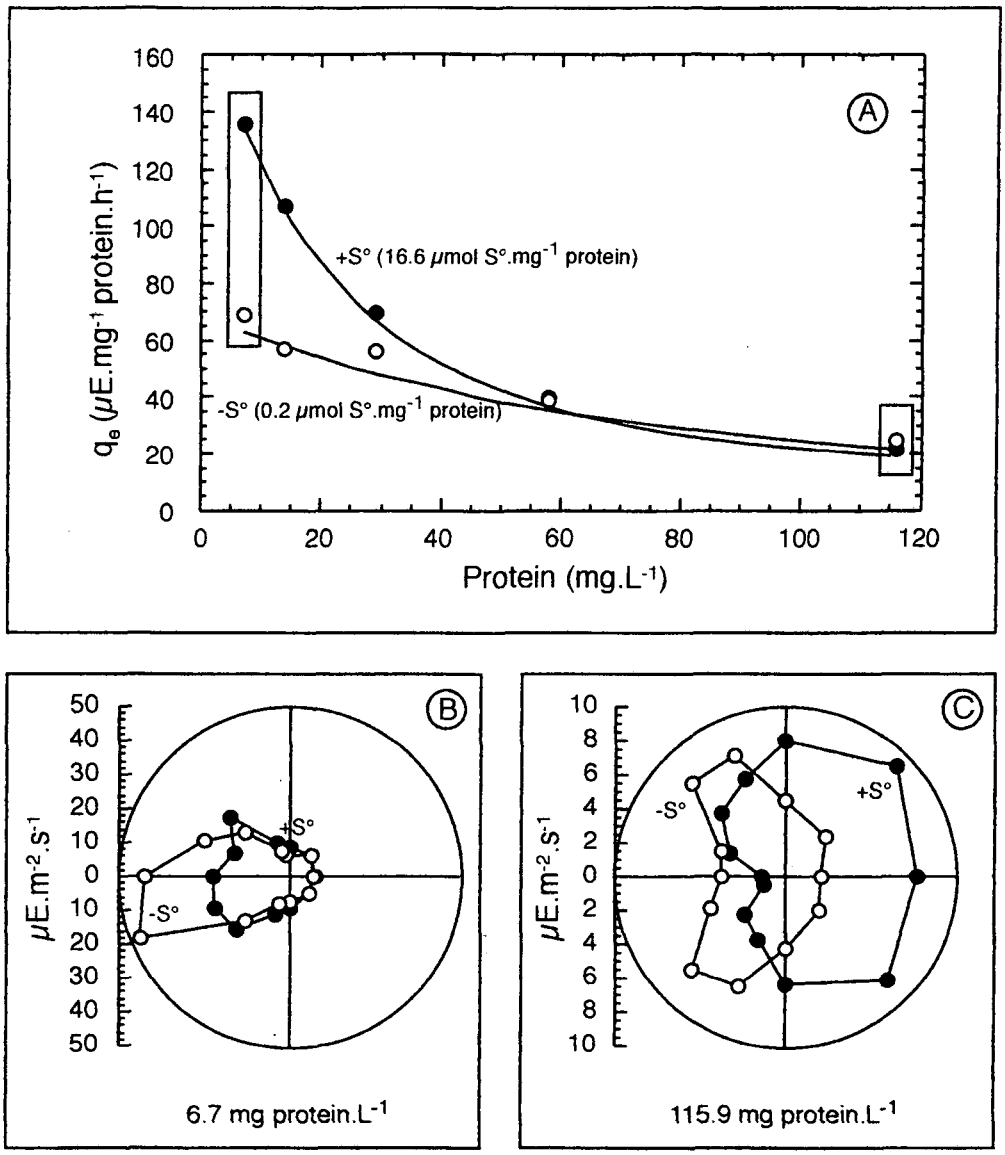

Fig. 3.6. (A) Specific rate of light uptake as a function of biomass (mg protein.L^{-1}) in cultures of *C. vinosum* depleted of sulfur (O) and in cultures containing 16.6 µmol S°.mg^{-1} protein (●).The points enclosed in rectangles at the right and left sides of the plot have been used to draw the light output distributions in Figs. 3.6B and 3.6C. (B) Distribution of the light output in diluted cultures of *C. vinosum* with a high (●) and a low (O) sulfur content. (C) Distribution of the light output in dense cultures of *C. vinosum* with a high (●) and a low (O) sulfur content. (-S°, 0.2 µmol S°.mg^{-1} protein; +S°, 16.6 µmol S°.mg^{-1} protein).

Fitting the model to experimental data. The equations described in Materials and Methods for the prediction of Q_e and q_e were fitted to the experimental data by non-linear regression. Different sets of data were fitted separately thus resulting in a number of independent estimates of the absorption coefficient (a^*) and the correction factor f. These estimates have been collected in Table 3.1 for each set of data. Two of the sets correspond to cultures with the same diameter (10.4 cm) and different specific contents of sulfur (16.6 and 0.2 μmol S°.mg^{-1} protein), in which biomass (c) was the independent variable. The values of a^* and f estimated from these data differed considerably between both sets. In sulfur-depleted cultures a^* averaged 0.000173 m^2.mg^{-1} protein while f was 1.350. On the other hand, cultures containing sulfur yielded an average a^* of 0.000393 m^2.mg^{-1} protein and an f value of 1.772. These differences, which can only be attributed to the presence of sulfur inclusions, indicate an increase in the absorbing capacity of the cells and suggest an increase of the average light path due to scattering. The third set of data was obtained from cultures with different diameters (Ø) and identical biomass. The average values of a^* and f (0.000146 m^2.mg^{-1} protein and 1.290) were very similar to the values derived from the sulfur-depleted culture described above. Coincidence between the estimates of a^* and f from both experiments agrees with what could be expected from cultures with similar specific contents of sulfur (0.2 and 0.4 μmol S°.mg^{-1} protein) and pigments (49.7 and 41.0 μg BChl a.mg^{-1} protein).

Table 3.1. Absorption coefficients (a^*) and correction factors (f) estimated from different sets of experimental data. The parameters were estimated after fitting equations 11 and 12 of the model by nonlinear regression. The standard errors of the estimates have also been included.

Independent variable	Sulfur content (μmol S°. mg^{-1} protein)	Pigment content (μg BChl a. mg^{-1} protein)	Dependent variable	a^* (x10^{-3}) (m^2.mg^{-1} protein)	f	r^2
biomass (c)	0.2	49.7	Q_e	0.163 ± 0.007	1.460 ± 0.049	0.997
			q_e	0.182 ± 0.007	1.240 ± 0.193	0.969
biomass (c)	16.6	49.7	Q_e	0.358 ± 0.021	1.837 ± 0.039	0.990
			q_e	0.427 ± 0.005	1.707 ± 0.028	1.000
diameter (Ø)	0.4	41.0	Q_e	0.115 ± 0.019	1.303 ± 0.196	0.824
			q_e	0.177 ± 0.026	1.276 ± 0.461	0.859

The model was fitted only in those cases in which the correction factor and the absorption coefficient were expected to remain constant during the experiment. As indicated above, the presence of sulfur inclusions has a strong effect on a^* and f. Thus, data from experiments in which the content of sulfur changed along time (Fig. 3.1B), were not used for the fitting.

Spatial distribution of the light output. Light output can be divided in three fractions, which indicate the relative amount of forward scattered, backward scattered and transmitted light. These fractions have been represented for three different experiments, as a function of the sulfur content (Fig. 3.7A), the size of the culture vessel (Fig. 3.7B) and the concentration of protein (Fig. 3.7C).

In Fig. 3.7A, as the content of refractile inclusions increased, the back scattered fraction rose substantially from a value of 600 $\mu E.m^{-2}.s^{-1}$.degree at low contents of sulfur up to 1500 $\mu E.m^{-2}.s^{-1}$.degree at high specific contents. On the contrary, transmitted and forward scattered light tended to decrease, although to a lesser extent. When biomass remained constant and the diameter of the vessel increased (Fig. 3.7B), more light was absorbed. As a consequence, transmitted light decreased considerably, from 1550 to 50 $\mu E.m^{-2}.s^{-1}$.degree. Forward and back scattered light also experienced a small decline. A similar situation was found when light output was plotted against biomass concentration (Fig. 3.7C) in sulfur-depleted cultures. As biomass increased, more light was absorbed and consequently, the amount of transmitted light decreased. The quantities of forward and back scattered light showed a slight reduction, although at high biomass they turned to be an important fraction of the total light output, totaling 70% of the exiting light. Summarizing, increases in biomass and in the diameter of the culture vessel invariably result in a reduction in the amount of transmitted light and have very little impact on the scattered fractions. Accumulation of refractile inclusions, on the contrary, seems to increase the amount of back scattered light, although this conclusion only holds for dense cultures.

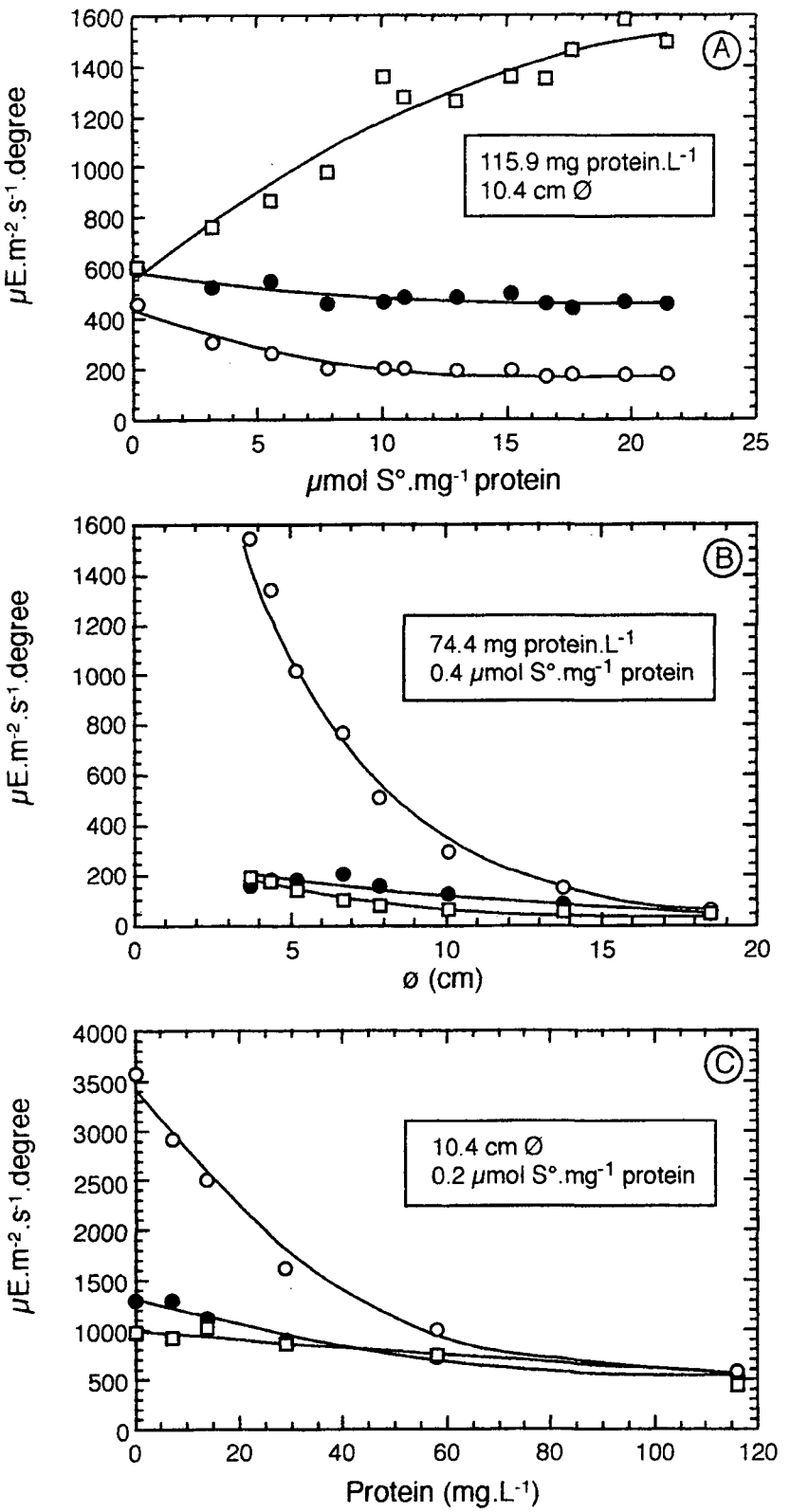

Fig. 3.7. Amounts of transmitted (O), forward scattered (●) and backward scattered (□) light output as a function of: (A) the specific content of sulfur, (B) the diameter of the culture vessel and (C) the protein concentration. The conditions for each set of experiments are specified in the plots.

DISCUSSION

The results indicate rather clearly that non absorbed light exits the culture vessel according to a spatial distribution which is considerably influenced by factors such as the concentration of organisms, the size of the vessel or the presence of refractile inclusions. In diluted cultures (Fig 3.4B) or in cultures with a small diameter (Fig 3.3A), as much as 80 % of the light output is transmitted. When either the biomass or the diameter of the culture increase, transmitted output (Fig 3.3A, Fig 3.4D) decreases to between 25 and 35% of the total output, the remaining fraction being distributed in roughly equal amounts between forward scattered light and backward scattered light. As a consequence, since light exits the vessel at directions other than opposite to the light source, point measurements of light irradiance at the front and at the back of the culture vessel result inappropriate to determine the amount of light absorbed by a culture. This phenomenon is even more apparent during sulfur accumulation (Fig. 3.2). When sulfur is accumulated, transmitted light decreases to about 8% of the total output, while backward scattered light (i.e. reflected light) increases to constitute up to 70% of the total output.

The theoretical model described in Materials and Methods fitted the experimental data reasonably well, allowing estimation of the absorption coefficient of the culture (a^*) and the parameter f, a factor which corrects for differences between the diameter of the vessel and the actual average length of the light path. Two values were obtained for each parameter depending on whether the set of data used corresponded to experiments with sulfur-depleted or sulfur-containing cultures (Table 3.1). For cultures without sulfur inclusions, a^* had an average value of 0.000163 $m^2.mg^{-1}$ protein (0.0035 $m^2.mg^{-1}$ Bchl a) and f was 1.320. In cultures containing sulfur (16.6 μmol $S^\circ.mg^{-1}$ protein), a^* increased to 0.000393 $m^2.mg^{-1}$ protein (0.0079 $m^2.mg^{-1}$ Bchl a) and f was 1.772. The more than twofold increase in the absorption coefficient in the presence of intracellular sulfur represents an unexpected finding and indicates that refractile structures affect dramatically the rate at which light can be absorbed by the cells. However, this effect can only be observed in diluted cultures. In nature, where the concentrations of microorganisms are relatively low, sulfur may increase the magnitude of light absorption and this fact would be especially relevant for populations living in a light-limited environment. In turn, it has been observed in the laboratory that cells of *C. vinosum* store sulfur under conditions of light limitation (Mas and van Gemerden 1995). In this conditions sulfur could behave as a storage of reducing power but also as a structure apt to increase light absorption, thus constituting an adaptive mechanism to improve light absorption in light-limited environments.

Overall, the coefficients obtained compare well with coefficients from the literature corresponding to other phototrophic organisms. The average value found in our experiments

for cultures without sulfur inclusions (0.0035 $m^2.mg^{-1}$ Bchl a) is similar to the values observed in *Phaeodactylum tricornutum* (0.0037-0.045 $m^2.mg^{-1}$ Chl a) (Geider et al 1986), *Thalassiosira weissflogii* (0.0038-0.0078 $m^2.mg^{-1}$ Chl a) (Dubinsky et al 1986), *Isochrysis galbana* (0.0090-0.0206 $m^2.mg^{-1}$ Chl a) (Dubinsky et al 1986), *Prorocentrum micans* (0.011-0.021 $m^2.mg^{-1}$ Chl a) (Dubinsky et al 1986), *Dunaliella tertiolecta* (0.0047-0.0088 $m^2.mg^{-1}$ Chl a) (Berner et al 1989) and *Tetraedron minimum* (0.017-0.026 $m^2.mg^{-1}$ Chl a) (Dubinsky 1992).

The second parameter (f) estimated with the model corrects the differences between the diameter of the culture and the actual length of the average light path. The model was developed having in mind an ideal system in which collimated light crossed a prismatic vessel with parallel walls containing a non-scattering suspension. In such a system, the length of the light path would coincide with the thickness of the vessel. However, in practice, culture vessels are cylindrical, and the thickness of the vessel, represented by the diameter, does not coincide with the length of the optical path. Besides, the existence of scattering complicates the situation further by changing the trajectory of the light beam. Since an analytical solution to this question seemed beyond our capabilities, we decided to treat the problem by defining the light path as the product between the diameter (Ø) and the correction factor f. Application of the model to the experimental data yielded different estimates of f which averaged 1.772 or 1.320 depending on whether the culture contained sulfur or not. In both cases, f is larger than 1, indicating that the average light path is longer than the diameter of the culture. Also, accumulation of sulfur increases f, suggesting that the presence of refractile structures increases the length of the light path.

The experiments described in this paper only analyze the effect of protein concentration and the size of the culture vessel on the rate of light absorption. Application of the theoretical model developed in Materials and Methods allows to formulate some additional predictions about the absorbing properties of illuminated cultures. Thus equations 11 and 12 indicate that both Q_e and q_e will increase linearly with increasing incident irradiance (E_0). However, the effect of variations in the distance to the light source (L_0) on q_e and Q_e is far from straightforward. When L_0 is very large (i.e., sunlight), the correction term $L_0^2/(L_0+Ø.f)^2$ approaches 1 and its contribution to equations 11 and 12 can be disregarded. Finite values of L_0 require a more elaborated analysis involving a numerical simulation. The simulation, which was carried out using equations 11 and 12, calculated Q_e and q_e as a function of biomass concentration and the diameter of the culture vessel for a culture placed at different distances from the light source. The output of the simulation has been represented in Fig. 3.8. Fig.3.8A shows how Q_e approaches an asymptotic maximum (Q_{emax}) when biomass increases, reflecting the fact that at high biomass all light is absorbed by the culture. An expression describing Q_{emax} can be derived from equation 11 assuming that biomass (c) approaches ∞:

$$Q_{e\max} = E_0 \cdot \varnothing \cdot h \cdot \frac{L_0^2}{(L_0 + \varnothing \cdot f)^2}$$

For very large values of L_0, Q_{emax} equals E_0 times the cross section of the culture vessel ($\varnothing$.h). Lower values of L_0 result in curves with the same shape but with a lower Q_{emax}. On the other hand, q_e (not shown) always approaches zero when biomass increases. This is due to the fact that q_e is defined as the amount of light absorbed (Q_e) divided by the biomass present in the culture. While Q_e remains constant at high concentrations, the increase in biomass results in a progressive reduction in q_e.

When Q_e is plotted against the diameter of the vessel (Fig. 3.8B) different curves can be observed. At low diameters Q_e increases exponentially due to the combined increase of both, the length of the light path and the cross-section of the culture vessel. At higher diameters, when the length of the light path is large enough to absorb most of the incident irradiance, the increase in Q_e is only due to the increase in the culture cross section. When the culture is placed at an infinite distance from the light source, Q_e increases linearly with the diameter. However, for finite values of L_0 the curve flattens and approaches a maximum which is determined by the geometric relationship between the vessel and the light source. As in the previous case, when diameter increases, q_e (not shown) declines approaching zero due to the fact that total biomass increases with the volume of the culture.

Overall, the observations gathered in this paper indicate how light absorption can be maximized by increasing the culture cross-section and/or the concentration of phototrophic biomass. Maximum light utilization, while of some relevance for the economy of applied processes, will necessarily result in a decrease of the specific rate of light uptake (q_e), this is, the amount of energy available for growth per unit biomass. Therefore, maximization of light absorption necessarily will be associated to a decrease in the biological activity and, as a consequence, optimization of phototrophic production in biological reactors will require a trade off between these two factors.

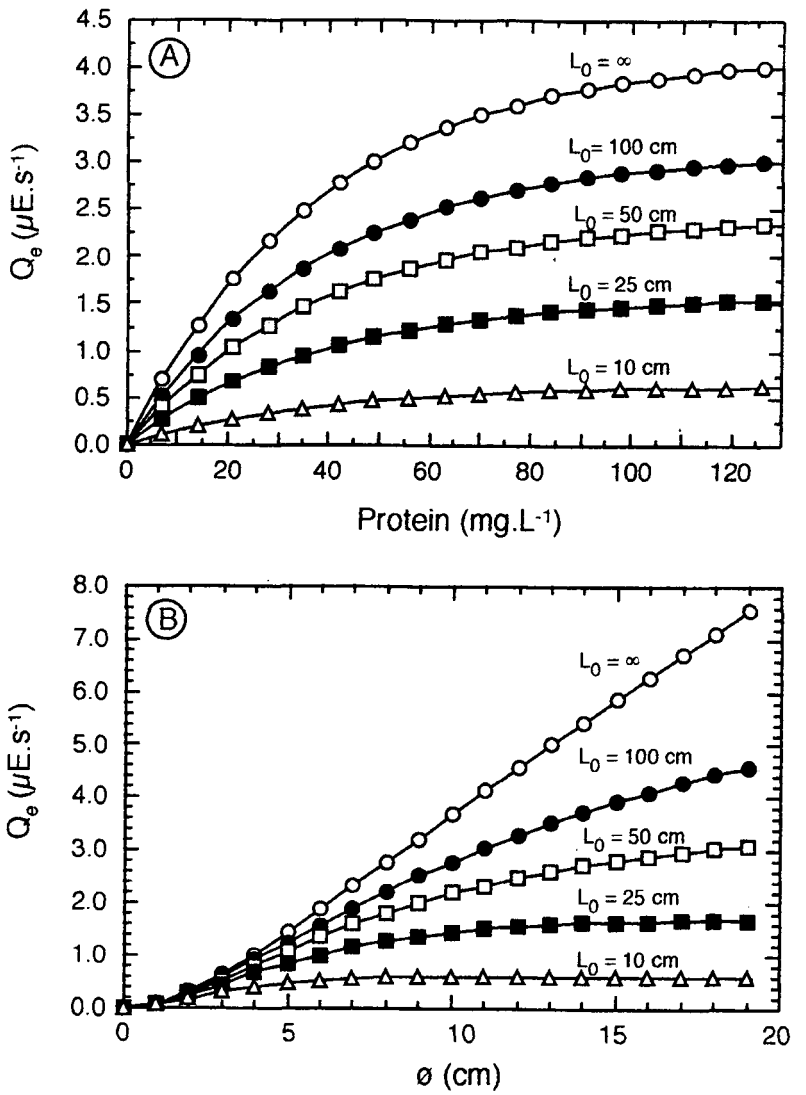

Fig. 3.8. (A) Rate of light absorption as a function of protein concentration in a culture placed at different distances from the light source (L_0). The data have been generated by means of a simulation carried out using equation 11 of the model and assuming a diameter of 10.4 cm. (B) Rate of light absorption as a function of the diameter of the vessel for a culture placed at different distances from the light source. Data predicted from equation 11. Biomass was set at 100 mg protein.L^{-1}. For both plots, E_0=200 μE.m^{-2}.s^{-1}, a*=0.00017 m^2.mg^{-1} protein, f=1.477 and h=20 cm.

NOMENCLATURE

Q_e rate of light absorption ($\mu E.s^{-1}$)

q_e specific rate of light uptake ($\mu E.mg^{-1}$ protein.s^{-1})

E_0 incident irradiance ($\mu E.m^{-2}.s^{-1}$)

E_x output irradiance ($\mu E.m^{-2}.s^{-1}$)

$E_{x'}$ output irradiance when there is no absorption ($\mu E.m^{-2}.s^{-1}$)

A culture cross-section (m^2)

c biomass concentration (mg protein.m^{-3})

x length of the optical path (m)

L_0 distance between the culture vessel and the light source (m)

a^* absorption coefficient ($m^2.mg^{-1}$ protein)

f correction factor for shape and scattering

$Ø$ diameter of the vessel (m)

h height of the vessel (m)

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CHAPTER 4

DESCRIPTION OF A REDOX-CONTROLLED SULFIDOSTAT FOR THE GROWTH OF SULFIDE-OXIDIZING PHOTOTROPHS

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CHAPTER 4

DESCRIPTION OF A REDOX-CONTROLLED SULFIDOSTAT FOR THE GROWTH OF SULFIDE-OXIDIZING PHOTOTROPHS

This paper describes a novel type of continuous culture for the growth of phototrophic sulfur oxidizers under constant concentrations of hydrogen sulfide. The culture maintains a constant concentration of sulfide despite possible variations in external factors likely to affect photosynthetic activity. Variations in biological activity lead to small departures from the steady-state concentration of hydrogen sulfide which result in variations of the redox potential. These changes in redox, monitored through a redox controller, modulate the rate at which the medium is pumped into the culture and therefore govern the dilution rate. As a result, when changes in external factors such as the light supply occur, the dilution rate of the culture adjusts to the new rate of sulfide oxidation, while maintaining a virtually constant concentration of hydrogen sulfide. The system has been successfully tested for an extended period of several weeks and under conditions of shifting illumination (868 to 113, 113 to 23, 23 to 7 $\mu E.m^{-2}.s^{-1}$). After changes in illumination, transition to a new dilution rate started immediately, reaching a new equilibrium in less than 3 h.

INTRODUCTION

Hydrogen sulfide, which is a toxic compound with an unpleasant smell, can be produced in relatively large amounts during the treatment of organic effluents, in tail gas from desulfurization plants, or during sulfide stripping from natural gas prior to combustion (Fischer 1988). Chemical oxidation of sulfide occurs spontaneously in the presence of oxygen, in a process which yields thiosulfate or sulfur as the final product, depending on the initial ratio between H_2S and O_2 (Chen and Gupta 1972). Alternatively, sulfide can be eliminated from gas streams by a variety of processes in which sulfide is removed by an absorbent that has to be periodically regenerated, often through expensive procedures (Parkinson et al 1994, Jensen and Webb 1995, Kwong and Meissner 1995). Anoxygenic phototrophic bacteria have been used as an alternative to chemical treatments for the removal of hydrogen sulfide from several types of industrial wastes. Thus, effluents from anaerobic waste treatment have been processed with cultures of *Chromatium* cells (Kobayashi and Kobayashi 1995), and cultures of *Chlorobium* cells have been successfully applied to the desulfurization of sour gas (Chen and Gupta 1972, Cork 1982, Cork

et al 1983, Kim et al 1992) in a process in which sulfide was oxidized anaerobically to elemental sulfur in the presence of CO_2.

Continuous operation of a sulfide oxidizing bioreactor requires an equilibrium between the rates at which sulfide is supplied and oxidized. When, as in the cases mentioned above, the organisms used are phototrophs, illumination must be provided at levels high enough to guarantee an adequate oxidizing activity. Failure to meet these levels will invariably result in a drop in activity which, if supply proceeds at the same rate, will result in a net accumulation of hydrogen sulfide. Since high concentrations of sulfide are toxic even for sulfide oxidizers (K_i between 2 and 4 mM) (Van Gemerden 1984), this accumulation will result in a further reduction of activity, which eventually will lead to a complete standstill of the oxidation process. To avoid this, some sort of feedback mechanism is required which adjusts the rate of sulfide supply to the rate at which this compound can actually be oxidized.

This paper describes a novel culture setup which is able to maintain a constant concentration of hydrogen sulfide inside the culture vessel under conditions of changing illumination. The culture operates as a sulfidostat in which the level of sulfide is maintained constant and biomass levels are determined by the concentration of residual sulfide. The system is regulated by means of a redox controller, and for every incident irradiance, the culture adjusts to a certain rate of sulfide supply. This setup has been tested for several irradiances. The results indicate how this method can be successfully used to couple sulfide supply to sulfide utilization in cultures of phototrophic sulfur bacteria subject to a variable light regimen.

MATERIALS AND METHODS

Organism. Experiments were performed with the purple sulfur bacterium *Chromatium vinosum* DSM 185.

Culture setup and growth conditions. The microorganism was grown on a mineral medium which contained carbonate as the only carbon source and hydrogen sulfide as electron donor (Mas and van Gemerden 1987). The medium was prepared as two separate solutions. One of them was alkaline (pH 12) and contained carbonate and sodium sulfide. The second solution was acidic (pH 5) and contained the remaining components. The two double strength solutions were pumped at equal rates into the culture vessel (1.4 liter), resulting in a slightly alkaline medium. Final adjustment of the pH was carried out with an independent titrator (Cole-Parmer pH/ORP controller 5652-10) connected to a pump (Masterflex 7554-60) which added a 1 M HCl solution at the rate required to maintain a pH of 7.5 (Fig 4.1A). Temperature control was

achieved with a glass vessel with a double wall through which water from a refrigerated water bath circulated at 25 °C. The culture vessel, placed in a dark room, was continuously illuminated from one side by two incandescent light bulbs. Incident irradiance (868, 113, 23 and 7 $\mu E.m^{-2}.s^{-1}$) was adjusted by changing the light source and its distance from the culture vessel. Irradiance was measured with a quanta meter (no. Li-185B; LiCor Inc.). The concentration of hydrogen sulfide was kept at 50 μM, well above the saturation constant (Table 4.1) to avoid sulfide limitation, but low enough to prevent inhibition. The culture was continuously stirred by means of a magnetic stirrer. Special care was taken to avoid abiotic losses. To maintain anaerobic conditions and to avoid the entrance of oxygen, all of the components of the culture were kept under oxygen-free nitrogen at a pressure of 0.1 bar (10^4 Pa). The culture vessel was completely filled, with no headspace, in order to prevent the escape of undissociated H_2S to the gas phase.

Analytical methods and control of the dilution rate. The culture was operated as a redoxstat under the assumption that the main factor controlling redox was the concentration of hydrogen sulfide.

Sulfide was measured in samples taken directly from the culture by the method of Pachmayr (1960) as described by Trüper and Schlegel (1964). The samples were taken directly into 20 ml of a Zn acetate solution. To this mixture, 10 ml of a solution of 4-amino-N,N-dimethylaniline sulfate in 20% H_2SO_4 and 1 ml of a solution of $FeNH_4(SO)_4$ in 20% H_2SO_4 were sequentially added. Water was added up to a final volume of 50 ml and the mixture was left for 15 min for the reaction to proceed. After this time, A_{670} was measured, and the concentration of sulfide was determined with a standard curve. For each sample, two blanks were carried out to correct for the absorbance of both bacterial cells and reagents.

The effect of pH fluctuations on the redox readings was minimized by adjusting the pH titrator to a narrow control interval (±0.1 pH unit). Redox was measured with a combined electrode (Schott PT6880) connected to a redox controller (Cole-Parmer pH/ORP controller 5652-10). Departure from a preestablished redox set point caused a proportional (4 to 20 mA) response in the controller. This output modulated the dilution rate through changes in the speed of the peristaltic pump (Watson Marlow 501U) supplying medium to the culture. The regulation of the system is represented in Fig. 4.1B. Photosynthetic activity (P) was governed by the amount of light that reached the culture vessel. When incident irradiance increased, microorganisms were more active and consumed more sulfide. Consequently, the redox of the culture increased and the difference between this value and the predetermined set point was higher, the pump speed increased and so did the dilution rate of the culture. The opposite occurred when irradiance decreased. The dilution rate was determined by collecting the overflow of the culture

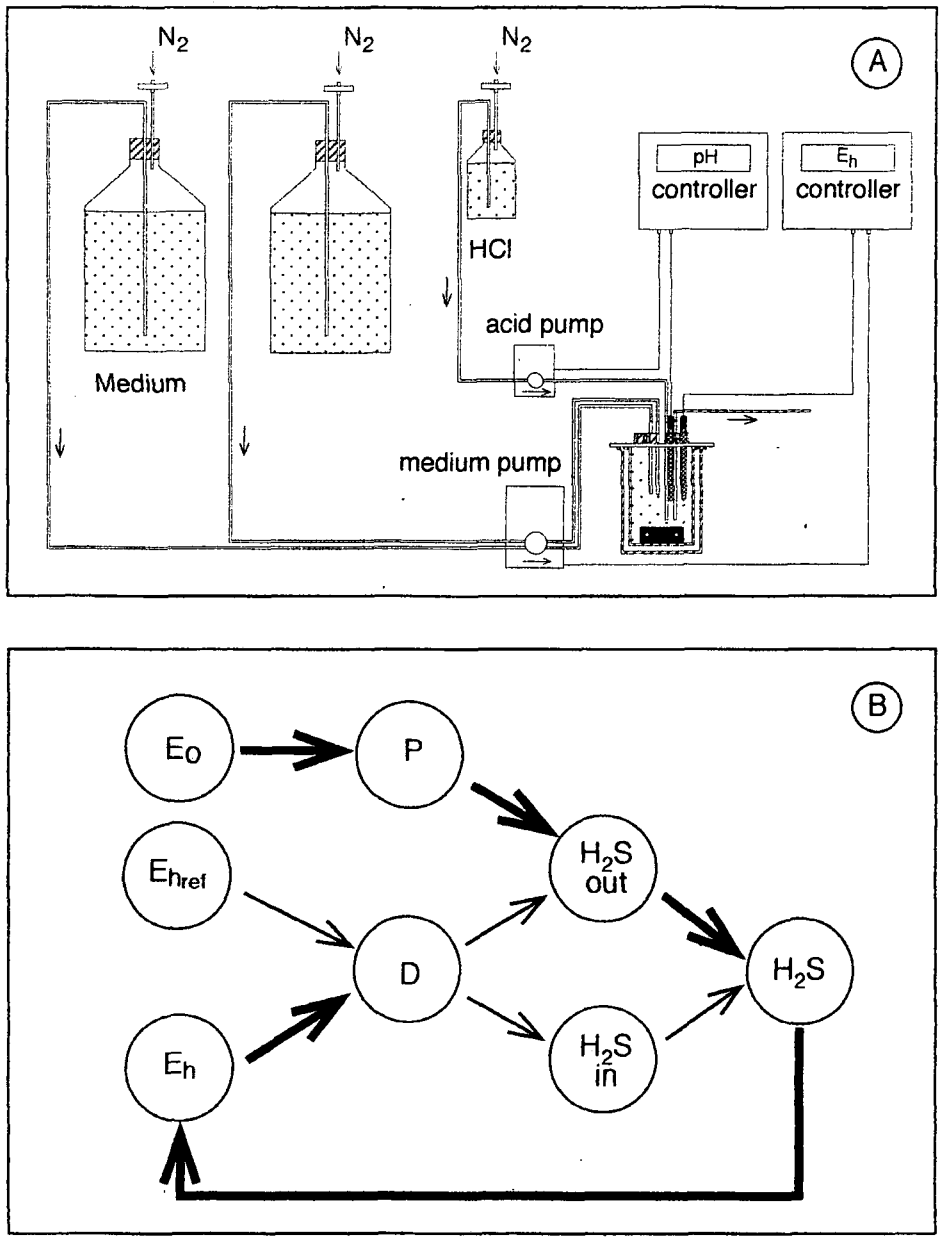

Fig. 4.1. (A) Diagram showing the different components of the redoxstat. The medium is prepared in two separated bottles and is pumped into the culture vessel. The pump speed (and hence the dilution rate of the culture) is set by the redox controller to a value proportional to the difference between the redox and a preestablished set point. The medium is neutralized as it enters the culture vessel through the addition of acid controlled by a pH titrator. (B) Outline of the mechanism involved in the regulation of the redoxstat. Thick arrows indicate the chain of events involved in the regulation. Irradiance (E_0) governs photosynthetic activity (P) which, in turn, contributes to sulfide removal from the vessel (H_2S_{out}). Variations in the rate of removal relative to the rate of supply (H_2S_{in}), affect the concentration of sulfide inside the culture vessel,and as the sulfide levels change, so does the redox potential (E_h). The difference between E_h and a preset reference value (E_{href}) finally determines the pump speed and the dilution rate of the system (D). The level of sulfide fixes the redox reading, and this controls the dilution rate of the system.

over an extended period of time. Redox potential and pH were continuously registered with a strip chart recorder.

Simulation. In order to predict the behavior of the system during transient states and to determine the time required for its stabilization, we carried out a numerical simulation with a simplified model which reflected the operating principles summarized above. The set of equations used in the simulation has been listed in Table 4.1 together with the values of the different parameters used and the values of the variables at the onset of the simulation. Equations 1 and 2 correspond to the classical equations defining the mass balance of the culture (Herbert et al 1956). These equations hold whether the culture is operated as a chemostat or as a turbidostat. Equation 3 describes the relationship between the specific growth rate (μ) of the organism and the magnitude of the limiting resource, either light (represented as the specific rate of light uptake, q_e) or sulfide (S). This equation combines a function relating photosynthetic activity to light availability with a hyperbolic function which accounts for sulfide limitation. For the description of the effect of irradiance on photosynthesis, we have chosen an exponential function (Webb et al 1974, Ley and Mauzerall 1982) which provides an adequate saturation response to high irradiances. The effect of sulfide limitation has been modeled with a hyperbola. Both equations have been combined by using an interactive model (Bader 1982). Equation 4 defines the specific rate of light absorption (q_e) as a function of biomass concentration (Mas and Sánchez submitted, Chapter 3). Equations 5 and 6, which have been determined experimentally from data in Fig. 4.2 and Fig. 4.3, predict the relative redox potential as a function of sulfide concentration and the dilution rate as a function of the difference between the redox reading and a preestablished set point. The simulation was carried out with a second-order Runge-Kutta algorithm with a time step of 0.01 h.

Table 4.1. Definition of the model used in the simulation.

	Element of the model	Reference
Equations		
(1)	$\frac{dS}{dt} = D \cdot (S_r - S) - \frac{\mu \cdot x}{Y}$	Herbert et al 1956
(2)	$\frac{dx}{dt} = (\mu - D) \cdot x$	Herbert et al 1956
(3)	$\mu = \mu_{max} \cdot \left(1 - e^{-\alpha \cdot q_e / \mu_{max}}\right) \cdot \left(\frac{S}{K_s + S}\right)$	This work
(4)	$q_e = \frac{L_0^2}{(L_0 + \varnothing \cdot f)^2} \cdot E_0 \cdot \left(1 - e^{-a^* \cdot x \cdot \varnothing \cdot f}\right) \cdot \frac{4}{x \cdot \pi \cdot \varnothing}$	Mas and Sánchez subm.[a]
(5)	*if* E_h - $E_{href} < 2.8097$ $D = 0$ *if* E_h - $E_{href} \geq 2.8097$ $D = -0.0154 + 0.0055 \cdot (E_h - E_{href})$	This work
(6)	$E_h = 226.16 - 68.13 \cdot \log S$	This work
Parameters		
	E_0 = $3.6e^6$ $\mu E.m^{-2}.h^{-1}$ (incident irradiance)	
	$\varnothing$ = 0.104 m (diameter of the culture vessel)	
	L_0 = 0.09 m (distance to light source)	
	S_r = 2000 μM (reservoir concentration of $S^=$)	
	E_{href} = 56 mV (redox set point)	
	a^* = 0.00035 $m^2.mg^{-1}$ protein (absorption coefficient)	Mas and Sánchez subm.[a]
	f = 1.8 (correction factor)	Mas and Sánchez subm.[a]
	α = 0.0006 mg protein. μE^{-1} (photosynthetic efficiency)	Mas and Sánchez subm.[b]
	μ_{max} = 0.195 h^{-1} (maximum specific growth rate)	Mas and Sanchez subm.[b]
	K_S = 7 μM (saturation constant for $S^=$)	Van Gemerden 1984
	Y = 23.6 mg protein.$mmol^{-1}$ $S^=$ (yield)	Unpublished
Variables		
	x = 44828 mg protein.m^{-3} (biomass concentration)	
	q_e = 98.2 $\mu E.mg^{-1}$ protein.h^{-1} (specific rate of light uptake)	
	S = 100 μM (concentration of hydrogen sulfide)	
	E_h = 90 mV (redox)	
	D = 0.173 h^{-1} (dilution rate)	
	μ = 0.173 h^{-1} (specific growth rate)	
	d = 34 mV (difference between E_h and E_{href})	

RESULTS

Relationship between E_h and sulfide concentration. In order to assess the empirical relationship between the redox reading and the concentration of sulfide, we performed an experiment in which the concentration of hydrogen sulfide in the culture vessel was gradually increased. For each concentration, the redox potential was recorded. The results, represented in Fig. 4.2, were used to fit a logarithmic equation. This equation has been used for the simulation described in this paper (Table 4.1, equation 6). Several replicates of the experiment (not shown) yielded curves with similar slopes (average of 68.9 ± 2.5 mV per decade), differing only in the offset of the equation. Since we were mainly interested in the difference E_h - E_{href} as a means of controlling the dilution rate, the redox values shown in Fig. 4.2 are relative. Calibration of the redox electrode was not completely reliable, because the reference element was poisoned by sulfide, which resulted in an certain drift (about 1%) after several days of continuous utilization. Because the electrode was permanently inserted in the culture, recalibration was not possible without a considerable risk of contamination. Therefore, we opted to carry out periodical measurements of the actual concentration of hydrogen sulfide. When the sulfide concentration departed from the preset value, the redox set point was slightly shifted to a new value. The setpoint increase was calculated as the product between the slope of the curve E_h versus sulfide (equation 6, Table 4.1) and the difference between the logarithms of the actual and the previous sulfide concentrations.

Relationship between D and the difference E_h - E_{href}. Regulation of this system is based on the fact that as the redox potential (E_h) departs from a preestablished setpoint (E_{href}), the controller generates a current proportional to the magnitude of the departure, which modulates the pump speed, and therefore, the dilution rate. Since E_h depends on the concentration of hydrogen sulfide (Fig. 4.2) and this, in turn, is affected by photosynthetic activity, the system generates a dilution rate proportional to the rate at which sulfide is being used by the organisms. Figure 4.3 represents the dilution rate (D) as a function of the difference E_h - E_{href} in a set of data extracted from a culture running during 46 days under several irradiance regimes. Data are clustered around four nuclei generated by each of the four irradiances used in the experiment. The straight line represented in Fig. 4.3 is the best fit obtained by linear regression and corresponds to equation 5 in Table 4.1. Although the actual values of the slope and the ordinate intercept depend largely on the characteristics and settings of the equipment being used, data from Fig. 4.3 indicate a predictable response over the range of conditions tested.

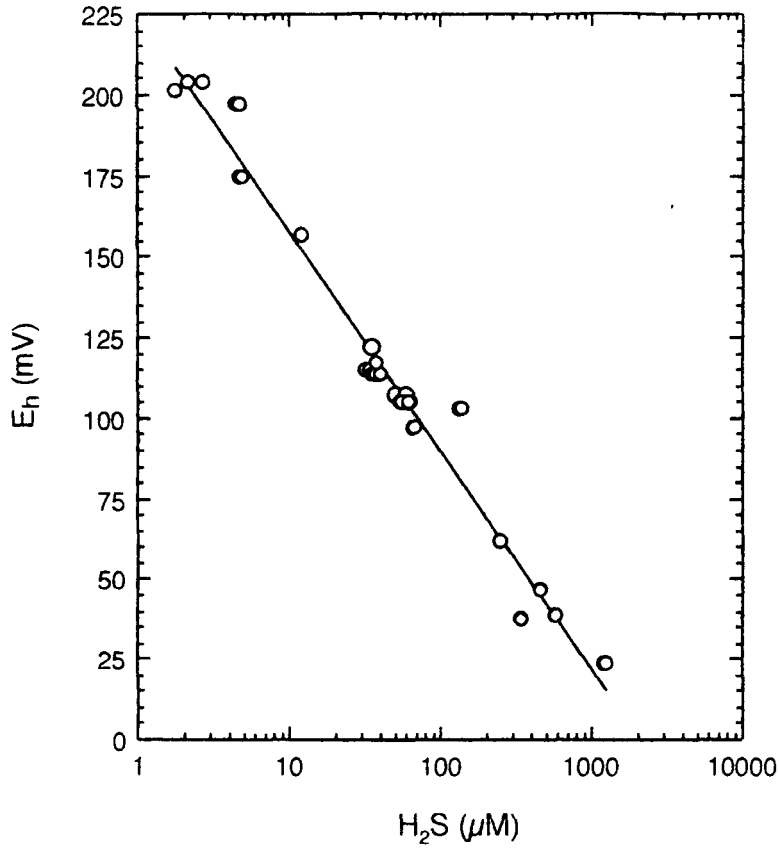

Fig. 4.2. Redox potential as a function of the concentration of hydrogen sulfide. The line corresponds to a logarithmic fit with the following equation: $E_h = 226.16 - 68.13 \log [H_2S]$.

Simulation of transient states. Application of the model described in Table 4.1 to a series of changes in illumination allowed prediction of the resulting sequence of events. The results of the simulation are represented as a function of time in Fig. 4.4. The first half of the figure reproduces the transition from a high to a low irradiance (1000 to 245 $\mu E.m^{-2}.s^{-1}$). The second half shows how initial conditions are restored after shifting to the initial light supply. Figure 4.4A indicates the variation in the concentration of sulfide throughout these transitions. When incident irradiance is reduced (t = 2h), the photosynthetic activity of the cells declines, and as a consequence, sulfide accumulates in the medium. As a result of sulfide accumulation, the redox potential drops (Fig. 4.4B) and the difference between E_h and E_{href} decreases from d_1 to d_2. This, in turn, decreases the dilution rate to a value at which the rate of sulfide supply equals the rate of sulfide utilization. The overall result is a culture with a lower activity and a lower rate of sulfide supply. An undesired side effect of this sequence of events is a temporary increase in the concentration of hydrogen sulfide. To restore the desired concentration of sulfide, the redox setpoint (E_{href}) is increased (t = 5h) yielding a transient decrease in the dilution rate (Fig.

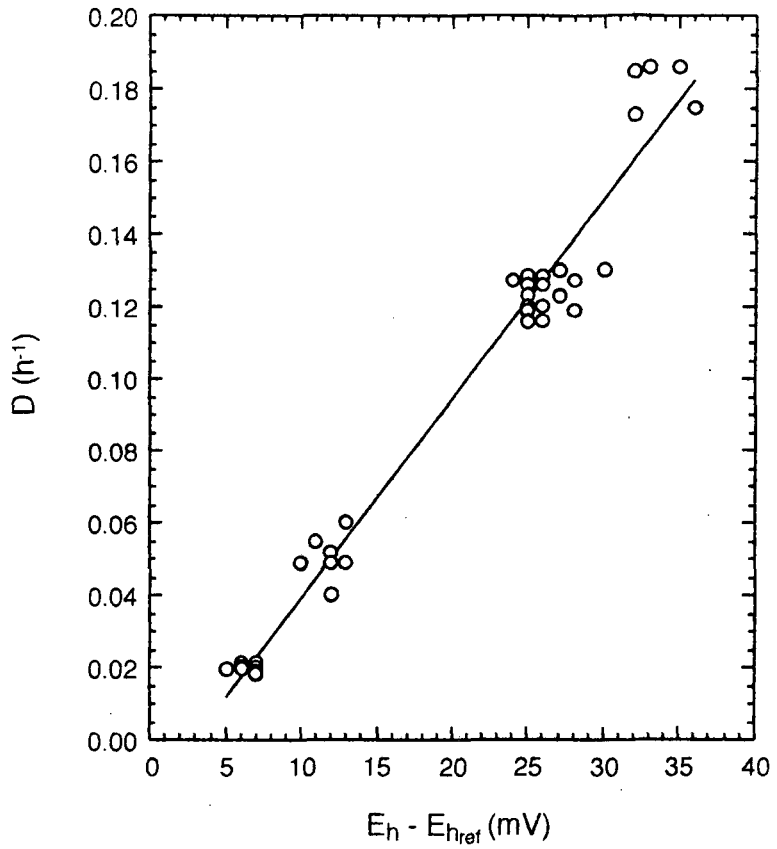

Fig. 4.3. Relationship between the dilution rate and the difference E_h - E_{href}. The line corresponds to the best linear fit: D = -0.0154 + 0.0055 (E_h - E_{href}).

4.4C). During this decrease, sulfide is used up faster than it is supplied. As a result, the concentration of sulfide returns to the initial value. From then on, the system remains stable until a new change is introduced.

The second half of Fig. 4.4 shows the sequence of events which results when irradiance is shifted up from 245 to 1000 $\mu E.m^{-2}.s^{-1}$ (t = 9h). As photosynthetic activity steps up and the culture consumes more sulfide, the redox potential increases, making the difference E_h - E_{href} larger and therefore increasing the dilution rate. In the new equilibrium, the enhanced sulfide supply, consequence of a higher dilution rate, matches the new photosynthetic activity, generating a stable situation with a higher turnover of this compound. As in the transient state described above, the concentration of hydrogen sulfide in this new equilibrium differs from the value initially established. To return the system to this concentration, the redox set point is lowered (t = 12h). This causes a temporary increase in the difference E_h - E_{href} and, as a consequence, in the dilution rate. During this short period, sulfide supply surpasses consumption, and therefore, its concentration rises to a new equilibrium.

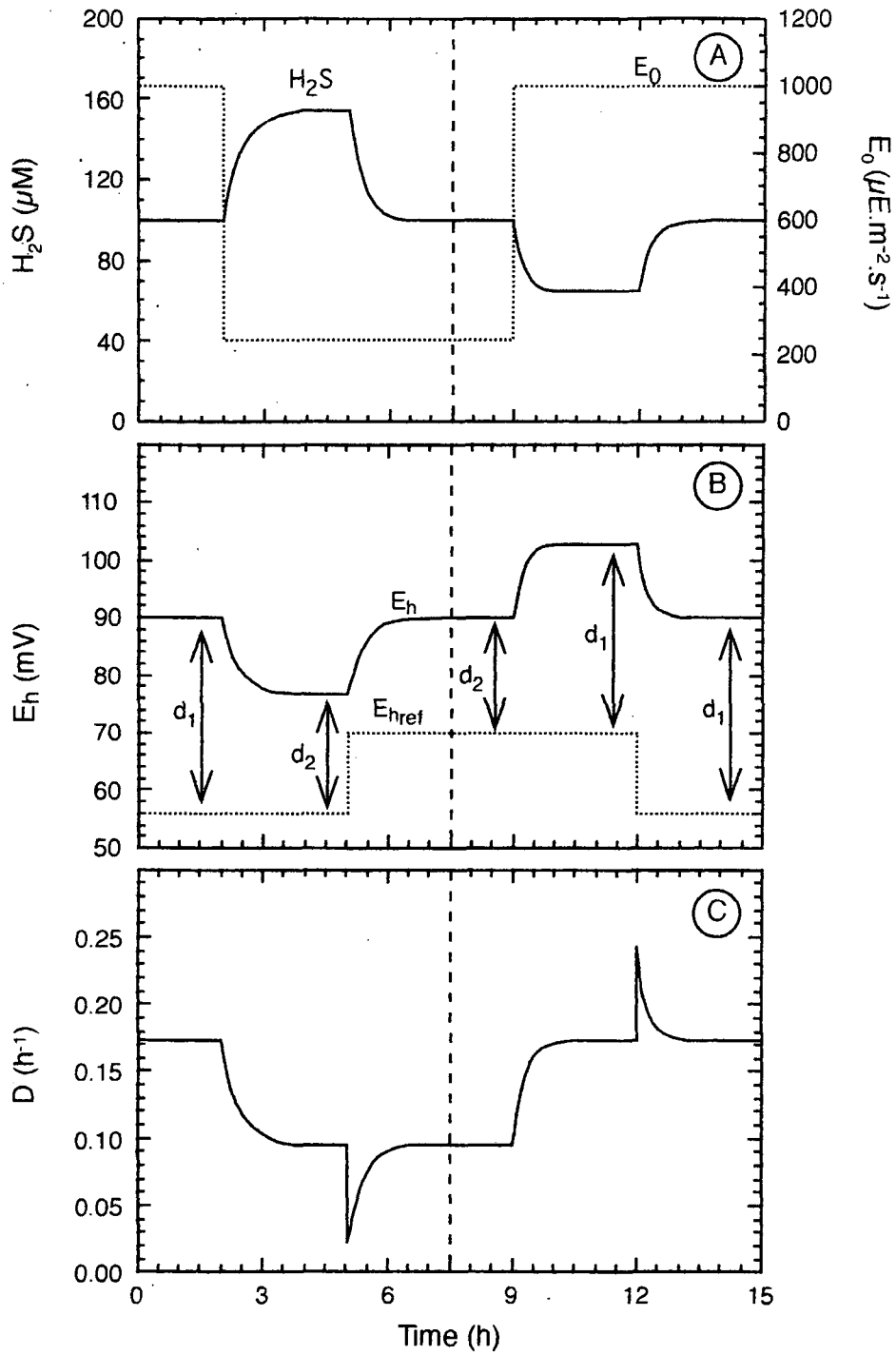

Fig. 4.4. Simulated time course of the transition from a high irradiance (1000 $\mu E.m^{-2}.s^{-1}$) to a low irradiance (245 $\mu E.m^{-2}.s^{-1}$) steady state (first half of the figure) and vice versa (second half of the figure). Data have been generated by numerical integration of the model described in Table 4.1. (A) Sulfide concentration (H_2S) and incident irradiance (E_0). (B) Redox (E_h) and reference redox (E_{href}). (C) Dilution rate.

Testing of the system. Although the simulation described above shows that the culture setup described in this paper is able to adjust to changes in irradiance, reaching a new equilibrium in a matter of hours, it was necessary to obtain experimental confirmation. To this end, we designed an experiment in which a culture of *C. vinosum* was subject to a series of step changes in incident irradiance. The experiment lasted for a total of 1125 h and, during this time, several variables (dilution rate, E_h, and sulfide concentration) were periodically determined. E_h and pH were also recorded continuously in order to provide an accurate account of the transient states.

One of these transient states, in which incident irradiance was shifted from a high value (113 $\mu E.m^{-2}.s^{-1}$) to a low value (23 $\mu E.m^{-2}.s^{-1}$), has been represented in Fig. 4.5. The transient state describes in detail the changes in pH and redox occurring between the second and the third steady states shown in Fig 4.6. Redox, which initially had a value of 113 mV, started to decrease at t = 0 reaching a new stable E_h of 84 mV after approximately 3 h. At t = 4 h, the redox set point was modified, and the culture readjusted to the initial redox potential. During the transition, the behavior of the system was the same as that predicted by the simulation in Fig. 4.4B. The complete transition required 8 h overall, well within the order of magnitude predicted by the model. pH, also represented in Fig. 4.5 was subject to oscillations with an amplitude caused by the hysteresis interval of the controller and a period determined by the dilution rate. When irradiance was high, pH oscillations had a short period, characteristic of a high dilution rate. After

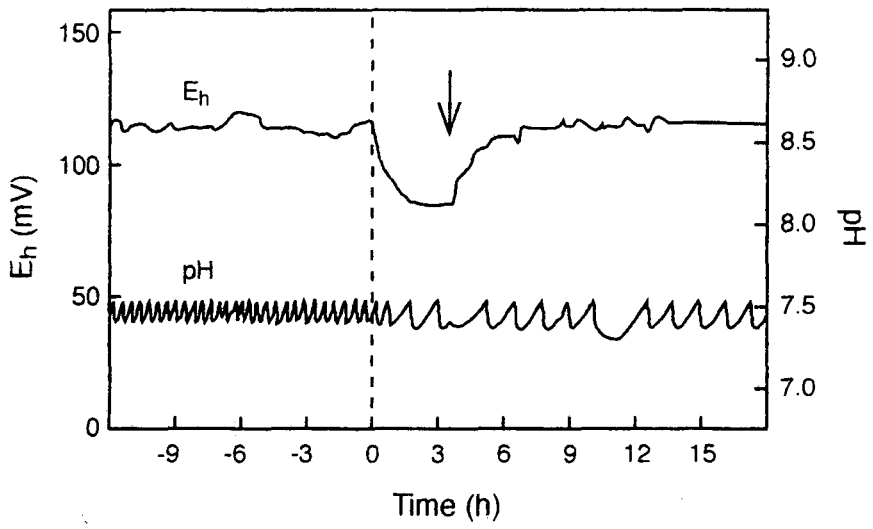

Fig. 4.5. Strip chart recording of pH and redox during the actual transition from a high irradiance (113 $\mu E.m^{-2}.s^{-1}$) to a low irradiance (23 $\mu E.m^{-2}.s^{-1}$) steady state. The change in incident irradiance occurs at t = 0. the position of the arrow indicates the point (t = 4) at which the set point was readjusted. Fluctuations in pH result from the discontinuous nature of the titration and are determined by the hysteresis interval of the titrator.

the shift down in irradiance the period increased immediately because of a virtually instantaneous drop in the dilution rate.

On a different time scale, periodic point measurements of sulfide concentration, dilution rate, and the difference E_h - E_{href} (Fig. 4.6) show that the system is stable during extended periods of time and, furthermore, provide additional indications that the new equilibrium can be reached in a relatively short period. As predicted by the simulation, the concentration of sulfide (S) showed little variation, remaining at approximately 50 μM for the length of the experiment (Fig. 4.6A). The same figure also shows the value of Sr (the reservoir concentration of sulfide) and the incident irradiance (E_0) for every steady state.

The difference E_h - E_{href}, which regulated the dilution rate of the system, has been represented in Fig. 4.6B as a function of time. This difference remained constant during each steady state, with average values of 34, 26, 12, and 6 mV. Transitions from a high to a low irradiance were very fast, and as a consequence, the new dilution rate (Fig. 4.6C) was established almost instantaneously, remaining constant for as long as irradiance was undisturbed.

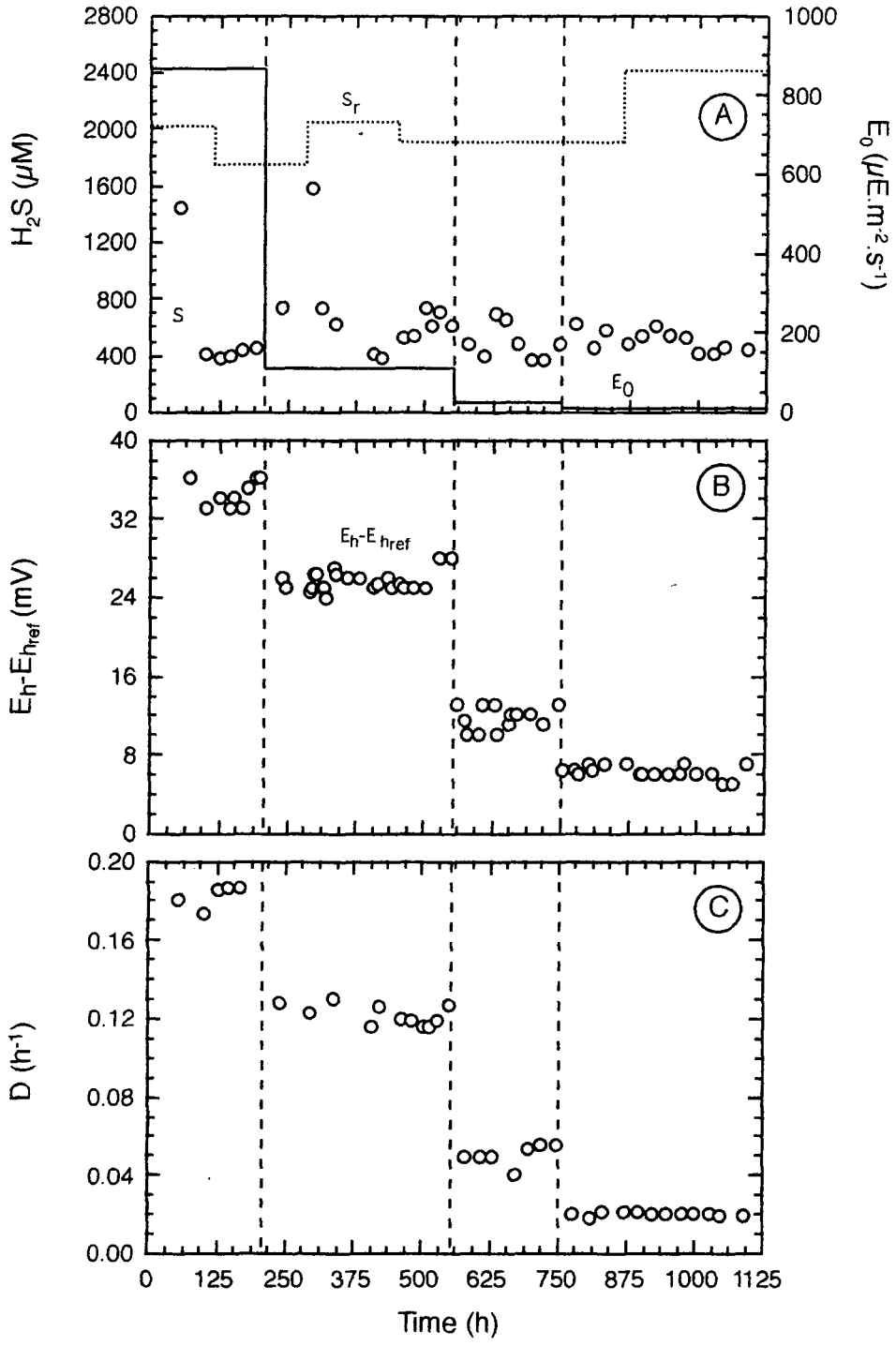

Fig. 4.6. Response of a culture of *C. vinosum* to a progressive series of step changes in irradiance. Vertical dashed lines indicate the points at which irradiance was decreased. (A) Incident irradiance (continuous line), concentration of hydrogen sulfide in the reservoir (dotted line), and concentration of hydrogen sulfide in the culture vessel (open circles, x10). (B) Difference between redox (E_h) and reference redox (E_{href}). (C) Dilution rate.

DISCUSSION

The system described in this paper allows the continuous operation of bioreactors of phototrophic sulfur bacteria while maintaining a constant concentration of hydrogen sulfide and coupling changes in the dilution rate to changes in the light supply. Although other types of culture, like chemostats and turbidostats, have been used in the past, neither of the two seems to provide an adequate solution for the control of bioreactors of phototrophic sulfur bacteria.

In chemostats, in which light input and dilution rate are not linked, the concentration of nutrients invariably changes when either one of the two variables is modified. When the dilution rate is kept constant and incident irradiance changes (Aiking and Sojka 1979), microorganisms keep growing at the same specific growth rate. A decrease in irradiance will automatically reduce biomass down to levels at which the specific rate of light uptake (the amount of light available per cell) (Sánchez and Mas 1996) is high enough to support growth at the established dilution rate. Because the specific growth rate and the amount of energy per cell remain constant, this approach does not result in different degrees of light limitation, but rather in different states characterized by different biomass levels, and as a consequence, different concentrations of residual nutrients in the medium.

If irradiance remains constant but the dilution rate changes (Eppley and Dyer 1965, Gons 1977, Aiking and Sojka 1979) biomass will change in order to adjust the share of energy per cell to a new specific growth rate. As in the previous case, changes in biomass give rise to variations in the concentration of nutrients in the culture medium.

In turbidostats, the concentration of substrate is indirectly kept constant through a control of the biomass levels (measured as optical density). Increases of optical density above a predetermined value result in an increase in the dilution rate of the culture, which then tends to decrease biomass down to the original levels. This system, although widely used (Beardall and Morris 1976, Raps et al 1983, Post et al 1984, Post et al 1985, Berner et al 1989) cannot be utilized with phototrophic sulfur bacteria. These organisms accumulate sulfur in important amounts (40 to 50% of their dry weight, Mas and Van Gemerden 1995) during their growth on reduced sulfur compounds. This sulfur increases absorbance dramatically, giving rise to a wide range of turbidity values for the same biomass. Besides, indirect control through optical density can only be applied to homogeneous suspensions of microorganisms, a serious drawback when dealing with cultures which often can contain immobilized populations.

Besides being useful for the control of sulfide-oxidizing bioreactors, the experimental setup described in this paper can be successfully utilized for the study of light limitation in

phototrophic sulfur bacteria. This system is able to generate stable conditions during extended periods of time while maintaining a constant concentration of hydrogen sulfide inside the culture vessel independently of the light supply. Thus, it is possible to obtain different steady states in which the physiological events observed are not a consequence of collateral effects due to the excess or limitation of substrate. Furthermore, the microorganisms grow at a rate imposed exclusively by the conditions of illumination.

This culture setup provides a reliable mechanism to maintain a low and constant concentration of hydrogen sulfide in cultures of phototrophic sulfide oxidizers, allowing highly stable steady states and avoiding washout of the system under conditions likely to affect photosynthetic performance. Thus, events such as a sudden increase in the sulfide load, a transient decrease in irradiance, or variations in temperature, dissolved organic matter, oxygen, or pH will only result in a temporary change in the dilution rate leaving the sulfide and biomass levels unaltered.

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CHAPTER 5

ACCLIMATION OF *Chromatium vinosum* TO LIGHT-LIMITING CONDITIONS: VARIATIONS IN PIGMENT CONTENT AND QUANTUM YIELD

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CHAPTER 5

ACCLIMATION OF Chromatium vinosum TO LIGHT-LIMITING CONDITIONS: VARIATIONS IN PIGMENT CONTENT AND QUANTUM YIELD

The photosynthetic response of the purple sulfur bacterium *Chromatium vinosum* DSM 185 has been analyzed. The microorganism was grown in continuous culture at four different degrees of illumination. Samples were taken from the effluent of the culture and incubated at different irradiances to determine the specific rate of sulfur oxidation as a measure of the photosynthetic activity of the organism. The activities obtained were plotted as a function of the specific rate of light uptake, and for each set of data a photosynthesis equation was fitted, which allowed the estimation of P_{max} (photosynthetic capacity), ϕ (quantum yield) and m (maintenance coefficient). The results indicate that light limitation induces an increase in the quantum yield for photosynthesis (ϕ), which in turn result in an increased photosynthetic efficiency (α). The photosynthetic capacity (P_{max}), on the contrary, remained constant for all the conditions of illumination tested, while the maintenance expenses (m) were higher under light limitation.

INTRODUCTION

Phototrophic sulfur bacteria develop in anoxic environments in which light penetration is often poor. As a consequence, light seems to be a limiting resource (Bergstein et al 1979, Parkin and Brock 1980, Guerrero et al 1985, Mas et al 1990), and it can be expected that these organisms will have developed mechanisms to compensate for this limitation and increase their chances for survival. Although adaptation to limiting irradiances has been extensively studied in planktonic algae (Beale and Appleman 1971, Gons 1977, van Liere 1979), little is known on whether and to what extent phototrophic sulfur bacteria are able to adapt to these situations. In general, when irradiance decreases below the saturating value for photosynthesis, the specific content of pigments increases. This phenomenon has been observed in green algae such as *Chlorella* (Myers 1946, Beale and Appleman 1971), *Scenedesmus protuberans* (Gons and Mur 1975), *Euglena* (Cook 1963), some marine diatoms (Yoder 1979), in cyanobacteria (Miers and Kratz 1955, van Liere and Mur 1978), and in anoxygenic phototrophs such as *Rhodopseudomonas sphaeroides* (Sistrom 1962), *Rhodospirillum rubrum* (Holt and Marr 1965, Uffen 1973), *Rhodomicrobium vannielii* (Trentini and Starr 1967), *Chlorobium* (Broch-Due et al 1978), and *Chromatium vinosum* (van Gemerden 1980).

Besides these variations in the pigment content, some organisms are also able to change their chlorophyll-carotenoids ratio. This has been demonstrated for the marine dinoflagelate *Glenodinium* by means of an increase in the peridinin/Chl a ratio (Prézelin 1976, 1981) and for some cyanobacteria as an increase in the ratio of phycocianin/Chl a (Foy and Gibson 1982, Post et al 1985). In *Chromatium minus*, it has been observed a dependence between the proportion carotenoids/BChl a and the specific content of BChl a. This proportion increases according to the specific content of BChl a (Montesinos 1982).

These changes in the pigment composition of the photosynthetic apparatus usually result in variations in the photosynthetic response (Halldal 1970). This response, defined as the relationship between photosynthetic activity and irradiance, adjusts to a saturation curve described by a number of different empirical equations (Smith 1936, Van Oorschot 1955, Steele 1962, Vollenweider 1965, Bannister 1974, Jassby and Platt 1976, Iwakuma and Yasuno 1983), which define two basic parameters: P_{max}, the maximum rate of photosynthesis, and α, the initial slope of the curve. A third parameter I_k is sometimes utilized, which in fact is a function of the previous two ($I_k = P_{max}/\alpha$). The parameter α indicates the photosynthetic efficiency of the organism and it depends on the light harvesting capacity (related to the specific content of pigments) and on the quantum yield for photosynthesis (which can be regarded as the conversion efficiency of absorbed light to biological activity). Under light-limiting conditions, the pigment content increases and so does α. Increases in pigment content can reflect either an increase in the total number of reaction centers (PSU or photosynthetic units) and their associated antennal pigment or an increase in the antennal pigments associated with a constant number of reaction centers. In the first case, the increase in the number of PSU would result in an enhanced photosynthetic capacity (P_{max}). This strategy can be found in marine phytoplankton organisms such as *Scenedesmus* (Senger and Fleischhacker 1978, Falkowski and Owens 1980), as well as in algae like *Chlorella* and *Dunaliella*, which grow in high-light environments. Changes in the size of PSU, on the contrary, would result in variations of α. The cyanobacterium *Oscillatoria* (Foy and Gibson 1982, Post et al 1985), an organism which can be found dominating waters with a high phytoplankton biomass and low-light conditions, seems to respond with this second strategy. Some organisms, like *Microcystis*, are able to change both P_{max} and α (Zevenboom and Mur 1984), suggesting that these species would be more adaptable in order to grow either in high (change in P_{max}) or in low-light environments (change in α).

This paper attempts to analyze the effect of light limitation on the characteristics of the photosynthetic apparatus of the purple sulfur bacterium *Chromatium vinosum* DSM 185 in order to understand the physiological mechanisms involved in the adaptation to light-limiting

irradiances. The information obtained should allow a better understanding of how purple sulfur bacteria adapts to the low-light environments they usually live in.

MATERIALS AND METHODS

Organism and growth conditions. The work was carried out with *Chromatium vinosum* DSM 185 growing in mineral medium with carbonate as the only carbon source and hydrogen sulfide as electron donor (Mas and Van Gemerden 1987). The experiments were performed in a redox-controlled sulfidostat designed to provide an automatic adjustment of the dilution rate, and constant sulfide and biomass concentrations when subject to changes in irradiance (Sánchez et al 1996, Chapter 4). The concentration of sulfide in the reservoir of the continuous culture ($S_{R\text{-sulfide}}$) was set at 2 mM. The culture was illuminated with a variable number of light bulbs arranged to provide different incident irradiances (868, 113, 23 and 7 $\mu E.m^{-2}.s^{-1}$). The specific rates of light uptake (q_e) determined for each of these irradiances (Sánchez and Mas 1996, Chapter 2) were 103, 30.9, 16.8 and 7.1 $\mu E.mg^{-1}$ protein.h^{-1}.

Sampling and analyses. Samples were collected from the effluent of the culture, in the dark and on ice, divided in subsamples and centrifuged. After discarding the supernatants, the pellets were frozen and kept at -20 °C until the analyses were performed.

The concentration of residual hydrogen sulfide was mesured in 1-ml samples taken directly from the culture vessel with a syringe, fixed with 20 ml of zinc acetate and assayed according to Pachmayr (1960) in a total volume of 50 ml, as described by Trüper and Schlegel (1964).

Bacteriochlorophyll a and sulfur were measured spectrophotometrically at 770 nm and 265 nm respectively, in methanol extracts. The actual concentrations of both compounds were calculated using the extinction coefficients provided by Stal et al (1984). Proteins were determined with the Folin phenol reagent (Lowry et al 1951) after extraction of the pellet with methanol to remove sulfur, and subsequent solubilization in 1 N sodium hydroxide for 10 min at 100 °C (Herbert et al 1971).

Measurement of photosynthetic activity. Photosynthetic activity was measured as the rate of sulfur oxidation in cultures depleted of sulfide. In the absence of sulfide, cells containing intracellular sulfur carry out the following reaction:

$$2S^{o} + 3CO_2 + 5H_2O + light \longrightarrow 2SO_4^{2-} + 3\langle CH_2O\rangle + 4H^{+} \qquad (1)$$

in which CO_2 is fixed to organic carbon using the reducing power released in the oxidation of sulfur to sulfate. Thus, because in sulfur containing cells incubated in the absence of sulfide photosynthesis relies exclusively on stored sulfur as electron donor, the specific rate of sulfur oxidation (P) provides a good estimate of the photosynthetic activity of the organism.

Thus, photosynthetic activity was measured for every steady state using the following procedure. Samples were directly extracted from the culture and dispensed into 60-ml screwcapped bottles . These bottles were kept for 10 min in the light under nitrogen pressure (incident irradiance of 40 $\mu E.m^{-2}.s^{-1}$) in order to allow for the depletion of the residual sulfide. After checking that sulfide was actually depleted, the samples were incubated at eight different irradiances (ranging from 1.5 to 130 $\mu E.m^{-2}.s^{-1}$ incident irradiance) using an incandescent light source. At regular time intervals, 5 ml subsamples were extracted from each bottle, replacing the volume with oxygen-free nitrogen. The samples were placed immediately in an ice-water bath at 0°C in order to stop metabolic activity and centrifuged. The pellets were stored at -20°C for later analysis of sulfur and protein.

From the protein and sulfur data, kinetics of sulfur consumption were plotted for every irradiance and a linear regression was applied. The slope of every series yielded the specific rate of sulfur oxidation (P) for each of the illuminations tested.

Photosynthesis-light curves. The parameters of the photosynthesis-light and the μ-light curves were estimated by fitting the following equation (derived from Webb et al 1974) to the data using a non-linear regression:

$$P = P_{\max} \cdot \left(1 - e^{-\phi \cdot (q_e - m) \cdot P_{\max}}\right) \qquad (2)$$

in which P is the estimated activity and P_{max} is the light-saturated activity. In the original equation, activity was a function of the incident irradiance, and two parameters were estimated: P_{max} and α, the initial slope of the curve. In our equation, activity is plotted as a function of the specific rate of light uptake (q_e), a measure of the amount of light available per cell, and the slope of the curve yields the quantum yield (ϕ). We have also added another parameter, m, the maintenance coefficient, which corresponds to the x-intercept and accounts for the maintenance expenses of the organism.

Determination of the absorption coefficient (a*). The absorption coefficient, also referred to as absorption cross-section, has dimensions of $m^2.mg^{-1}$ protein and relates the photon flux ($\mu E.m^{-2}.s^{-1}$) to the rate at which light is absorbed per unit biomass ($\mu E.mg^{-1}$

protein.h^{-1}). The larger the cross-section, the higher the amount of light that can be harvested per unit biomass in a certain time interval. In practice, the cross-section can be calculated as the quotient between the specific rate of light uptake (q_e) and the incident irradiance E_0.

RESULTS

Effect of light irradiance on the specific growth rate and the content of bacteriochlorophyll. In order to determine the effect of light limitation on growth, *C. vinosum* was grown in a continuous culture at irradiances ranging from 7 to 868 $\mu E.m^{-2}.s^{-1}$ until reaching steady state. For each irradiance, the culture adjusted to a characteristic dilution rate (Sánchez et al 1996, Chapter 4) which in steady state equalled the specific growth rate of the organism. When plotting the specific growth rate against incident irradiance, a characteristic saturation curve appeared (Fig. 5.1), showing that light limitation started below 300-400 $\mu E.m^{-2}.s^{-1}$. Although light limitation was attained by changing the incident irradiance, this is not a good measure of the energy actually available to the organisms because it does not take into account the effect of self shading. Light limitation is better characterized by the specific rate of light uptake (q_e) (Sánchez and Mas 1996, Chapter 2) which has been used as the independent variable in the following figures. However, we have represented μ vs E_0 because it is still the most habitual representation in these kind of works, and thus, provides a curve which can be compared to other experiments from the literature.

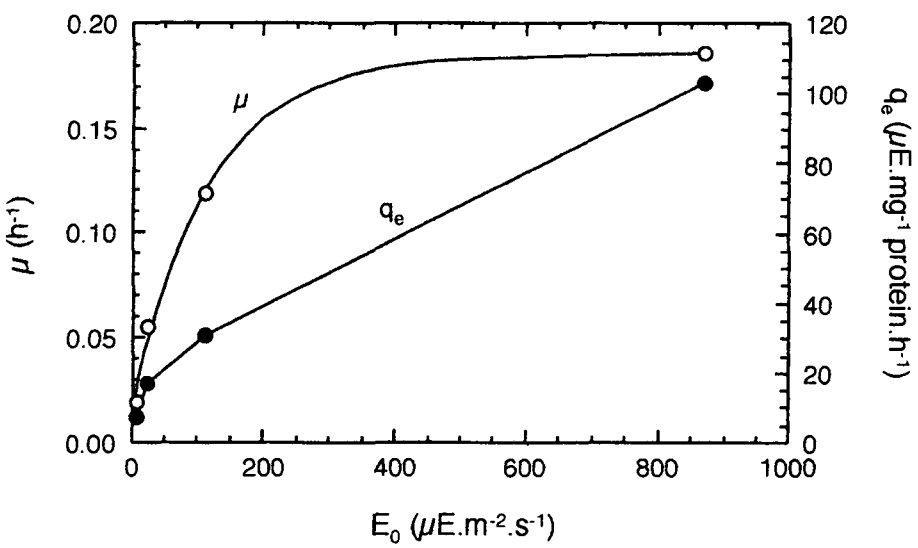

Fig. 5.1. Specific growth rate (μ) and specific rate of light uptake (q_e) as a function of incident irradiance in steady-state cultures of *C. vinosum*.

Fig. 5.1 also shows the relationship between the specific rate of light uptake (q_e) and the incident irradiance. As could be expected, q_e increased as the amount of light reaching the culture vessel was higher, but the specific rate at which light is absorbed depends also on other factors such as the biomass of the culture, the presence of refractile structures within the cells, and the size of the vessel (Mas and Sánchez submitted, Chapter 3).

Fig. 5.2A shows the relationship between the specific growth rate and q_e in steady-state cutures of *C. vinosum*. Two parameters can be estimated from this plot: μ_{max}, the maximum specific growth rate, and μ_e, or cell maintenance rate constant, which can be obtained from the extrapolation of the μ-q_e plot towards the ordinate. μ_e eliminates the necessity to express the maintenance energy in units of irradiance, since it has units of h^{-1}. In this work, it has been found a value of μ_e for *C. vinosum* of -0.025 h^{-1}, which compares well with μ_e values reported for other purple sulfur bacteria, which range between -0.01 and -0.04 h^{-1} (Holt and Marr 1965, Göbel 1978, Van Gemerden 1980, Veldhuis and Van Gemerden 1986). μ_{max} has a value of 0.195 h^{-1}, somewhat higher than other values previously found for this microorganism (Van Gemerden 1980).

The specific content of BChl a has also been represented as a function of q_e (Fig. 5.2B). As the light supply decreased, the Bchl a contents increased from 24.0 to 85.6 μg BChl a.mg^{-1} protein. Variations in a similar range (from 5 to 85 μg BChl a.mg^{-1} protein) have already been described for other purple sulfur bacteria (Takahashi et at 1972, Matheron 1976, Van Gemerden 1980, de Wit 1989, Mas and Van Gemerden 1992).

Photosynthesis-light curves. In order to determine the photosynthetic response of *C. vinosum*, samples were taken directly from the culture and incubated at eight different irradiances ranging from 1.5 to 130 $\mu E.m^{-2}.s^{-1}$ for a short-term experiment. Subsamples were taken at regular intervals for the measurement of the specific content of sulfur. The kinetics of sulfur consumption were plotted and a linear regression was adjusted. The slope of each plot provided the specific rate of sulfur oxidation in μmol S°.mg^{-1} protein.h^{-1} for every irradiance. These photosynthetic activities were plotted against q_e and represented in Fig. 5.3. The four plots corresponded to saturation curves. The model described in Material and Methods (equation 2) fitted well to experimental data and allowed the determination of the quantum yield (ϕ), the photosynthetic capacity (P_{max}) and the maintenance coefficient (m), which are summarized in Table 5.1. The specific content of pigments for every steady state and the standard error of each measure are also indicated. The quantum yield, which was 0.23 μmol S°.μE^{-1} under light saturation, increased almost three times, up to 0.68 μmol S°.μE^{-1} when the light supply became limiting.

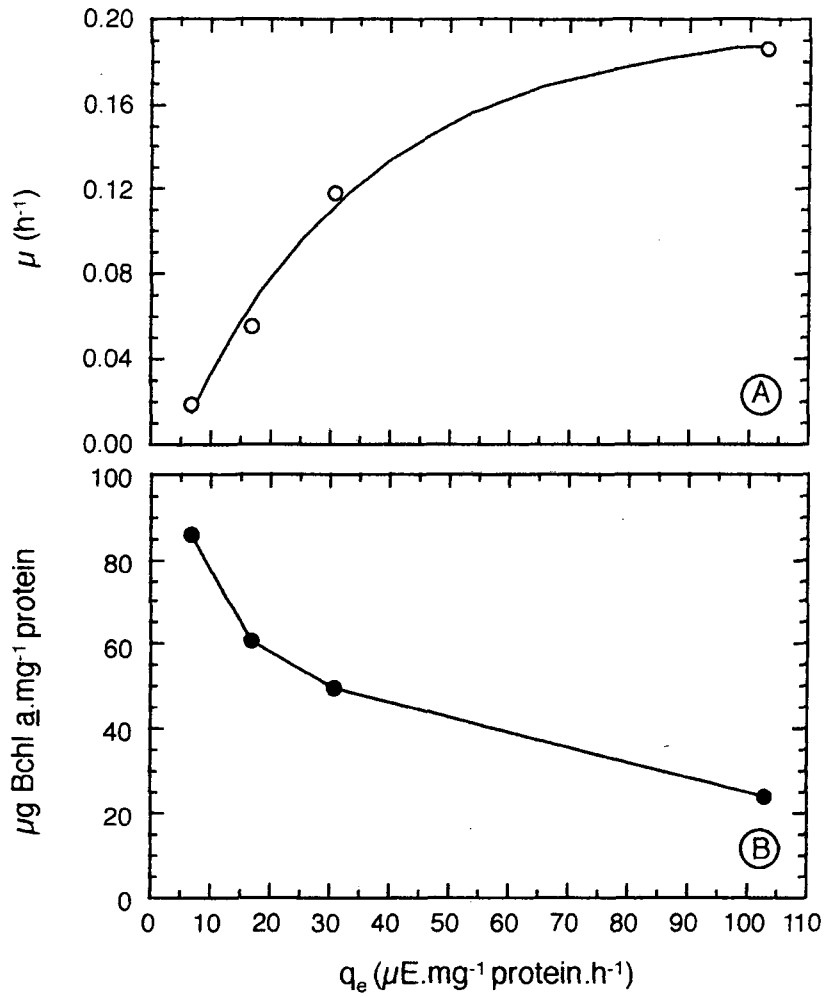

Fig. 5.2. Variation of the specific growth rate (A) and the specific pigment content (B) as a function of the specific rate of light uptake (q_e) in steady-state cultures of *C. vinosum*.

P_{max} is a measure of the photosynthetic capacity of the organism and it depends on the number of reaction centers in the photosynthetic apparatus, as well as on their reaction time (time required to absorb and process a photon). In our experiments, P_{max} turnt to be practically constant and it was not affected by the conditions of illumination.

The maintenance coefficient m, which indicates the quantity of energy that the microrganism requires for maintenance processes (turnover of cell material, osmotic equilibrium, motility, etc.) reached the highest value (3.37 μE.mg^{-1} protein.h^{-1}) in cells grown at the lowest irradiance.

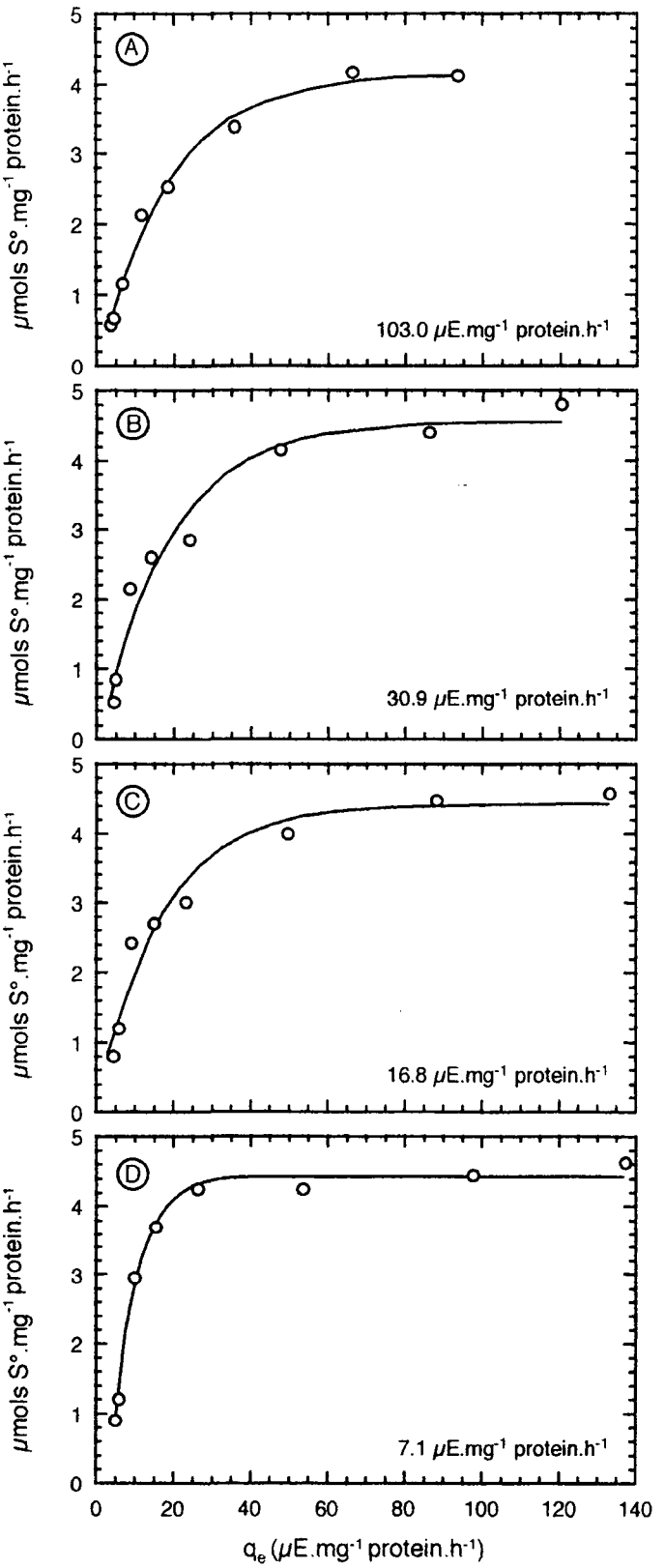

Fig. 5.3. Relationship between the photosynthetic activity (P), measured as μmol S° oxidized per mg of protein per hour, and the specific rate of light uptake (q_e) from steady-state samples of *C. vinosum*.

Table 5.1. Estimates of the parameters ϕ, P_{max} and m obtained by non-linear regression from the photosynthesis-q_e curves from cultures of *C. vinosum* grown at different light supplies. The standard errors have also been indicated.

q_e (μE.mg^{-1} protein.h^{-1})	BChl a/Protein (μg.mg^{-1})	ϕ (μmol S°.μE^{-1})	P_{max} (μmol S°. mg^{-1} protein.h^{-1})	m (μE.mg^{-1} protein.h^{-1})
103.00	23.96 ± 1.17	0.229 ± 0.025	4.150 ± 0.133	0.696 ± 0.760
30.87	49.67 ± 2.18	0.251 ± 0.059	4.550 ± 0.265	0.552 ± 1.830
16.80	60.90 ± 4.93	0.266 ± 0.062	4.417 ± 0.226	0.011 ± 1.995
7.06	85.62 ± 1.20	0.684 ± 0.070	4.429 ± 0.084	3.372 ± 0.315

Absorption coefficient versus specific rate of light uptake. Fig. 5.4 shows the relationship between the absorption coefficient a* and q_e. Two curves have been represented depending on whether a* is expressed as a cross-section in units of m^2 per mg protein or in units of m^2 per mg Bchl a. When a* is expressed as the protein cross-section, an increase can be observed at low values of q_e. The opposite occurs when a* is expressed as a Bchl a cross-section. This apparently contradictory observation results from the fact that, at low irradiances, the specific content of Bchl a per mg of protein is higher, and therefore, these proteins are able to absorb more light. However, these increased Bchl a contents result in mutual shading between pigment molecules, which translates into a lower absorption coefficient for the bacteriochlorophyll (Dubinsky 1992).

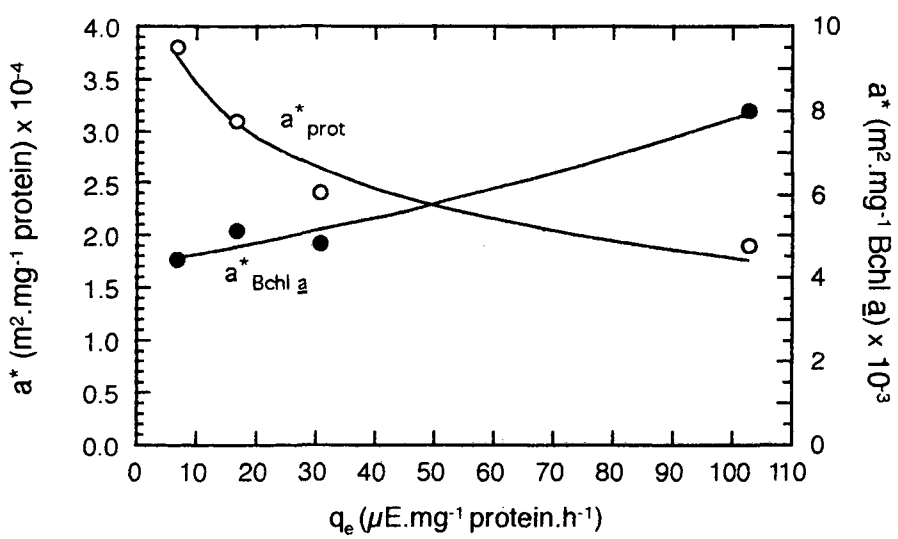

Fig. 5.4. Absorption coefficients, expressed as $m^2.mg^{-1}$ protein (a^*_{prot}) and as $m^2.mg^{-1}$ Bchl a ($a^*_{Bchl\ a}$), as a function of the specific rate of light uptake (q_e) in steady-state cultures of *C. vinosum*.

DISCUSSION

The data shown above indicate that *C. vinosum* has different mechanisms to cope with light limitation. These mechanisms include shifts in pigment content and changes in the photosynthetic activity of the light harvesting system.

The specific content of pigments changed between 24 and 85 μg BChl a.mg^{-1} protein. These values are comparable with other specific contents described previously in the family Chromatiaceae, which range between 5 and 85 μg BChl a.mg^{-1} protein (Takahashi et al 1972, Matheron 1976, van Gemerden 1980, de Wit 1989, Mas and van Gemerden 1992). Other phototrophic bacteria (Rhodospirillaceae and Chloroflexaceae), have specific contents in the same order (Trentini and Starr 1967, Aiking and Sojka 1979, Schmidt 1980), except for the family Chlorobiaceae, where higher specific contents (between 60 and 1557 μg BChl a.mg^{-1} protein) have been found (Cohen-Bazire 1963, Matheron 1976, Broch-Due et al 1978, Schmidt 1980, Montesinos 1982, Overmann et al 1992). This can be ecologically relevant since high specific contents could allow these organisms to proliferate in environments with even lower irradiances.

Data obtained from the short-term incubations carried out in the present work show that *Chromatium* changes the characteristics of its photosynthetic apparatus in order to maximize the photosynthetic response at light-limiting irradiances. The fact that the increase in pigment content does not affect P_{max} suggests that light-limited bacteria would augment the pigments associated to their reaction centers rather than changing the number of PSU, a strategy reported for the cyanobacterium *Oscillatoria*, usually found in systems with a high biomass and a low irradiance (Foy and Gibson 1982, Post et al 1985).

It is also quite remarkable that the maintenance coefficient (m) increased when the light supply was very low. Stouthamer et al (1990) proposed that, when organisms grow slowly, the ratio protein/RNA increases. Given that the turnover of protein is more expensive energetically than that of RNA, m increases at low μ. On the contrary, when bacteria are not light-limited, as they grow faster, they have a comparatively lower protein/RNA ratio and their maintenance expenses decrease.

While P_{max} was constant at all the irradiances tested, the quantum yield increased considerably under light limitation from 0.23 to 0.68 μmol S°.μE^{-1}. Since quantum yields found in the literature are usually expressed as μmol C per μE absorbed, comparison between both could only be achieved after converting our data to the same units. Conversion was carried out using a factor of 1.5 μmol C fixed per μmol S° oxidized, derived from the stoichiometry of reaction 1. The

converted values, ranging between 0.34 and 1.03 μmol C.μE^{-1} were much higher (almost ten times) than the yields previously reported in the literature. Thus, Bannister (1974) estimated that ϕ_{max} can't surpass 0.10 μmol C.μE^{-1} absorbed and it can't fall below 0.04 μmol C.μE^{-1} absorbed. Dubinsky and Berman (1976) estimated a value for the maximum quantum yield of 0.07 μmol C.μE^{-1} and Platt and Jassby (1976) found values for the quantum yield between 0.048 and 0.064 μmol C.μE^{-1} for marine plankton. Geider and Osborne (1986) reported a mean value of 0.09 μmol C.μE^{-1} (assuming a photosynthetic quotient of 1.4 mol O_2.mol^{-1} CO_2) for *Nannochloris atomus*, which compared well with their previous observations in the diatom *Phaeodactylum tricornutum*. Other observations show even lower quantum yields (Dubinsky et al 1984, Lewis et al 1985, Morel et al 1987).

This remarkable difference in quantum yields is a consequence of the fact that quanta meters only measure light in the interval between 400 and 700 nm, while purple sulfur bacteria absorb light well beyond this interval, up to 900 nm. The result is an underestimation of the actual amount of light absorbed by the organisms. In order to determine the fraction of light absorbed and not taken into account, we carried out two new steady states in which illumination was provided by incandescent light bulbs placed behind a heat reflecting mirror (Edmund Scientific) that reflected light above 700 nm. The mirror eliminated the wavelengths not detected by the light meter, but which could have been used by the microorganisms. With the data obtained, it was possible to estimate the magnitude of the difference between the actual and the measured rates of light uptake. It turned out that light beyond 700 nm contributed with 86% of the quanta absorbed by the organism, while only 14% came from the interval between 700 and 400 nm. The quantum yields recalculated using this information had values between 0.032 and 0.095 μmol S°.μE^{-1}, which correspond to 0.048 - 0.140 μmol C.μE^{-1}. These estimates compare very well with the data reported above for algae, although the higher value (0.140 μmol C.μE^{-1}) is somewhat larger than the maximum value of 0.10 μmol C.μE^{-1} observed in algae. This difference could be explained by the fact that oxygenic phototrophs need energy to fix CO_2 and to obtain reducing power from water. Purple sulfur bacteria still need to fix CO_2, but they obtain reducing power from the oxidation of reduced sulfur compounds, a process energetically less expensive than splitting a water molecule. Besides, oxygenic phototrophs can have significant losses due to photorespiration, a process which does not occur in anoxygenic phototrophs.

The absorption coefficients found for *C. vinosum* (a^*_{prot} = 0.00019 - 0.00038 m^2.mg^{-1} protein, $a^*_{Bchl\ \underline{a}}$ = 0.0044 - 0.0080 m^2.mg^{-1} BChl <u>a</u>) compare very well from those reported in the literature for algae. Thus, the chlorophyll cross-section of *Phaeodactylum tricornutum* ranged between 0.0037 and 0.0045 m^2.mg^{-1} Chl <u>a</u>, while the protein cross-section (calculated from the Chl:C ratio, assuming that carbon, as proteins, constitutes 50% of the dry weight) changed

between 0.00030 and 0.00032 $m^2.mg^{-1}$ protein (Geider et al 1986). For *Nannochloris atomus*, the chlorophyll and protein cross-sections ranged between 0.003-0.006 $m^2.mg^{-1}$ Chl a, and 0.00012 - 0.00032 $m^2.mg^{-1}$ protein respectively (Geider and Osborne 1986). Morel et al (1987) reported chlorophyll cross-sections between 0.0084 and 0.0189 $m^2.mg^{-1}$ Chl a in *Chaetoceros protuberans*; the calculated protein cross-sections for this organism ranged between 0.00027 and 0.00059 $m^2.mg^{-1}$ protein. In summary, similar values are found for the absorption coefficients for different phototrophic organisms, no matter the structure of their photosynthetic apparatus. This suggests that the capacity of acclimation of *C. vinosum* to low-light climates is similar to the capacity of the above mentioned organisms.

In general, it can be concluded that *C. vinosum* increases the photosynthetic efficiency in order to improve survival at low irradiances. This higher photosynthetic efficiency is a consequence of an increase in the quantum yield for photosynthesis and also of an enhanced absorption coefficient (protein cross-section), which resuls from the increase in the specific content of photosynthetic pigments.

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CHAPTER 6

UTILIZATION OF REDUCING POWER IN LIGHT-LIMITED CULTURES OF *Chromatium vinosum* DSM 185

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CHAPTER 6

UTILIZATION OF REDUCING POWER IN LIGHT-LIMITED CULTURES OF *Chromatium vinosum* DSM 185

This paper examines how the phototrophic organism *Chromatium vinosum* copes with light limitation by optimizing the resources obtained from the reducing power initially present in the medium as hydrogen sulfide. Under all the conditions of illumination tested, sulfur was the preferred storage compound, while glycogen was accumulated preferentially at saturating irradiances. Analysis of the electron budget shows that under light-limited conditions there is an important fraction of reducing power not included as storage products or structural cell material. The results suggest that the microorganism could be excreting an organic compound.

INTRODUCTION

Phototrophic microorganisms commonly live in illuminated aquatic ecosystems at depths where light penetration is severely modified and irradiance is very low. Therefore, they have developed several mechanisms to cope with light limitation. Changes in the characteristics of the photosynthetic apparatus have been reported to occur in algae and in some cyanobacteria. Organisms can adapt by changing their photosynthetic capacity (P_{max}) (Senger and Fleischhacker 1978, Falkowski and Owens 1980), their photosynthetic efficiency (α) (Foy and Gibson 1982, Post et al 1985) or both (Zevenboom and Mur 1984). Furthermore, variations in their pigment content have also been reported. In general, phototrophic organisms adapt to light limitation by increasing their specific pigment content (Myers 1946, Myers and Kratz 1955, Sistrom 1962, Cook 1963, Holt and Marr 1965, Trentini and Starr 1967, Beale and Appleman 1971, Uffen 1973, Gons and Mur 1975, Broch-Due et al 1978, van Liere and Mur 1978, Yoder 1979, van Gemerden 1980). On the other hand, changes in the chlorophyll-carotenoid ratio have been demonstrated in algae and in some cyanobacteria (Prézelin 1976, 1981, Foy and Gibson 1982, Post et al 1985). However, little is known about their ability to adapt their particular cellular composition to light-limited conditions.

Phototrophic sulfur bacteria fix CO_2 utilizing light as energy source and reduced sulfur compounds, mainly sulfide, as electron donors for their biosynthesis. During their growth, they can accumulate zero-valence sulfur (S°), glycogen, poly(3-hydroxialkanoates) (PHA) and

polyphosphate, and their different resources can be directed to different cell fractions depending on the growth conditions. Thus, the amount and type of storage products synthesized can be modulated by the environment in which the bacteria are developing. In general, storage compounds are synthesized whenever substrates are present in excess. Elemental sulfur is produced as an intermediate in the oxidation of sulfide to sulfate and it accumulates extracellularly in green sulfur bacteria as well as in purple sulfur bacteria belonging to the family Ectothiorhodospiraceae, and intracellularly in the family Chromatiaceae. Glycogen is stored in batch cultures of phototrophic organisms growing on sulfide/CO_2 for as long as sulfide is present. Furthermore, the presence of organic compounds such as malate results in the synthesis of glycogen, while intracellular PHA is accumulated in the presence of acetate and other organic acids (Liebergesell et al 1991).

This work, carried out with the model microorganism *Chromatium vinosum* DSM 185, attempts to find out whether, an to what extent, this organism can change its composition when challenged with growth-limiting irradiances. The results will provide information about how these organisms optimize their resources under light limitation but also will provide the information needed to interpret cellular composition as an indicator of the physiological state (i.e. type and magnitude of stress) for the study of phototrophic organisms in natural environments.

MATERIALS AND METHODS

Organisms and growth conditions. Experiments were performed with *Chromatium vinosum* DSM 185 growing in mineral medium containing carbonate as the only carbon source and hydrogen sulfide as electron donor (Mas and Van Gemerden 1987). The work was carried out in continuous culture, with an redox-controlled setup specially designed for the maintenance of a constant concentration of hydrogen sulfide inside the culture vessel despite variations in the conditions of illumination (Sánchez et al 1996, Chapter 4). Concentration of sulfide in the reservoir of the continuous culture ($S_{R\text{-sulfide}}$) was set to 2 mM. Illumination was provided by incandescent light bulbs. Light irradiance was measured using a quanta meter (no. Li-185B; LiCor Inc.). The following incident irradiances were used in each of the steady states: 868, 113, 23 and 7 $\mu E.m^{-2}.s^{-1}$.

Sampling and analyses. Samples for the analyses of cell constituents were collected from the effluent of the culture, in the dark and on ice, divided in subsamples and centrifuged. The supernatants were discarded except in a few cases in which they were frozen for further analysis of possible excretion products. The pellets were frozen and kept at -20 °C until the analyses were performed.

The residual concentration of hydrogen sulfide was measured in 1-ml samples taken directly from the culture vessel with a syringe, fixed with 20 ml of zinc acetate and assayed using the method of Pachmayr (1960) as described by Trüper and Schlegel (1964) in a total volume of 50 ml.

Bacteriochlorophyll a and sulfur were measured spectrophotometrically at 770 nm and 265 nm respectively, in methanol extracts. The actual concentrations of both compounds were calculated using the extinction coefficients provided by Stal et al (1984). Proteins were determined with the Folin phenol reagent (Lowry et al 1951) after extraction of the pellet with methanol to remove sulfur, and subsequent solubilization of the samples in 1 N sodium hydroxide for 10 min at 100 °C (Herbert et al 1971). Total sugars were assayed with the anthrone reagent (Herbert et al 1971) in samples in which sulfur had been previously extracted with methanol. The values obtained were used to estimate the concentration of glycogen, assuming a content of 0.02 mg structural sugars per mg protein (measured in glycogen-depleted cultures of the same organism). Poly-ß-hydroxybutyrate (PHB) was assayed spectrophotometrically as crotonic acid according to Law and Slepecky (1961). Particulate (POC) and dissolved organic (DOC) and inorganic (DIC) carbon were determined as CO_2 in a carbon analyzer (Shimadzu TOC 500). For the analysis of POC the pellet was resuspended in 5 ml of distilled water and injected in the analyzer. Dissolved carbon, both organic and inorganic was determined on the supernatant fraction. Glycolate was also assayed in some of the supernatants following the procedure described by Calkins (1943).

Total cell numbers and average cell volumes were determined by means of a Coulter Counter (ZM) connected to a Channelyzer 256 (Coulter Electronics LTD). The cells had been fixed previously with formaldehyde (4% final concentration) and diluted in counting medium at pH 7.5 containing 35 mg of K_2HPO_4 and 26 ml of 37% formaldehyde in a total volume of 1L. Latex beads with known diameters were used for size calibration.

Calculation of reducing equivalents. In order to assess how the reducing power initially present as sulfide is distributed between the different cell fractions, the concentrations of structural cell material estimated as protein, glycogen and sulfur must be converted into electron equivalents. This conversion allows the calculation of an electron balance, useful to monitor whether any reducing power ends up in fractions not included in the initial measurements. Calculation of the electron equivalents was done using the following electron yields: 8 mmol e^- $\cdot mmol^{-1}$ H_2S, 6 mmol $e^-.mmol^{-1}$ S°, 0.133 mmol $e^-.mmol^{-1}$ glycogen (Van Gemerden and Beeftink 1978) and 0.36 mmol $e^-.mg^{-1}$protein (see Results).

Calculation of metabolic rates. Metabolic rates of sulfide oxidation (v_1), sulfur oxidation (v_2), and glycogen accumulation (v_6) were calculated from the dilution rate, and the steady state concentrations of sulfur, sulfide, glycogen and proteins according to the procedure described by Van Gemerden and Beeftink (1978). In order to make the rates comparable, calculations were done after converting the concentrations mentioned above to their reducing power equivalents, and the resulting specific rates were all expressed in the same units (h^{-1}).

RESULTS

Although light limitation was applied by changing the incident irradiance, this is not a good measure of the energy actually available to the organisms. Light limitation is better characterized by the specific rate of light absorption (q_e) (Sánchez and Mas 1996, Chapter 2) which should be used as the independent variable in the next figures. In spite of that, the graphs have been plotted as a function of the specific growth rate. The reason for this decision was double, first, the specific growth rate is a good indicator of the degree of limitation, and second, data expressed as a function of μ can be easily compared with data from other experiments which study the effect of factors other than light.

In order to study the physiological effects of light limitation it is important to ensure that light is the only factor involved. The chemostat setup used in the experiments (Sánchez et al 1996, Chapter 4) was designed to provide different growth rates at different irradiances while maintaining a constant concentration of residual sulfide. The specific rate of light absorption, which is a measure of the energy supply, and the concentration of hydrogen sulfide, which is the only electron donor, have been represented in Fig. 6.1A as a function of the specific growth rate of the organism. Because light is the growth limiting factor, low values of q_e correspond to low values of μ. The specific rates of light absorption (q_e) determined for each incident irradiance were 103.0, 30.9, 16.8 and 7.1 μE.mg^{-1} protein h^{-1}. The concentration of hydrogen sulfide, however, remained constant (50 μM) at all values of μ, ruling out any possible effect of sulfide in the physiological variations experienced by the organism.

Total cell number and protein concentration have been represented in Fig. 6.1B. While cell number changed little between the different steady states (average value of 1.4x10^7 cells.ml^{-1}), the concentration of protein increased from 17.0 mg.L^{-1} to 29.2 mg.L^{-1} when decreasing the degree of light limitation. This indicates a larger amount of protein per cell at higher specific growth rates. Sulfur, which was present at high concentrations (741 μM) when the culture was light-limited, decreased as more light was available and the culture grew faster. The concentration of glycogen (Fig. 6.1C), also affected by light limitation, increased from 1.5 to

12.0 mg.L^{-1} when increasing light availability. Poly-ß-hydroxybutyrate (not shown) was analyzed and found to be zero in all the steady states.

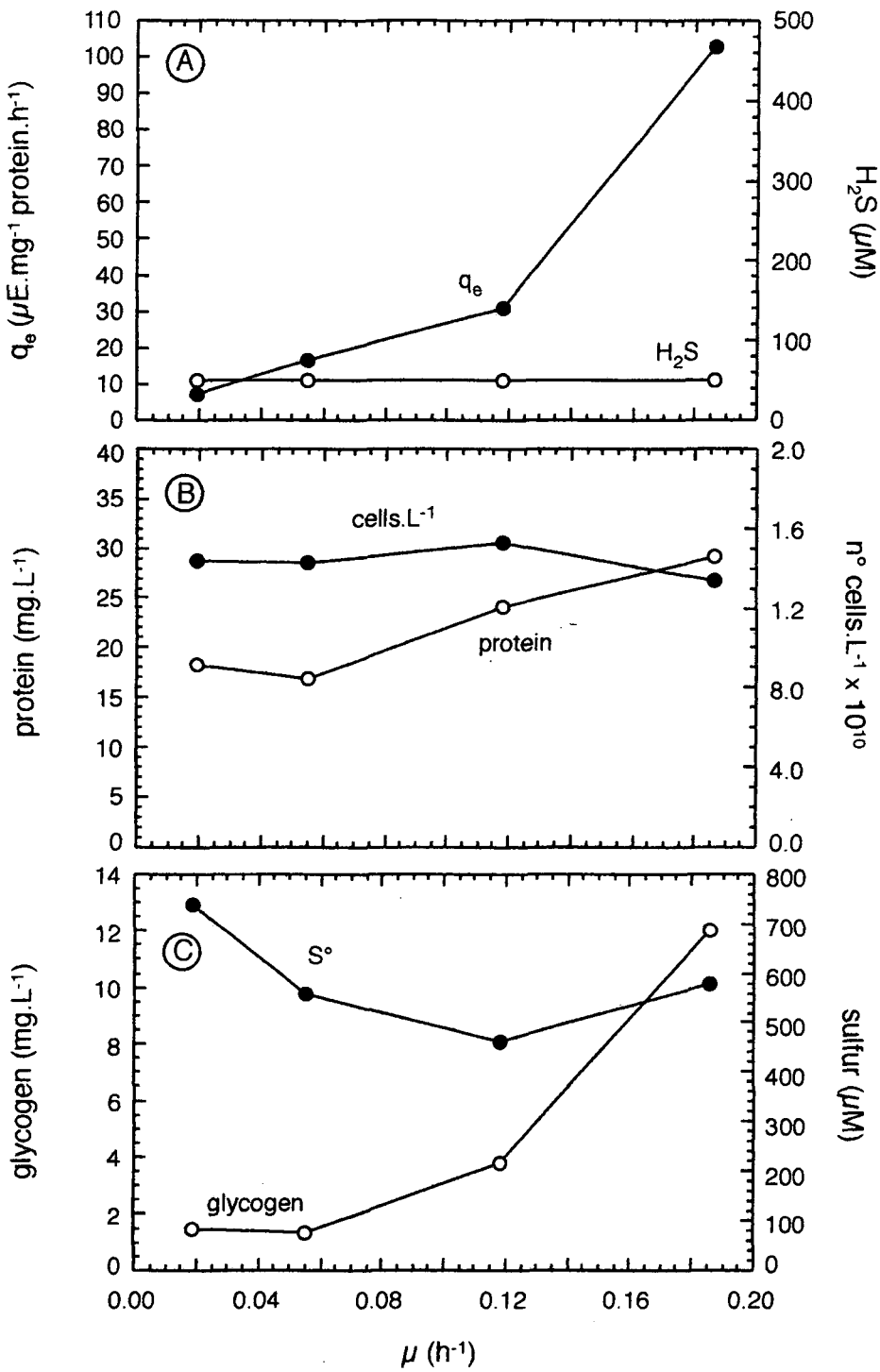

Fig. 6.1. Effect of the specific growth rate (μ) on steady-state cultures of *C. vinosum*. (A) Specific rate of light uptake (q_e) and concentration of hydrogen sulfide. (B) Protein concentration and cell number. (C) Concentration of storage compounds. Every point is the average of several samples. Standard errors are, in all the cases, less than 5% of the mean.

Accumulation of storage inclusions. Data from Fig. 6.1 have been used to calculate the specific contents of glycogen and sulfur. The specific content of sulfur (Fig 6.2) increased at low specific growth rates, when light limitation was more severe, reaching 40.7 μmol S°.mg^{-1} protein. The specific content of glycogen, on the contrary, was 82.0 μg.mg^{-1} protein under light limitation and increased up to 411.5 μg.mg^{-1} protein at higher irradiances, when the organism was growing faster. Thus, while glycogen was stored in large amounts under conditions of full illumination, sulfur was accumulated preferentially under light limitation. When the specific contents are expressed in terms of electrons (Fig. 6.3) it can be observed that sulfur is, by far, the preferred electron store under all conditions. Glycogen, on the contrary is virtually absent when light restriction is severe, and constitutes about 1/3 of the stored electrons when irradiance is saturating. Overall, storage of reducing power decreases at higher growth rates, when the organisms are less light-limited, because a higher amount of reducing power is invested in biosynthesis and growth.

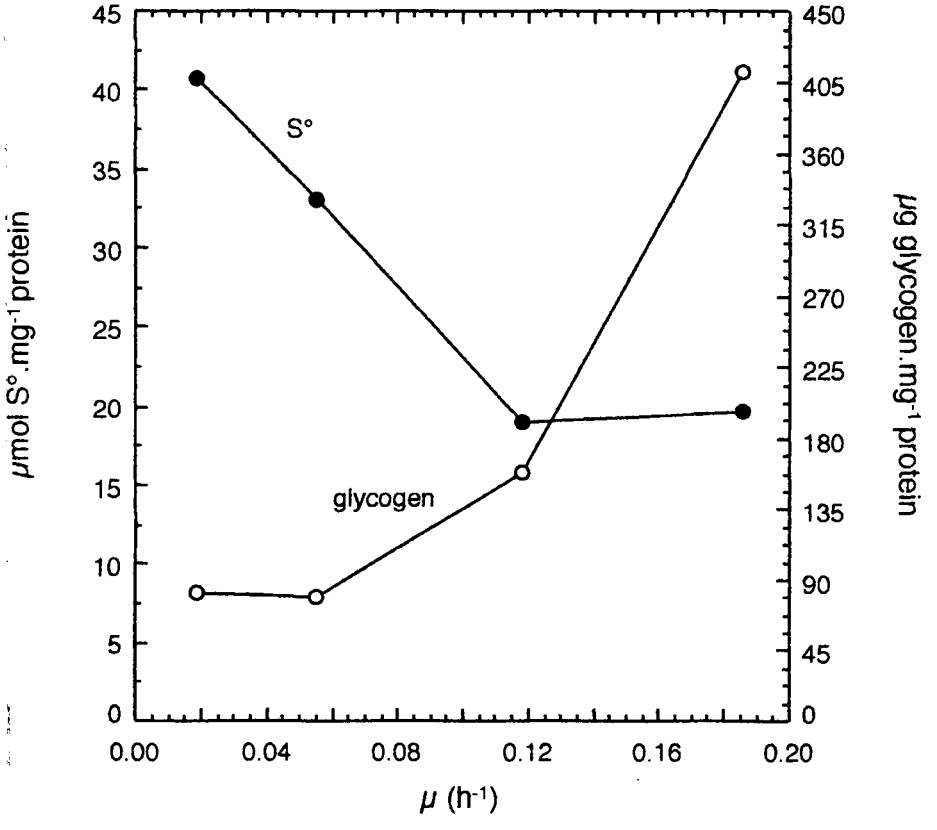

Fig. 6.2 Specific contents of sulfur (S°) and glycogen as a function of the specific growth rate (μ) in steady-state cultures of *C. vinosum*.

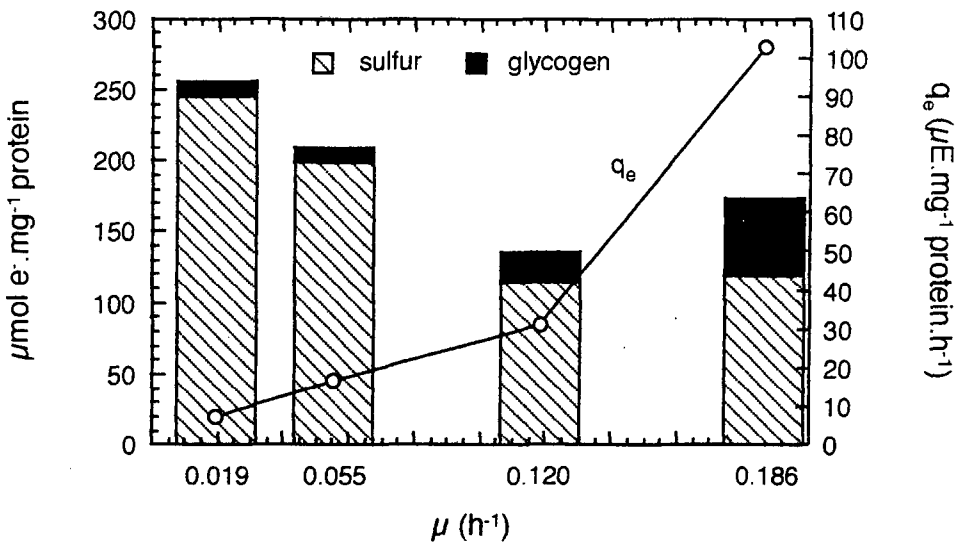

Fig. 6.3. Storage of reducing power as sulfur and glycogen (expressed as μmol of electrons per mg of protein) as a function of the specific growth rate (μ) in steady-state cultures of *C. vinosum* . Every steady state corresponds to a certain specific rate of light uptake (q_e).

Distribution of reducing power and electron budget. The reducing power initially present in the culture medium as sulfide is taken up by the organism and derived, partially to the synthesis of structural cell material, and partially to the synthesis of storage products. The relative amount allocated to each of these fractions changed depending on the illumination provided.

The difference between the reducing power initially available as sulfide (Sr) and the reducing power found as residual sulfide, sulfur and glycogen allowed to calculate the reducing equivalents which theoretically should be found as structural cell material (protein). This quantity, divided by the concentration of protein, should give the electron yield for structural cell material. Determination of this electron yield gave four different numbers, which ranged from 0.36 to 0.78 mmol e^-.mg^{-1} protein depending on the degree of light limitation. This variation in the electron yield for structural cell material could be due to the existence of differences in the composition of structural material from cells grown at different irradiances (change in lipid and membrane composition). A second interpretation could be made considering a constant electron yield and assuming the excretion of a reduced compound not detected in the fractions analyzed.

The presence of excretion products was therefore checked by quantifiying the different carbon fractions in samples from the most light-limited steady state. The results indicate the existence of 5.25 mM dissolved organic carbon, 10.83 mM dissolved inorganic carbon, and 2.75 mM

particulate carbon. The existence of a large organic fraction (around 28% of the total carbon) supports the idea that an important part of the electrons derived from sulfide were conducted to the synthesis of excretion products. These products, so far have not been identified. Total carbon, estimated after adding all the fractions, was 18.83 mM, a value which compares extremely well with the concentration of carbonate (18.87 mM) initially present in the culture medium.

From these results, we assumed that the electron yield for structural cell material was constant for all the conditions of illumination tested. From them on, the electron yield for structural cell material was calculated as the value that allowed the adjustment of the light-saturated electron budget to zero. The value obtained, 0.36 mmol $e^-.mg^{-1}$ protein, was subsequently utilized to calculate the electron budget of the remaining steady states. Table 6.1 summarizes the final electron budget of the experiment.

Table 6.1. Allocation of reducing power in a culture of *C. vinosum* DSM 185 growing at different degrees of light limitation. Data have been expressed as absolute values (in μmols of electrons per liter), and also as percentage of the electrons initially present as sulfide (Sr).

q_e ($\mu E.mg^{-1}$ protein.h^{-1})	Sr (μmol e^-. $liter^{-1}$)	S^{2-} (μmol e^-. $liter^{-1}$)	S° (μmol e^-. $liter^{-1}$)	Protein (μmol e^-. $liter^{-1}$)	Glycogen (μmol e^-. $liter^{-1}$)	Budget (μmol e^-. $liter^{-1}$)
103.0	16124.8	400.0 (2.5%)	3462.0 (21.5%)	10458.5 (65.7%)	1602.7 (9.9%)	0 (0%)
30.9	15276.6	400.0 (2.6%)	2761.9 (18.1%)	8650.4 (56.6%)	509.3 (3.3%)	2955.0 (19.3%)
16.8	15276.6	400.0 (2.6%)	3342.0 (21.9%)	6025.9 (39.4%)	177.3 (1.2%)	5331.4 (34.9%)
7.1	19256.0	400.0 (2.1%)	4446.0 (23.1%)	6523.6 (33.9%)	198.66 (1.0%)	7957.7 (41.3%)

When light was saturating (q_e = 103.0 $\mu E.mg^{-1}$ protein.h^{-1}) 65.7% of the reducing power was allocated to the synthesis of biomass (estimated as protein), 21.5% was stored as elemental sulfur, and 9.9% ended up as glycogen. Leftover residual sulfide constituted 2.5% of the total reducing power. When the culture was light limited (q_e = 7.1 $\mu E.mg^{-1}$ protein.h^{-1}) the situation was significantly different. The fraction allocated to growth (i.e. protein) was only 33.9% of the total reducing power, 23.1% was stored as sulfur, about 1% was stored as glycogen and 2.1% remained as residual sulfide. Addition of this fractions only accounted for 60.3% of the reducing power initially present as sulfide, leaving 41.3% of the electrons undetected. Such a

discrepancy clearly indicates that under these conditions a considerable amount of reducing power was derived towards the formation of compounds not included in the initial budget.

Metabolic rates. The specific metabolic rates of sulfide oxidation (v_1), sulfur oxidation (v_2), glycogen synthesis and degradation (v_6) and growth (v_8) have been represented as a function of the specific growth rate in Fig. 6.4 for each of the steady states. Data are shown as an area plot in which the different rates are accumulated providing the total specific rates at which reducing power is obtained and utilized. The lower part of the graph contain v_1 and v_2, the rates of electron-donating reactions, which have a negative sign. Both rates increase approximately five times as the organism grows faster. v_1 increases from -0.014 h^{-1} to -0.070 h^{-1} and v_2 also increases from 0.028 h^{-1} to 0.147 h^{-1}. The upper part of the plot represents the electron-consuming rates, v_6 and v_8, which have a positive sign. Thus, the difference between electron-donating rates (v_1 and v_2) and electron-consuming rates (v_6 and v_8) should yield a value of zero. However, this is not the case because there is an important fraction of electrons utilized by the organism which are not included in structural cell material nor glycogen inclusions. The rate at which reducing power is excreted from the cell has been referred to as the rate of spilling, and it

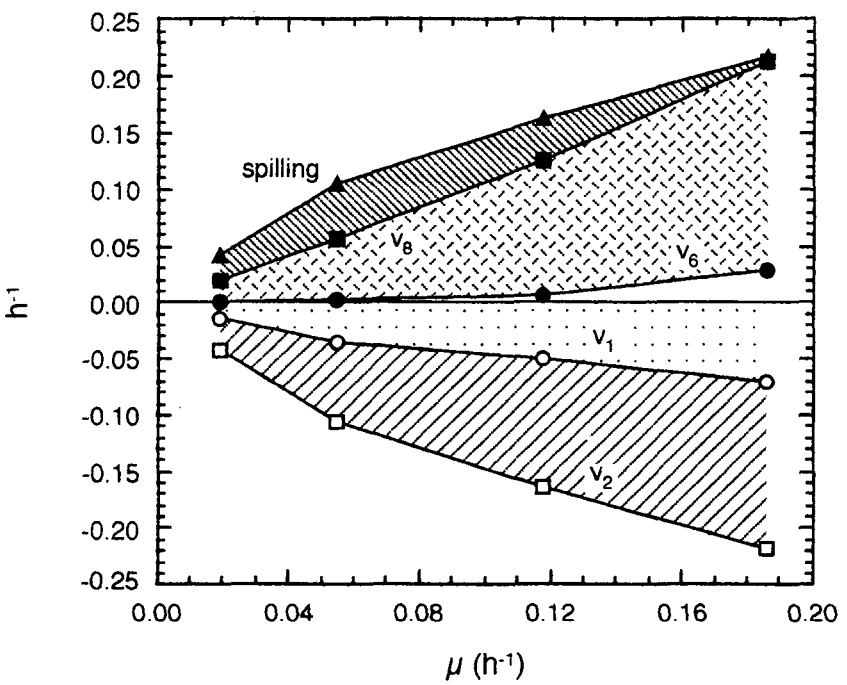

Fig. 6.4. Accumulated area plot with the specific metabolic rates of the electron-donating (open symbols) and electron-consuming (black symbols) reactions as a function of the specific growth rate (μ). Data correspond to steady-state cultures of *C. vinosum* (v_1, specific rate of sulfide oxidation; v_2, specific rate of sulfur oxidation; v_6, specific rate of glycogen storage; v_8, specific growth rate).

varies widely depending on the degree of light limitation imposed. The lowest value corresponds to light saturating conditions (0.003 h^{-1}) and the highest value (0.048 h^{-1}) occurs at a μ rate of 0.055 h^{-1}. Although the maximum amount of undetected electrons corresponds to the most severe light-limited steady state, the rate of spilling turns out to be lower (0.022 h^{-1}) due to the fact that the organism is growing more slowly (μ = 0.020 h^{-1}).

Variations in cell volume. Cellular volume changes as a function of the specific growth rate and as a function of the content of storage inclusions of the organisms (Guerrero et al 1984, Mas 1985, Mas and van Gemerden 1987). Therefore, cell size is a variable which summarizes the effects of several environmental factors and could, therefore, be used as an indicator of the physiological state of the organisms. The size distribution of *C. vinosum* was analyzed in samples from each of the steady states, in order to explore the existence of a trend which could be used as an indication of light limitation. The results of the analyses, have been represented in Fig. 6.5. At low illuminations (q_e = 7.1 and 16.8 $\mu E.mg^{-1}$ protein.h^{-1}) the distribution was clearly unimodal, characteristic of a slow-growing population. At higher values of q_e (30.9 and 103.0 $\mu E.mg^{-1}$ protein.h^{-1}), the distribution was more spread towards higher values, with the appearance of a shoulder characteristic of populations in which a large number of organisms is about to undergo division. The average volume, represented by a vertical line, decreases from 6.4 to 4.8 μm^3 when decreasing the light supply, increasing again up to 5.3 at very low values of q_e.

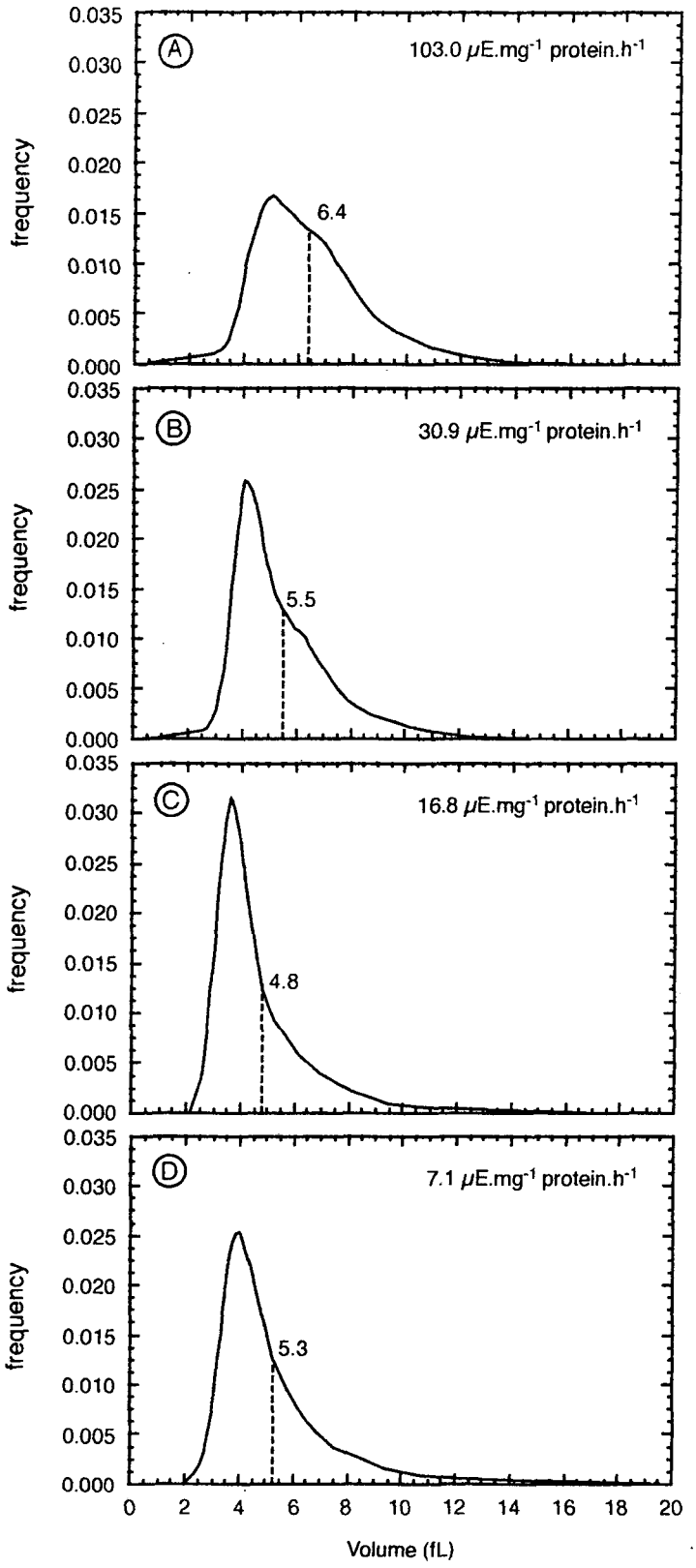

Fig. 6.5. Cell size distribution in steady-state cultures of *C. vinosum* grown at different light supplies. The mean volume has been indicated with a dotted line.

DISCUSSION

Our results show that sulfur was the preferred storage compound under all the conditions of illumination tested, although maximum amounts were found in light-limited cultures (as much as 95.7 % in terms of μmol $e^-.mg^{-1}$ protein). Glycogen was virtually absent under conditions of severe light limitation, but constituted a 31.6 % (in μmol $e^-.mg^{-1}$ protein) under light-saturating conditions. This fact can be easily explained provided that low-light limits CO_2 fixation and, subsequently, the production of a surplus of sugars needed for glycogen synthesis. The maximum sulfur storage found in the present work reached a value of 40% of the dry weight, a little higher than other values calculated from the literature for the same organism, which range between 30-35% of the dry weight (van Gemerden 1968, 1974, 1986, van Gemerden and Beeftink 1978, Mas and van Gemerden 1987, 1992, van Gemerden et al 1990). Other environmental situations lead also to the accumulation of storage compounds. Thus, under sulfide-limiting conditions, *C. vinosum* stores mainly glycogen, while an excess of electron donor results in sulfur accumulation (van Gemerden et al 1990). Both products can also be stored during severe phosphate-limiting conditions and excess of sulfide (Mas and van Gemerden 1992). Besides, environmental factors such as pH or temperature can also affect the accumulation of sulfur (Van Gemerden et al 1990).

The electron budget, calculated assuming a constant conversion factor for structural cell material of 0.36 mmol $e^-.mg^{-1}$ protein shows that, under light limiting conditions, there is an important fraction of electrons which is not included in storage products neither structural cell material. This fraction increases while increasing the degree of light limitation up to values which virtually represents half the total reducing power initially present in the culture. If this were not the case, a different conversion factor should be applied for every steady state (0.36, 0.48, 0.68 and 0.78 mmol $e^-.mg^{-1}$ protein from light-saturating to light-limiting conditions). However, analysis of particulate and dissolved organic and inorganic carbon in the supernatant fraction of the most light-limited culture suggests that *Chromatium* could be excreting an organic compound. Analysis of glycolate, protein and carbohydrates in the supernatant of the culture yielded zero values for all these compounds. PHB, which was analyzed in the particulate fraction, could not be detected in any of the cases. There are evidences from the literature which show that the non-sulfur bacteria *Rhodopseudomonas acidophila* could also be excreting carbon compounds under autotrophic growth, since it was found an extracellular carbon concentration in the culture (not bicarbonate) which equaled 4.7 % of the carbon fixed in cell material (Göbel 1978).

Our value for the structural material conversion factor (0.36 mmol $e^-.mg^{-1}$ protein) is very similar to the average value determined by Van Gemerden and Beeftink (1978) for the same organism

(0.33 mmol $e^-.mg^{-1}$ protein, assuming a 16% of N_2 content in protein). For *Thiocapsa roseopersicina*, the conversion factors reported were 0.46 (de Wit, 1989) and 0.44 mmol $e^-.mg^{-1}$ protein (Visscher 1992).

The values obtained in this work for the actual rates of sulfide oxidation (v_1), sulfur oxidation (v_2) and glycogen formation (v_6) compare well with the rates calculated by Beeftink and van Gemerden (1979) for *C. vinosum* in sulfide-limited cultures. They found that v_1 ranged between -0.005 and -0.050 h^{-1} for dilution rates of -0.010 and 0.116 h^{-1} respectively, while v_2 changed between -0.010 and -0.090 h^{-1}. v_6 ranged between 0 and 0.020 h^{-1}, reaching a maximum potential value (0.050 h^{-1}) upon growth inhibition. The difference between electron-donating rates (v_1 and v_2) and electron-consuming rates (v_6 and v_8) allows the calculation of a rate of electron spilling, which has a positive sign. This rate depends on the specific growth rate of the organism but under light-limited conditions, can account for a 52.4% of the electron-consuming rates.

Changes in the average cell volume were also observed in cultures of *Chromatium* subject to different degrees of illumination. Mas and van Gemerden (1987) reported that sulfur accumulation in this microorganism had an important effect on cell volume and buoyant density, both increasing accordingly to an increase in the specific content of sulfur. They also observed that variations in the specific content of glycogen and Bchl a did not appear to be related to changes in volume and density. In addition to the accumulation of sulfur, the average cell volume also varied with the specific growth rate. Using the model described by Mas and Van Gemerden (1987), the volume due to sulfur inclusions and that due to structural cell material were estimated for every dilution rate. These calculations are summarized in Table 6.2. They indicate that the fraction of the total volume occupied by sulfur inclusions tends to increase (from 34.4 % to 49.1%) when cells are light-limited and consequently, growing more slowly. This result is consistent with the fact that under light-limited conditions the storage of reducing power increases. On the contrary, the volume occupied by structural cell material decreases with growth rate (from 65.6% to 50.9% of the total cell volume). These calculations are not directly comparable with those made by Mas and van Gemerden (1987) because their data correspond to steady-state cultures of *C. vinosum* growing in sulfide-limited conditions, in which at low dilution rates the concentration of residual sulfide inside the culture vessel was virtually zero. Our data, on the contrary, refer to cultures growing at a saturating sulfide concentration (50 μM).

From an ecological point of view, the accumulation of sulfur may be advantageous since it represents a source of reducing power. Furthermore, sulfur could behave as a structure apt to increase light absorption (Mas and Sánchez submitted, Chapter 3). However, its storage is

disadvantageous due to the increase in total cell volume and buoyant density, which can increase the sinking speed of the organisms.

Table 6.2. Steady-state values of the specific rate of light uptake (q_e), specific growth rate (μ), specific content of sulfur and total cell volume for *C. vinosum* grown at different light supplies. The structural cell volume V_C has been determined from the equation described below (Mas and van Gemerden 1987). The volume occupied by sulfur inclusions results from the difference between V_t and V_C. The contribution (%) of sulfur inclusions and structural cell material to the total volume has also been indicated.

q_e (μE.mg^{-1} protein.h^{-1})	μ (h^{-1})	S°/cell (pg.cell^{-1})	V_t (μm^3)	V_S (μm^3)	V_C (μm^3)
103.0	0.186	1.37	6.4	2.2 (34.4%)	4.2 (65.6%)
30.9	0.120	0.97	5.5	1.5 (27.3%)	4.0 (72.7%)
16.8	0.055	1.25	4.8	2.0 (41.7%)	2.8 (58.3%)
7.1	0.020	1.64	5.3	2.6 (49.1%)	2.7 (50.9%)

$$V_C = V_t - \frac{R}{D_g} \cdot W_s$$ (Mas and van Gemerden 1987)

V_t: total cell volume
V_S: cell volume occupied by sulfur globules
V_C: volume of structural cell material
W_S: specific content of sulfur
R: ratio between weight of globule and weight of sulfur in globule (2.1 cell^{-1})
D_g: density of the sulfur globule (1.308 pg.μm^{-3})

Overall, our results show that *C. vinosum* can increase considerably sulfur accumulation (more than twice) under conditions of light limitation. When light is limiting, the amount of energy available for CO_2 fixation is severely reduced, and thus, the reducing power formerly available from the oxidation of sulfide to sulfate (8 electrons per sulfide oxidized) cannot be used. In this situation, the organism could drastically reduce sulfide oxidation, but what happens, at least in the case of *C. vinosum*, is that sulfide is partially oxidized to sulfur in a reaction that yields only 2 electrons. The byproduct of the process, sulfur, is stored intracellularly, and in this way the surplus of reducing power is made unavailable to other organisms. When light is saturating, sulfur is still accumulated, although at lower levels. In this case, though, accumulation of a

sizable amount of energy collected by the organism allows CO_2 conversion into carbohydrate at a rate higher than the rate at which it can be used for growth.

These variations show that *C. vinosum* can modulate the allocation of reducing power to different cell fractions in order to optimize the storage of whatever resource is in excess at that moment. The results also suggest that the specific contents of sulfur and glycogen could be used to monitor the physiological state of phototrophic sulfur bacteria in field samples. Further studies in this direction, analyzing the response of the organism to other types of environmental stress will no doubt provide the information required for this purpose.

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CHAPTER 7

OVERVIEW OF THE THESIS

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The aim of this thesis was the study of the physiological mechanisms through which *Chromatium vinosum* adapts to light-limiting conditions. *C. vinosum* was chosen as a model organism representative of purple sulfur bacteria, a group of anoxygenic phototrophs developing in anaerobic natural environments, often under conditions of low-light supply. While the physiology of the adaptation to low-light environments was well documented in oxygenic phototrophs, both algae and cyanobacteria, the information on anoxygenic phototrophs was scarce. This thesis attempts to fill this void.

To fulfill this objective it was necessary to address two methodological issues. First, a method was needed to assess the actual amount of light the cells were receiving. Previous literature referred activity data to light irradiance at the surface of the culture. This approach was not correct because the light climate experienced by the cells depended not only on the incident irradiance, but also on the size and biomass of the culture.

Second, the experiments had to be carried out in a continuous culture under steady state. The system required the possibility to change the light supply while keeping constant the remaining variables (biomass, pH, residual sulfide, and nutrients). In conventional chemostats, variations in the light supply invariably led to changes in all these variables, and as a consequence, it was virtually impossible to discriminate between the effect of light limitation and the effect of changes in the variables mentioned above, specially the concentrations of nutrients and sulfide. Therefore, it became necessary to maintain constant levels of nutrients and sulfide while undergoing a sequence of changes in the light supply.

7.1. MEASUREMENT OF LIGHT ABSORPTION (CHAPTERS 2 AND 3)

In the procedure developed in this study, light absorption was measured as the difference between the integrated light output from the surface of a culture vessel when it contained an active culture and the integrated output from a blank vessel filled only with culture medium. The specific rate of light uptake (q_e) was calculated after dividing the rate of light absorption by the total biomass present in the culture (Chapter 2, p 29).

Determination of light outputs at different positions of the culture surface provides valuable information about the exiting light field, which can be related to the optical properties of the suspension. The maximum possible output was obtained when the culture contained only growth medium (Fig 2.2A, p 32). As there were no particles in suspension, absorption was minimal and scattering was absent. In this case, light exited the culture vessel preferentially at 180°, opposite to the light source. When the vessel contained a dense culture suspension (Fig. 2.2B, p 32), outward irradiance was higher at 135 and at 225° due to scattering by the cell suspension. In the presence of cells containing refractile structures, such as sulfur (Fig. 2.2C, p 32), light was predominantly reflected, and output was maximum between 270 and 90°.

The method developed for the measurement of light absorption takes into account light outputs through the top an the bottom of the culture. When the culture vessel contained only growth medium, as much as 92.6% of the light exited through the sides (Table 2.1, p 33). However, different results where obtained when cells were present, and the magnitude of these changes depended on the biomass. However, the presence of refractile structures increases light losses through the top and bottom. Thus, it is important to consider vertical outputs through the top and bottom of the culture vessel when measuring the distribution of the exiting light field. However, differences were less evident when q_e was calculated (Table 2.2, p 34). In dense cultures, the error caused by calculating q_e without taking into account vertical outputs was very small (approximately 2%), no matter whether the culture contained sulfur. In diluted cultures, the error became much more important in the presence of sulfur (43%). These results indicated that the measurement of q_e could be simplified by measuring exclusively lateral light outputs, but only in dense cultures, especially in those cases in which no refractile structures were present.

Light absorption was considerably influenced by factors such as the concentration of organisms, the size of the culture vessel and the presence of refractile inclusions. In Chapter 3, the effect of these factors on the rate of light absorption and the specific rate of light uptake was analyzed.

As the cells of *C. vinosum* accumulated refractile sulfur inclusions, scattering increased and light output changed progressively from the back to the front of the culture vessel (Fig. 3.2, p 49). Overall, the total light output increased very little, and the consequence was a small decline in the rate of light absorption (Q_e) with a parallel reduction in the specific rate of light uptake (q_e) (Fig. 3.1, p 48). In diluted cultures, however, the result was the opposite, and sulfur accumulation appeared to intensify light uptake by the organisms.

The effect of changes in the biomass and size of the culture were also analyzed. Increases in culture size resulted in higher rates of light absorption and in a considerable decrease of the specific rate of light uptake (Fig. 3.3B, p 51). Total light output decreased steadily as the diameter of the vessel increased, due to a higher absorption by the culture (Fig. 3.3A, p 51). The effect of biomass was also analyzed in serially diluted cultures of *C. vinosum*. Determinations were carried out in cultures depleted of sulfur and in cultures containing 16.6 μmol S°.mg^{-1} protein (approximately 20% of the dry weight). In the sulfur-depleted cultures, light output decreased as biomass increased and the distribution of the output also changed. At low biomass, light exited the culture vessel preferentially at 180°, and as biomass increased there was a relative increase of the outputs at 135 and 225°, as a consequence of forward scattering within the culture (Fig. 3.4, p 53). The rate of light absorption (Q_e) increased with biomass (Fig. 3.5, p 54), attaining a maximum value when virtually all light was absorbed by the culture. On the contrary, q_e decreased approaching zero.

In sulfur-containing cultures, light output also decreased as biomass increased, but the presence of refractile inclusions affected dramatically the amount of back scattered light, resulting in a distribution of the exiting light field radically different from that found in sulfur-depleted cultures (Figs. 3.6B and 3.6C, p 55). In this case, the specific rate of light uptake also declined as biomass increased. However, diluted cultures of sulfur-containing cells presented values of q_e strictly different from those of sulfur-depleted cells (Fig. 3.6A, p 55). As a consequence of scattering, the overall light output decreased in diluted cultures and q_e showed a substantial increase when sulfur was present. This conclusion does not hold for dense cultures, in which structures apt to increase scattering seemed to have little effect on the specific rate of light uptake.

In order to interpret the experimental observations observed, a theoretical model was developed (Chapter 3, p 44). This model allows prediction of both the rate of light absorption (Q_e) and the specific rate of light uptake (q_e) as a function of the size of the culture vessel and the concentration of biomass. It includes a correction term for distance-dependent attenuation, not related to light absorption, and a correction term for shape and scattering, which takes into account changes in the average length of the light path. The model is constituted by the two following equations (p 47):

$$Q_e = \frac{L_0^2}{(L_0 + \varnothing \cdot f)^2} \cdot E_0 \cdot \left(1 - e^{-a^* \cdot c \cdot \varnothing \cdot f}\right) \cdot \varnothing \cdot h$$

$$q_e = \frac{L_0^2}{(L_0 + \varnothing \cdot f)^2} \cdot E_0 \cdot \left(1 - e^{-a^* \cdot c \cdot \varnothing \cdot f}\right) \cdot \frac{4}{c \cdot \pi \cdot \varnothing}$$

in which Ø is the diameter of the culture vessel, c is the concentration of biomass, E_0 is the incident irradiance, L_0 is the distance to the light source, a* is the absorption coefficient, f is the correction factor for shape and scattering, and h is the height of the culture vessel.

These equations, which fitted the experimental data reasonably well, allowed the estimation of two parameters, the absorption coefficient a* and the correction factor f, for each set of data (Table 3.1, p 56). In diluted cultures, the presence of sulfur inclusions gave place to a more than twofold increase in the absorption coefficient, indicating that this structure affects dramatically the rate at which light can be absorbed by the cells, and may act increasing the magnitude of light absorption. Overall, the coefficients obtained compared well with coefficients corresponding to other phototrophic organisms. The second parameter, f, corrects the differences between the diameter of the culture vessel and the actual length of the average light path, and was larger than 1 in all the experiments. This indicated that the average light path was longer than the diameter of the vessel. Furthermore, as f was higher in sulfur-containing cultures, it can be concluded that the presence of refractile structures increased the length of the light path.

Light absorption can thus be maximized by increasing the culture cross section and/or the concentration of phototrophic biomass. This increase, however, will lead to a decrease in the amount of energy available for growth per unit biomass, that is, q_e.

7.2. REDOX-CONTROLLED SULFIDOSTAT (CHAPTER 4)

The second methodological approach developed in this work was the application of a novel type of chemostat which allowed to maintain a growing culture of *C. vinosum* under constant concentration of hydrogen sulfide, despite variations in the light supply. The system was controlled by changes in redox, which were monitored through a redox controller. These changes modulated the pump speed, and therefore, the dilution rate. Several shifts in illumination were carried out, and the system behaved as expected, proving to be a valuable tool for the study of light limitation in phototrophic sulfur bacteria. Periodical measurements of sulfide concentration, dilution rate and redox showed that the system was stable during extended periods of time (Fig. 4.6, p 81). Furthermore, transitions to new dilution rates revealed that it readjusted immediately to the new conditions, avoiding changes in sulfide concentration.

Thus, with this new setup, it was possible to obtain different steady states in which the physiological events observed were not a consequence of collateral effects due to changes in the concentration of hydrogen sulfide, and were only related to the conditions of illumination imposed.

7.3. ACCLIMATION OF *C. vinosum* TO LIGHT LIMITATION (CHAPTERS 5 AND 6)

In order to study the ability of *C. vinosum* to adapt to low irradiances, four different light levels were tested, and for each of them, the culture was allowed to run until reaching steady state. Samples taken from each steady state were utilized for the assessment of the photosynthetic response and the particular cellular composition of *C. vinosum*. The specific rate of sulfur oxidation was used as a measure of photosynthetic activity (P) (Chapter 5, p 91), and different photosynthesis-light curves were obtained (Fig. 5.3, p 96), in which P was plotted as a function of q_e. Adjustment of a model describing the photosynthesis-light relationship to these data (Chapter 5, p 92) yielded three different parameters, which allowed the characterization of the photosynthetic response of the organism: (i) P_{max}, which gives the maximum photosynthetic rate the organism can achieve when light is saturating, (ii) ϕ, the quantum yield for photosynthesis, and (iii) m, the maintenance coefficient, which indicates the quantity of energy the microorganism requires for maintenance processes (Table 5.1, p 97). The results indicated that, while P_{max} was virtually constant in all the conditions of illumination tested, ϕ and m increased with the degree of light limitation. The values obtained for ϕ (0.048 - 0.140 μmol C.μE^{-1}) compared well with other quantum efficiencies reported for algae. Overall, the organism copes with light-limiting conditions by maximizing the photosynthetic response. This occurs through changes in quantum yield and increases in the absorption coefficient (a*), which result from changes in the specific pigment content at low irradiances (from 24 and 85 μg Bchl *a*.mg^{-1} protein) (Fig. 5.2, p 95).

Cellular composition in terms of sulfur, protein and total sugars was also analyzed for each steady state (Chapter 6). Calculation of the specific contents of glycogen and sulfur showed that glycogen was the main storage compound under light-saturating conditions, while sulfur was accumulated preferentially under light limitation (Figs. 6.2-6.3, pp 114-115). Conversion of the different cell fractions (structural cell material as protein, glycogen and sulfur) into electron equivalents allowed the calculation of an electron balance, useful to monitor the relative amount of reducing power initially present as sulfide allocated in each of these fractions (Table 6.1, p 116). The electron budget showed that, as the degree of light limitation increased, there was a considerable amount of reducing power not included as structural material, glycogen or sulfur. Under the most severe light limitation, this fraction constituted up to 41% of the initial reducing

power. Quantification of particulate, organic and inorganic carbon indicated the existence of a large organic fraction in the supernatant of the culture. This supports the idea that an important part of the electrons derived from hydrogen sulfide were conducted to the synthesis of excretion products.

Preferential accumulation of sulfur under conditions of light limitation makes ecological sense. Complete oxidation of sulfide to sulfate would provide 8 electrons to be used in CO_2 fixation. When energy for CO_2 fixation is in short supply, the organism does no longer require the reducing power, and therefore, faces two alternatives: either reduce the rate of sulfide oxidation, in which case sulfide would remain unused in the medium but perhaps available to other organisms; or carry out a partial oxidation of sulfide to sulfur, a reaction which provides only 2 electrons, and accumulate the resulting sulfur in intracellular inclusions, thus making the remaining reducing power unavailable to other organisms. *C. vinosum* seems to participate of this second strategy, and while the rate of sulfide oxidation is considerably reduced at low-light levels, the content of sulfur increases more than twice.

When light is saturating, glycogen accumulates to significant levels, indicating that the rate at which carbon is fixed exceeds the rate at which can be used for the synthesis of cell material.

The data obtained on the adaptability of the photosynthetic apparatus indicate that *C. vinosum* can improve the photosynthetic efficiency through simultaneous changes in the quantum yield and in the protein cross-section. However, the magnitude of the changes observed is similar to what is found in oxygenic phototrophs present in well illuminated environments. This suggests that *C. vinosum* is not particularly well adapted to conditions of extreme light limitation.

CONCLUSIONS

CONCLUSIONS

The conclusions of the work presented in this thesis are:

1. Incident irradiance does not indicate the actual light available for growth in cultures of phototrophic microorganisms, since it does not consider factors such as the biomass or the size of the culture vessel. A better variable to describe light availability is the specific rate of light uptake, which has been determined as the amount of light absorbed per unit biomass.

2. As a consequence of scattering and absorption in suspensions of phototrophic microorganisms, light might exit the vessel at directions other than opposite to the light source. Thus, an adequate characterization of the light field at different positions of the culture surface is necessary to obtain a correct measure of the light absorbed by the culture.

3. Absorption of light by suspensions of phototrophic microorganisms depends on changes in the biomass of the culture, the size of the culture vessel, and the presence of refractile structures within the cells. Increases in the biomass and the size of the culture maximize light absorption but decrease the amount of light available per cell. Refractile structures reduce specific light absorption in dense cultures, but in diluted cultures the effect is the opposite as they seem to increase q_e.

4. Increases in biomass and the diameter of the culture vessel reduce the transmitted light output due to absorption by the organisms, while sulfur inclusions increase the percentage of back scattered light.

5. The experimental system utilized allows the growth of sulfide-oxidizing microorganisms in continuous culture while maintaining a constant concentration of hydrogen sulfide inside the culture vessel. Changes in external factors such as light modulate the biological activity of the organisms, and in turn, the dilution rate of the system, generating new stable conditions.

6. There is a saturation-type relationship between the specific growth rate (μ) and the specific rate of light uptake (q_e) in cultures of *Chromatium vinosum*, in which μ_{max} attains a value of 0.195 h^{-1}. The cell maintenance rate constant, μ_e, has a value of -0.025 h^{-1}.

7. Changes in the light supply induce variations in the photosynthetic response of *C. vinosum*. Increases in the specific content of Bchl a (from 24 to 85 μg Bchl a .mg^{-1} protein), and

quantum yield for photosynthesis (from 0.032 to 0.095 μmol S°.μE^{-1}) have been observed in light-limited cultures. This results in an enhanced photosynthetic efficiency for the organism. The photosynthetic capacity (P_{max}), on the contrary, remains unchanged, while the organism increases its maintenance coefficient (m) under light limitation.

8. The increase in the specific pigment content results in a higher protein cross-section ($m^2.mg^{-1}$ protein), while the chlorophyll cross-section (m^2 Bchl a.mg^{-1} protein) declines because of the mutual shading between pigment molecules.

9. *C. vinosum* adapts the distribution of reducing power to shifts in light conditions. At low irradiances, most of the reducing power is stored as sulfur (95.7% of the stored reducing power), while glycogen is virtually absent. At saturating irradiances, on the contrary, an important part of the reducing power initially present as sulfide is destined to glycogen inclusions (31.6% of the stored reducing power). PHB inclusions could not be detected in any of the conditions tested.

10. The electron budget shows the existence of an important amount of reducing power not present as sulfur, glycogen or structural cell material. This fraction increases up to 41% of the total electrons in light-limited cultures. The presence of dissolved organic carbon in the medium suggests that *C. vinosum* could be excreting an organic compound.

CONCLUSIONS

Les conclusions del treball presentat en aquesta tesi són:

1. La irradiancia incident no indica la llum disponible pel creixement en cultius d'organismes fototròfics, ja que no té en compte factors com la biomassa i la mida del recipient de cultiu. La taxa específica d'absorció de llum és una variable millor per descriure la disponibilitat de llum i s'ha determinat com la quantitat de llum absorbida per unitat de biomassa.

2. Com a conseqüència de la dispersió i l'absorció de llum en suspensions de microorganismes fototròfics, la llum pot sortir del recipient de cultiu en direccions diferents a l'oposada a la font d'il.luminació. Per tant, és necessària una adequada caracterització del camp de llum en diferents posicions de la superfície del cultiu per obtenir una mesura correcta de la llum absorbida pel cultiu.

3. L'absorció de llum per suspensions de microorganismes fototròfics es veu afectada per canvis en la biomassa del cultiu, la mida del recipient de cultiu i la presència d'estructures refringents a les cèl.lules. Increments en la biomassa i la mida del recipient de cultiu maximitzen l'absorció de llum però disminueixen la quantitat de llum disponible per cèl.lula. Les estructures refringents redueixen l'absorció específica de llum en cultius densos, però en cultius diluïts tenen un efecte contrari i incrementen q_e.

4. Increments en la biomassa i el diàmetre del recipient de cultiu redueixen la llum transmesa degut a l'absorció pels organismes, mentre que les inclusions de sofre incrementen el percentatge de llum reflectida cap endavant.

5. El sistema experimental utilitzat permet el creixement de microorganismes oxidadors de sulfhídric en cultiu continu mentre manté una concentració constant de sulfhídric en l'interior del recipient de cultiu. Canvis en factors externs com la llum modulen l'activitat biològica del organismes i, en conseqüència, varien la taxa de dilució del sistema, generant noves condicions estables.

6. La relació entre la taxa específica de creixement (μ) i la taxa específica d'absorció de llum (q_e) en cultius de *Chromatium vinosum* és del tipus saturació, on μ_{max} assoleix un valor de 0.195 h^{-1}. La taxa específica de manteniment, μ_e, té un valor de -0.025 h^{-1}.

7. Canvis en el subministrament de llum indueixen variacions en la resposta fotosintètica de *C. vinosum*. S'han observat increments en el contingut específic de Bchl a (de 24 a 85 μg Bchl a .mg^{-1} protein), i en el rendiment quàntic de la fotosíntesi (de 0.032 a 0.095 μmol S°.μE^{-1}) en cultius limitats per la llum. La capacitat fotosintètica (P_{max}), pel contrari, roman constant, mentre que l'organisme augmenta el quoficient de manteniment (m) quan es troba limitat per la llum.

8. L'increment en el contingut específic de pigments produeix un augment en el quoficient d'absorció de les proteïnes ($m^2.mg^{-1}$ protein), mentre que el quoficient d'absorció de la clorofil.la (m^2 Bchl a.mg^{-1} protein) disminueix degut a l'autoombrejat entre molècules de pigments.

9. *C. vinosum* adapta la distribució del poder reductor a canvis en les condicions d'il.luminació. A baixes irradiàncies, la major part del poder reductor s'acumula en forma de sofre (95.7% del poder reductor magatzemat), mentre que no hi ha pràcticament glicogen. A irradiàncies saturants, pel contrari, una part important del poder reductor present inicialment com a sulfhídric és destinat a inclusions de glicogen (31.6% del poder reductor magatzemat). No es van detectar inclusions de PHB en cap de les condicions d'il.luminació.

10. El balanç d'electrons mostra l'existència de una quantitat important de poder reductor que no està present en forma de sofre, glicogen o material estructural. Aquesta fracció s'incrementa fins a un 41% del total d'electrons en cultius limitats per la llum. La presència de carboni orgànic dissolt en el medi suggereix que *C. vinosum* podria estar excretant algun compost orgànic.

CONCLUSIONES

Las conclusiones del trabajo presentado en esta tesis son:

1. La irradiancia incidente no indica la luz disponible para el crecimiento en cultivos de organismos fototróficos, ya que no tiene en cuenta factores como la biomasa y la medida del recipiente de cultivo. La tasa específica de absorción de luz es una variable mejor para describir la disponibilidad de luz y se ha determinado como la cantidad de luz absorbida por unidad de biomasa.

2. Como consecuencia de la dispersión y la absorción de luz en suspensiones de microorganismos fototróficos, la luz puede salir del recipiente de cultivo en direcciones diferentes a la opuesta a la fuente de iluminación. Por tanto, es necesaria una adecuada caracterización del campo de luz en diferentes posiciones de la superficie del cultivo para obtener una medida correcta de la luz absorbida por el cultivo.

3. La absorción de luz por suspensiones de microorganismos fototróficos se encuentra afectada por cambios en la biomasa del cultivo, la medida del recipiente de cultivo y la presencia de estructuras refringentes en las células. Incrementos en la biomasa y la medida del recipiente de cultivo maximizan la absorción de luz pero disminuyen la cantidad de luz disponible por célula. Las estructuras refringentes reducen la absorción específica de luz en cultivos densos, pero en cultivos diluídos tienen un efecto contrario, incrementando q_e.

4. Incrementos en la biomasa y el diámetro del recipiente de cultivo reducen la luz transmitida debido a la absorción por los organismos, mientras que las inclusiones de azufre incrementan el porcentaje de luz reflejada hacia adelante.

5. El sistema experimental utilizado permite el crecimiento de microorganismos oxidadores de sulfhídrico en cultivo continuo mientras mantiene una concentración constante de sulfhídrico en el interior del recipiente de cultivo. Cambios en factores externos como la luz modulan la actividad biológica de los organismos y, en consecuencia, varían la tasa de dilución del sistema, generando nuevas condiciones estables.

6. La relación entre la tasa específica de creixemento (μ) y la tasa específica de absorción de luz (q_e) en cultivos de *Chromatium vinosum* es del tipo saturación, en que μ_{max} alcanza un valor de 0.195 h^{-1}. La tasa específica de mantenimento, μ_e, tiene un valor de -0.025 h^{-1}.

7. Cambios en el suministro de luz inducen variaciones en la respuesta fotosintética de *C. vinosum*. Se han observado incrementos en el contenido específico de Bchl a (de 24 a 85 μg Bchl a $.mg^{-1}$ protein) y en el rendimiento quántico de la fotosíntesis (de 0.032 a 0.095 μmol $S^{\circ}.\mu E^{-1}$) en cultivos limitados por la luz. La capacidad fotosintética (P_{max}), por el contrario, permanece constante, mientras que el organismo aumenta el coeficiente de mantenimiento (m) cuando se encuentra limitado por la luz.

8. El incremento en el contenido específico de pigmentos produce un aumento en el coeficiente de absorción de las proteínas ($m^2.mg^{-1}$ protein), mientras que el coeficiente de absorción de la clorofila (m^2 Bchl a.mg^{-1} protein) disminuye debido al autosombreado entre moléculas de pigmentos.

9. *C. vinosum* adapta la distribución de poder reductor a cambios en las condiciones de iluminación. A irradiancias bajas, la mayor parte del poder reductor se acumula en forma de azufre (95.7% del poder reductor almacenado), mientras que no se observa prácticamente glicógeno. A irradiancias saturantes, por el contrario, una parte importante del poder reductor presente inicialmente como sulfhídrico es destinado a inclusiones de glicógeno (31.6% del poder reductor almacenado). No se detectaron inclusiones de PHB en ninguna de las condiciones de iluminación.

10. El balance de electrones muestra la existencia de una cantidad importante de poder reductor que no está presente en forma de azufre, glicógeno o material estructural. Esta fracción aumenta hasta un 41% del total de electrones en cultivos limitados por la luz. La presencia de carbono orgánico disuelto sugiere que *C. vinosum* podria estar excretando algún compuesto orgánico.

AGRAÏMENTS

AGRAÏMENTS

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