# PHOTOSYNTHESIS AND CALCIFICATION IN THE COCCOLITHOPHORE *EMILIANIA HUXLEYI* AND TWO HERMATYPIC CORALS, *PORITES PORITES* AND *ACROPORA* SP.

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## Abstract

Most global calcification is carried out by organisms which are also photosynthetic. In this study, the coccolithophore *Emiliania huxleyi* (Lohmann) Hay and Mohler and two species of hermatypic coral were used to:

- examine the effect of dissolved inorganic carbon (DIC) and light on photosynthesis and calcification; and
- determine the extent to which these two processes interact.

A novel method of producing coccolith-less (non-calcifying) cells from calcifying cells of the same strain of *E. huxleyi* was developed thus allowing photosynthesis and calcification to be studied separately. The kinetics of photosynthesis in both types of cell, and of calcification in coccolith-bearing cells, were shown to be biphasic with respect to DIC concentration. The hiatus in all three cases was located at 1 mM DIC. This unusual pattern was shown to be the product of two carbon uptake mechanisms: an anion exchanger working at all DIC concentrations and an external carbonic anhydrase active only at low DIC concentrations.

In contrast to the commonly-held view, this study demonstrated that calcification did not promote photosynthesis in *E. huxleyi*. Nevertheless, there was clearly strong biological control of calcification in this alga since DIC uptake was mediated by an anion transporter and a dehydroxylating enzyme.

This work also showed that in *E. huxleyi*, DIC addition enhanced photosynthesis at both limiting and saturating photon flux densities and that bicarbonate affected photochemical processes directly. Photosystem II activity was stimulated and non-photochemical quenching was reduced, possibly protecting the photosynthetic apparatus from damage by light.

In the two corals; *Porites porites* and *Acropora* sp., strong biological control of calcium carbonate precipitation was also evident. Again, calcification did not stimulate photosynthesis. Calcification rates of *Acropora* sp. were monitored in the dark and although these were lower than in the light, they still increased dramatically with bicarbonate addition. This showed that high concentrations of the bicarbonate ion can compensate for the lack of light. Hence, it seems that in hermatypic corals, light-dependence of calcification may be facultative and not obligate. It is therefore clear from the results of this study that calcification and photosynthesis are not as closely coupled as has been previously thought.

In neither *E. huxleyi,* nor in the hermatypic corals, were photosynthetic and calcification rates saturated at the present ambient DIC concentration of seawater.

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# Abbreviations

| | |
|---|---|
| AZ | acetazolamide |
| CA | carbonic anhydrase |
| Chl. | Chlorophyll |
| CPC | coccolith production compartment |
| DIC | dissolved inorganic carbon |
| DIDS | 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid |
| ETC | electron transport chain |
| ETR | electron transport rate |
| EZ | ethoxyzolamide |
| FMS | fluorescence monitoring system |
| $\phi$PSII | quantum efficiency of PSII |
| $G^0$ | standard free energy |
| LHC | light harvesting complex |
| MAP cycle | Mehler-Ascorbate-Peroxidase cycle |
| $P_{680}$ | photosystem II phototrap |
| $P_{700}$ | photosystem I phototrap |
| $pCO_2$ | partial pressure of $CO_2$ |
| PFD | photon flux density |
| PSI | photosystem I |
| PSII | photosystem II |
| qNP and NQP | non-photochemical quenching parameters given by the FMS |
| $q_P$ | photochemical quenching |
| $q_N$ | non-photochemical quenching |

| | |
|---|---|
| $q_E$ | energy-dependant non-photochemical quenching |
| $q_I$ | slow induction non-photochemical quenching |
| $q_T$ | transition type non-photochemical quenching |
| PQ | plastoquinone |
| SEM | scanning electron microscope |
| SITS | 4-Acetamido-, 4'-isothiocyanostilbene-2,2'-disulfonic acid |
| TA | total alkalinity |
| WSC | water splitting complex |
| NPS | net photosynthesis |

# Chapter 1 :

# Introduction

Traditionally, the Earth has been divided into 'spheres' : the lithosphere in its central part and at its surface the atmosphere, hydrosphere and biosphere. The hydrosphere is made up of all the forms of water (fresh, salt, ice and vapour) that are present at the surface of the planet. Water molecules originate from the interior of the Earth, through the process of de-gassing, which began when the planet was formed. De-gassing is the consequence of active convection movements of the Earth's mantle, caused by the extreme heat generated by the radioactive elements, which comprise the planet's central part (Bearman 1995). Because of the exponential decay of radioactivity, the central core of the Earth is now much cooler than in early geological times, so that the rate of degassing has massively decreased. Consequently, the current ratio of mass to land is more or less constant. Nowadays, the oceans dominate the hydrosphere and cover 70.8 % of the Earth's surface (Mason 1966).

Seawater consists of an assemblage of chemical species present in aqueous and gaseous phases (Stumm and Morgan 1981). A series of chemical reactions and physiological processes maintain the current chemical composition of natural seawater. Indeed, in addition to the intrinsic chemical reactions within the water system, seawater constituents can also pass from the water to the surrounding systems, such as the atmosphere, the sediments, and the biota...

Photosynthesis is one of the major processes by which seawater constituents are transferred to the biota; in fact, 40 % of the Earth's productivity takes place in the ocean (Falkowski and Raven 1997). During this process,

organisms transform the dissolved inorganic carbon (DIC) of seawater into organic carbon as illustrated by the following equation :

$$2H_2A + CO_2 + \text{light} \longrightarrow CH_2O + H_2O + 2A \qquad \text{Equation 1.1}$$

In equation 1.1, the letter A represents an element that has a large electron donor capacity. In anaerobic photosynthetic bacteria, this compound can be an atom of sulphur (Blankenship *et al.* 1995). However, most photosynthetic organisms, including cyanobacteria, prochlorophytes, eukaryotic algae and higher plants, evolve oxygen. Thus, equation 1.1 can be modified as followed :

$$2H_2O + CO_2 + \text{light} \longrightarrow CH_2O + H_2O + O_2 \qquad \text{Equation 1.2}$$

Equation 1.2 shows that in aerobic photosynthesis, the light energy is used to reduce water molecules and produce oxygen. The mechanism of the water splitting process, whose emergence was a major turning point in the evolution of life, will be discussed in detail in Chapter 4.

Although anaerobic and aerobic photosynthesis use different electron donors, both processes chemically reduce $CO_2$ to form organic molecules. DIC does not contain any biologically usable energy and can only be transformed into useful carbon through chemical and biochemical reactions. Photosynthesis is the most important process to do this (Falkowski and Raven 1997).

The DIC of seawater originates from minerals and from the atmosphere (Stumm and Morgan 1981). Substantial amounts of the seawater DIC comes from the weathering of rocks, while $CO_2$ addition to the atmosphere is primarily derived from volcanic eruption and fossil fuel combustion, but also from respiration and organic matter oxidation. Although $CO_2$ is the major form of inorganic carbon in the seawater gas phase, the DIC system of seawater is complex, because DIC is present as four distinct species : $CO_3^{2-}$, $H_2CO_3$, $CO_2$ and $HCO_3^-$ (Stumm and Morgan 1981). Equation 1.3 shows that when atmospheric $CO_2$ dissolves in water it reacts with it to form carbonic acid, which in turn dissociates to form bicarbonate and carbonate ions.

$$CO_2 + H_2O \longleftrightarrow H_2CO_3 \longleftrightarrow HCO_3^- + H^+ \longleftrightarrow H^+ + CO_3^{2-} \qquad \text{Eq. 1.3}$$

Bicarbonate dehydration is also aided by the following equation :

$$CO_2 + OH^- \longleftrightarrow HCO_3^-$$

The reaction of the uncatalysed hydration and dehydration of $CO_2$ is relatively slow (1-2 minutes), while the others occur instantaneously (Stumm and Morgan 1996). The equilibrium is mainly dependent on the pH of seawater, but is also affected by temperature and salinity. At high pH, the equilibrium is shifted towards the right of equation 1.3, while at low pH, it is moved toward the left. Figure 1.1 shows the variation in the chemical species

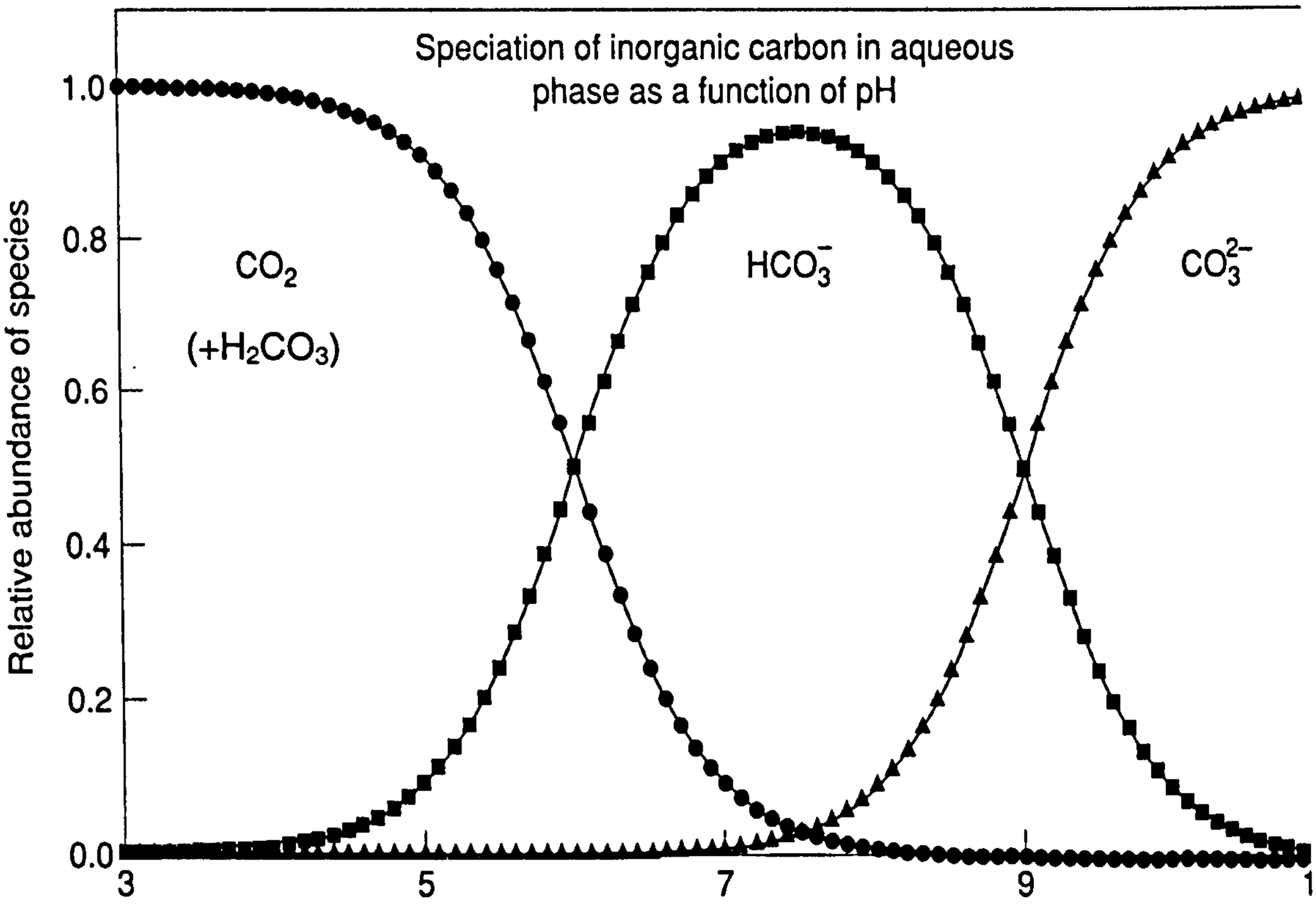


Figure 1.1 : The distribution of $CO_2$, $HCO_3^-$ and $CO_3^{2-}$ as a function of pH (from Falkowski and Raven 1997).

composition of DIC in seawater with different pH. Since the current pH of seawater is 8.0-8.2, most of the carbon is in the form of bicarbonate.

Bicarbonate ions are also used in another important biological process; calcification. This has however the opposite effect of photosynthesis on global carbon cycling. Indeed, equations 1.4 and 1.5 show that, during $CaCO_3$ and $MgCO_3$ precipitation, aquatic organisms sequester DIC into mineral structures (Raven and Falkowski 1999).

$$2\,HCO_3^- + Ca^{2+} \longrightarrow CaCO_3 + CO_2 + H_2O \quad \text{Equation 1.4}$$

$$2\,HCO_3^- + Mg^{2+} \longrightarrow MgCO_3 + CO_2 + H_2O \quad \text{Equation 1.5}$$

Calcium carbonate precipitation is the most common form of calcification in marine organisms. The diagram in figure 1.2 demonstrates that $CaCO_3$ precipitation has been a very active process over geological time, because now most of the carbon is located in the lithosphere in the form of calcium carbonate. In fact, in oceanic sediments, 85 % of the total carbon is in the form of calcium carbonate, while only 15 % is organic carbon (Westbroek *et al.* 1994). At present, there is 2000 times more carbon in inorganic deposits than in the $CO_2$ of the atmosphere (Holland 1984). This massive difference illustrates the high efficiency of calcification to sequester carbon. It is clear that calcification is a crucial process when considered over geological time, but over short periods its importance might be overrated since total calcification is much lower than total photosynthesis. In fact, Westbroek *et al.* (1993) reported that 30-40 Gt of carbon

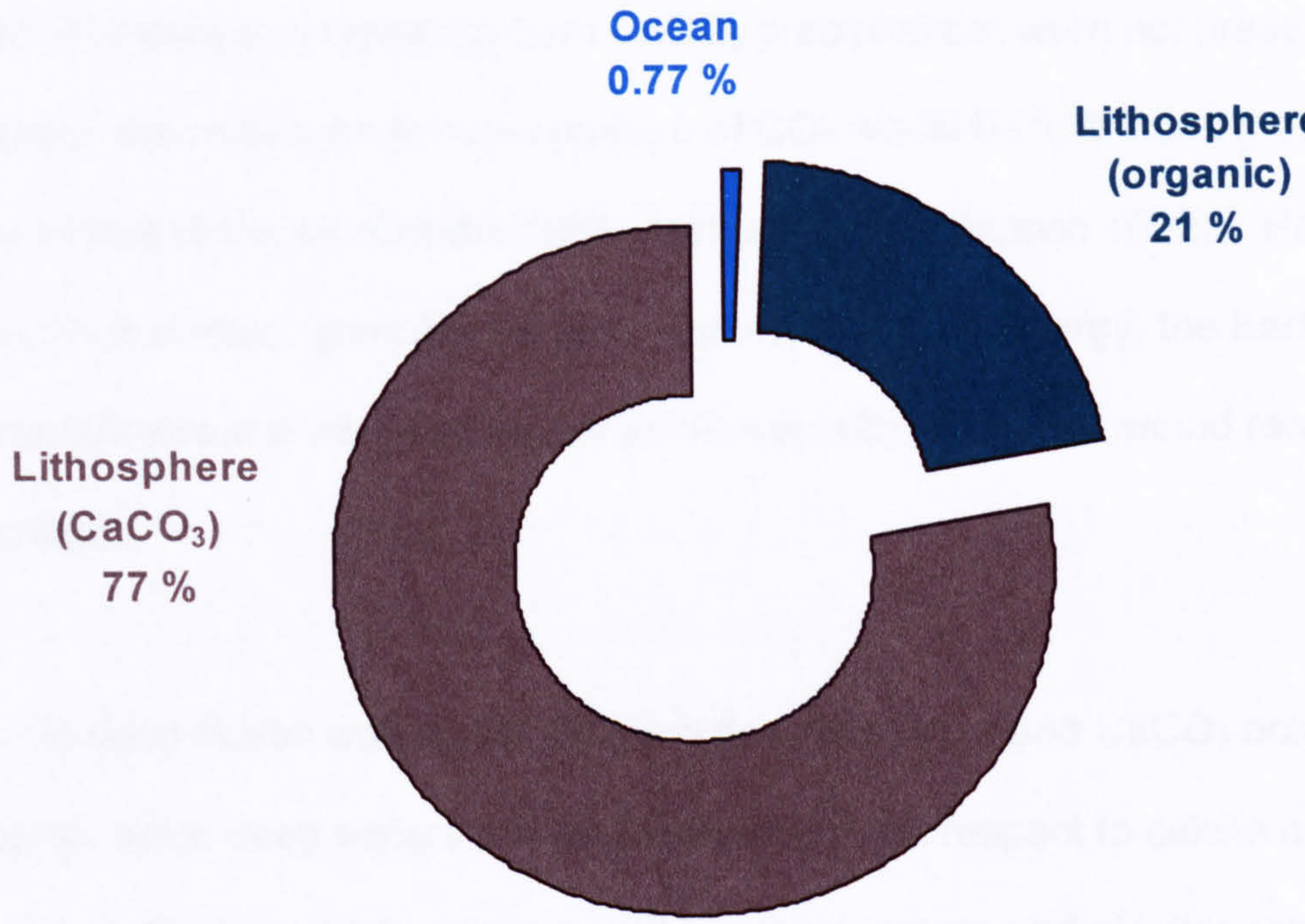


Figure 1.2 : Diagram showing the distribution of carbon on Earth.

was removed each year by aquatic photosynthetic organisms, while only 1-1.3 Gt of calcium carbonate was produced annually.

Calcification is, however, an essential process that sustains life on Earth. Indeed, if limestone, originating from $CaCO_3$ precipitation, were not present on the planet, the atmospheric concentration of $CO_2$ would be 100 times greater, i.e. similar to that of Venus (Condie 1989, Jastrow and Thompson 1972). Hence, since $CO_2$ is a major greenhouse gas, by absorbing heat energy, the Earth's surface temperature would be as hot as Venus (425 °C), which would render life impossible.

In deep ocean waters, calcification does not occur and $CaCO_3$ actually dissolves, since deep waters are undersaturated with respect to calcite and aragonite. Owing to high pressure and low temperature and pH, the solubility of $CaCO_3$ increases with depth. The depth at which the water becomes just saturated with calcium carbonate is around 4.6 km in the North Atlantic and 0.8 Km in the North Pacific. However, at these depths sediments are still found, because settling time is faster than dissolution time. Below these depths, there are no calcium carbonate sediments, and this is called the carbonate compensation point (Whitfield and Watson 1982). In order to describe the state of saturation of $CaCO_3$ minerals, the ion solubility product is compared to the solubility product. The saturation index (SI) of calcium carbonate is defined by the following equation:

$$SI_{\text{calcium carbonate}} = \log \left( \left( \{Ca^{2+}\} \{CO_3^{2-}\} \right) / K_{s0} \right)$$

If the actual ion solubility product is higher than the solubility product ($K_{s0}$), then the solution is oversaturated; if it is lower than $K_{s0}$, the solution is undersaturated; if it is equal to $K_{s0}$, the solution is in equilibrium (saturated) (Stumm and Morgan 1981). It is interesting to note that although surface seawater is saturated with respect to calcite and aragonite (Gattuso *et al.* 1999, Stumm and Morgan 1981), $CaCO_3$ precipitation does not occur spontaneously, but is biologically driven (Kempe and Kazmierczak 1994).

To sum up, while carrying out photosynthesis and calcification, aquatic organisms actively participate in the global cycling of carbon on Earth. For a few decades, the central question has been the extent to which human alteration of the carbon cycle affects these two processes.

Over the last 250 years, increases in the human population and in industrialisation had lead to a rise in the rate of fossil fuel burning and deforestation (Raven and Falkowski 1999). These anthropogenic activities have caused a rise in the atmospheric $CO_2$ concentration ($[CO_2]_{atm}$). The present $[CO_2]_{atm}$ has reached 365 μmol $mol^{-1}$, whereas it was 280 μmol $mol^{-1}$ 2000 years before the Industrial Revolution. Although on a century to millennium time scale the $[CO_2]_{atm}$ is controlled by the DIC concentration of seawater, the net flux of $CO_2$ from ocean to atmosphere is now inverted (Raven and Falkowski 1999). Because the $CO_2$ concentration of surface seawater co-varies with atmospheric $CO_2$ levels, the last 2.5 centuries have been marked by an increase of the surface seawater $CO_2$ partial pressure ($_pCO_2$) (Broeker and Peng 1982). Since the beginning of the Industrial Revolution, oceans have absorbed 30 % of the

additional atmospheric $CO_2$ (Houghton *et al.* 1996). Within the next centuries, a doubling of the $_pCO_2$ is expected, which will correspond to the highest level recorded in the last 24 million years (Gattuso and Buddemeier 2000).

As long as seawater remains supersaturated with respect to $CaCO_3$, $CO_2$ exchange does not affect alkalinity because it does not cause the dissolution of $CaCO_3$ (Stumm and Morgan 1981). Alkalinity is defined as the sum of the charges of all anions of weak acid (see Chapter 6 for details). Thus, a doubling in $_pCO_2$ would simply result, under equilibrium conditions, in a concomitant increase of the $CO_2$ concentrations of seawater and a diminution of the seawater pH by 0.279 units (Stumm and Morgan 1981).

It is relatively easy to predict the way in which ocean chemistry changes in response to $_pCO_2$ increases, but biological responses are very difficult to foresee because of our limited understanding of physiology and of the relationship between photosynthesis and calcification (Gattuso and Buddemeier 2000).

On land, increased $[CO_2]_{atm}$ will probably have a fertilising effect, since terrestrial plants take up and use $CO_2$ for photosynthesis. However, $HCO_3^-$ is the form of DIC taken up by most photosynthetic aquatic organisms (Gattuso and Buddemeier 2000). In these marine plants, subsequent internal $HCO_3^-$ dehydration occurs in order to provide $CO_2$ molecules to the main enzyme of the carbon reduction cycle, ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Hence, if the alkalinity of seawater remains constant under increased $_pCO_2$ levels, little direct effect should be noticed on the photosynthetic rates of

most marine organisms, except for a few mainly $CO_2$ users such as seagrasses (Gattuso and Buddemeier 2000). On the other hand, calcification is likely to be inhibited by the predicted higher concentration of $CO_2$ of seawater. Indeed, several studies have shown that the calcification rate of marine organisms is repressed by enhanced $CO_2$ concentrations (Riebesell *et al.* 2000, Langdon *et al.* 2000, Leclercq *et al.* 2000, Gattuso *et al.* 1999).

However, the biological effects of increased $_pCO_2$ may not be as straightforward as described above. Firstly, $_pCO_2$ is not the only parameter that is expected to change in the future. In fact, increased seawater temperature and lower pH are also predicted. These factors will most certainly affect photosynthetic and calcification rates. Secondly, if photosynthesis depends on calcification, although increased $_pCO_2$ levels are not expected directly to affect photosynthesis, indirect effects could occur through inhibition of calcification. An understanding of the nature of the relationship between photosynthesis and calcification is therefore essential in order to make any predictions of the biological effects of increased $CO_2$ levels. Thirdly, long-term effects and possible adaptations are currently difficult to appreciate.

Several authors argue that $CO_2$ production during calcification (equation 1.4) can potentially be used for photosynthesis, so that calcification and photosynthesis enhance one another (Gattuso *et al.* 1999, Buitenhuis *et al.* 1999, Nimer *et al.* 1996, Sikes *et al.* 1980). Calcification has been strongly associated with photosynthesis primarily because of its dependence on light. High calcification rates are indeed only measured in the light. Photosynthesis could

promote calcification, because when using $CO_2$ molecules, photosynthesis drive equation 1.3 to the right, hence locally increasing the carbonate concentration. Although calcification and photosynthesis seems to be linked, the exact nature of this interaction remains controversial.

The aim of the present thesis is to improve our knowledge on this subject by examining the link between photosynthesis and calcification and by establishing the physiological effect of bicarbonate on these two processes. The coccolithophore *Emiliania huxleyi* (Lohmann) Hay & Mohler and the hermatypic corals, *Porites porites* (Link) and *Acropora* sp. (Oken), were chosen for this study, because these two groups are the main photosynthetic organisms that produce calcium carbonate. The map on figure 1.3 was computed by Gattuso and Buddemeier (2000) and demonstrates that together coral reefs and coccolithophores cover most of the world's coastal waters. Because of their widespread distribution, any physiological changes concerning these organisms, caused by increased $CO_2$ concentrations, are likely to strongly affect the biogeochemistry of the ocean (Gattuso and Buddemeier 2000) and possibly of the whole planet.

*E. huxleyi* and hermatypic corals have also been extensively studied because they are useful tools in the study of past climates, an understanding of which is essential in order to predict future climate changes. Corals reefs are long lived and although coccolithophore blooms are transient, they occur annually. The analysis of coral skeletons or of coccoliths (i.e. plates of $CaCO_3$) embedded in sediments affords a glimpse at past climates.

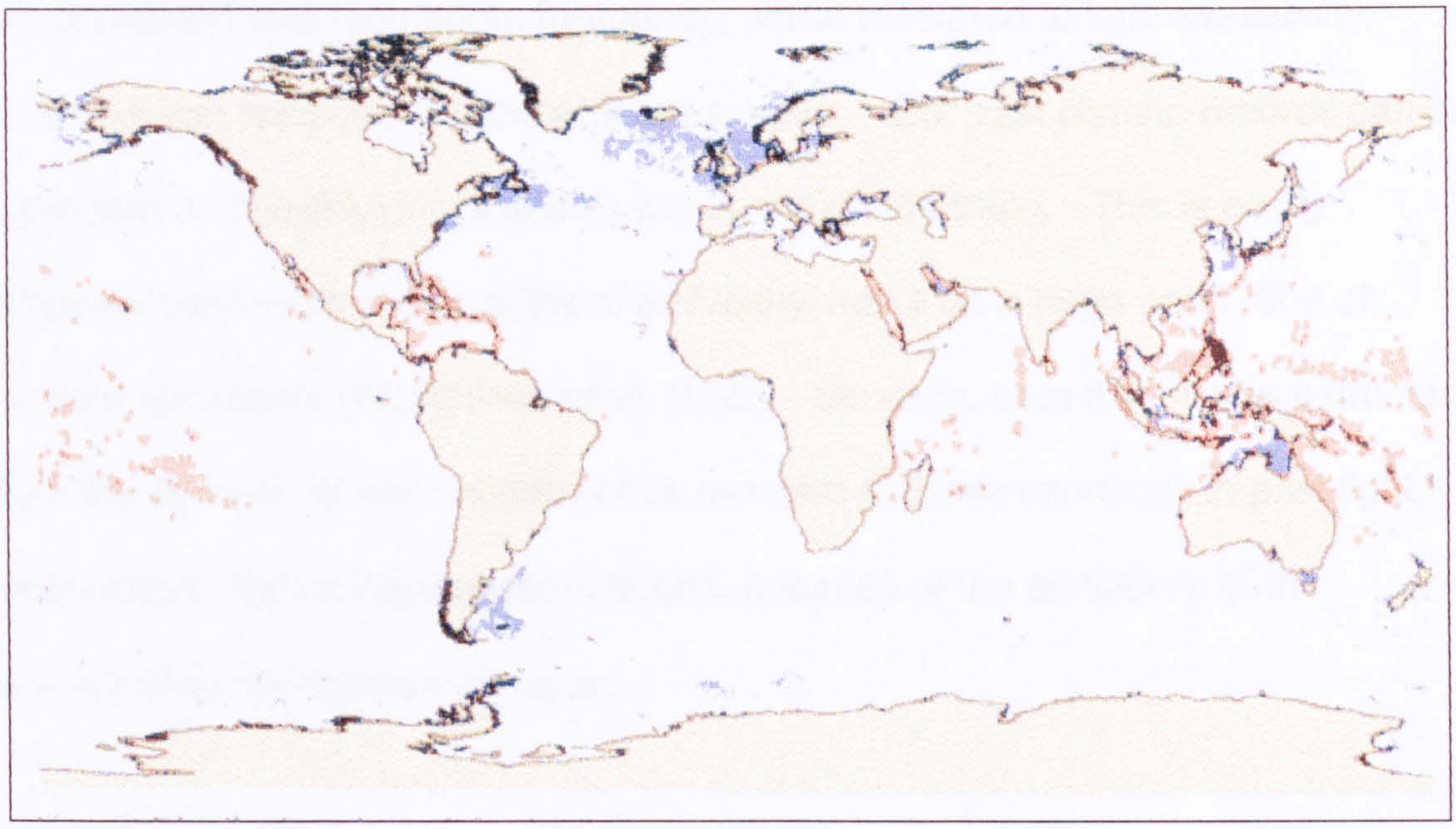

Figure 1.3 : The distribution of coccolithophore blooms (blue dots) and coral reefs (red dots) in the ocean (from Gattuso and Buddemeier 2000).

Barnes and Lough (1993) stressed that the International Geosphere-Biosphere Program (1989-1990) has emphasised our need to understand past and future climate variation. Large quantities of coccoliths of *E. huxleyi* within sediments are correlated with high boom frequency, which is related to light availability, water column temperature and nutrient supply. So, past climate records can be estimated by quantifying coccoliths within sediments traps. This is easily achieved because the coccoliths of *E. huxleyi* make up a large proportion of modern sediments (Westbroek *et al.* 1986). Similarly, data from cores extracted from the skeleton of hermatypic corals can also indicate variations in past light, temperature and nutrient concentrations, because of the sensitivity of the calcification rate to these variables.

*E. huxleyi* is currently the most abundant coccolithophore (Honjo 1976). This group of phytoplanktonic algae belongs to the class Prymnesiophyceae, which is essentially marine and characterised by the production of external organic scales or of calcite plates. Fossil records have established that the emergence of the Prymnesiophyceae dates from the Carboniferous, approximately 300 million years ago. The global domination of coccolithophores in coastal and oceanic water ended with the massive Cretaceous extinction event (144 millions years ago), which gave rise to the present worldwide supremacy of diatoms. *E. huxleyi* is however a relatively young species, since it evolved in tropical waters approximately 270,000 years ago. Even more recently (80,000 years ago) *E. huxleyi* replaced *Gephyrocapsa caribbeanica* as the dominant coccolithophore (Thiestein *et al.* 1977). The global importance of *E. huxleyi* might have been overrated (see Paasche 2001), hence in this study, this alga was treated as a representative of the coccolithophorid group as a whole.

*E. huxleyi* is a pelagic microalga which lives in temperate regions and carries out internal crystallisation of calcium carbonate in the form of calcite (see more detail in Chapter 5). These characteristics differ from those of the other large $CaCO_3$ producer, the hermatypic corals. These live on the benthos of tropical seas, and calcium carbonate is deposited at the base of the animal tissue in the form of aragonite. The differences between aragonite and calcite lie in the organisation and the orientation of the carbonate molecules (Falini *et al.* 1996). In hermatypic corals, aragonite crystallisation occurs externally to the plasmalemma of the symbiotic alga, so that unlike *E. huxleyi*, photosynthesis and calcification do not take place at the same location.

Hermatypic corals are a good example of a symbiosis between an animal and a plant. The invertebrates harbour microalgae called zooxanthellae within their tissues (see Chapter 6 for more details). The animal part of this association is a cnidarian. The most characteristic features of this phylum are given below. These animals display radial symmetry, which is created by the tentacles that encircle the mouth located at one end of the body. This anus / mouth is the connection between the outside seawater and a vascular digestive cavity called coelenteron. These are two-layered organisms, with a band of mesogloea separating two tissues : the ectoderm (epiderm) and the endoderm (gastroderm). These cnidarians produce stinging organelles (nematocysts) containing a paralysing toxin, which enables them to adopt predatory behaviour. Finally, their body can take two different forms, either a pelagic medusa, or a benthic polyp.

The class anthozoa includes hermatypic corals and is characterised by large and complex polyps. The coelenteron of hermatypic corals contains vertical divisions, called mesentery. Figure 1.4 shows the structure of a typical polyp of hermatypic corals. Although these invertebrates appear quite primitive, they form reefs that are the largest structure ever made by an animal, including humans. They also have an ancient origin, as fossil remains of cnidarians have been traced back to the Precambrian. Nevertheless, the way in which this phylum relates to other animals is still unclear, so that many consider cnidarians to be a monophyletic group that is an evolutionary dead end (Moore 2001).

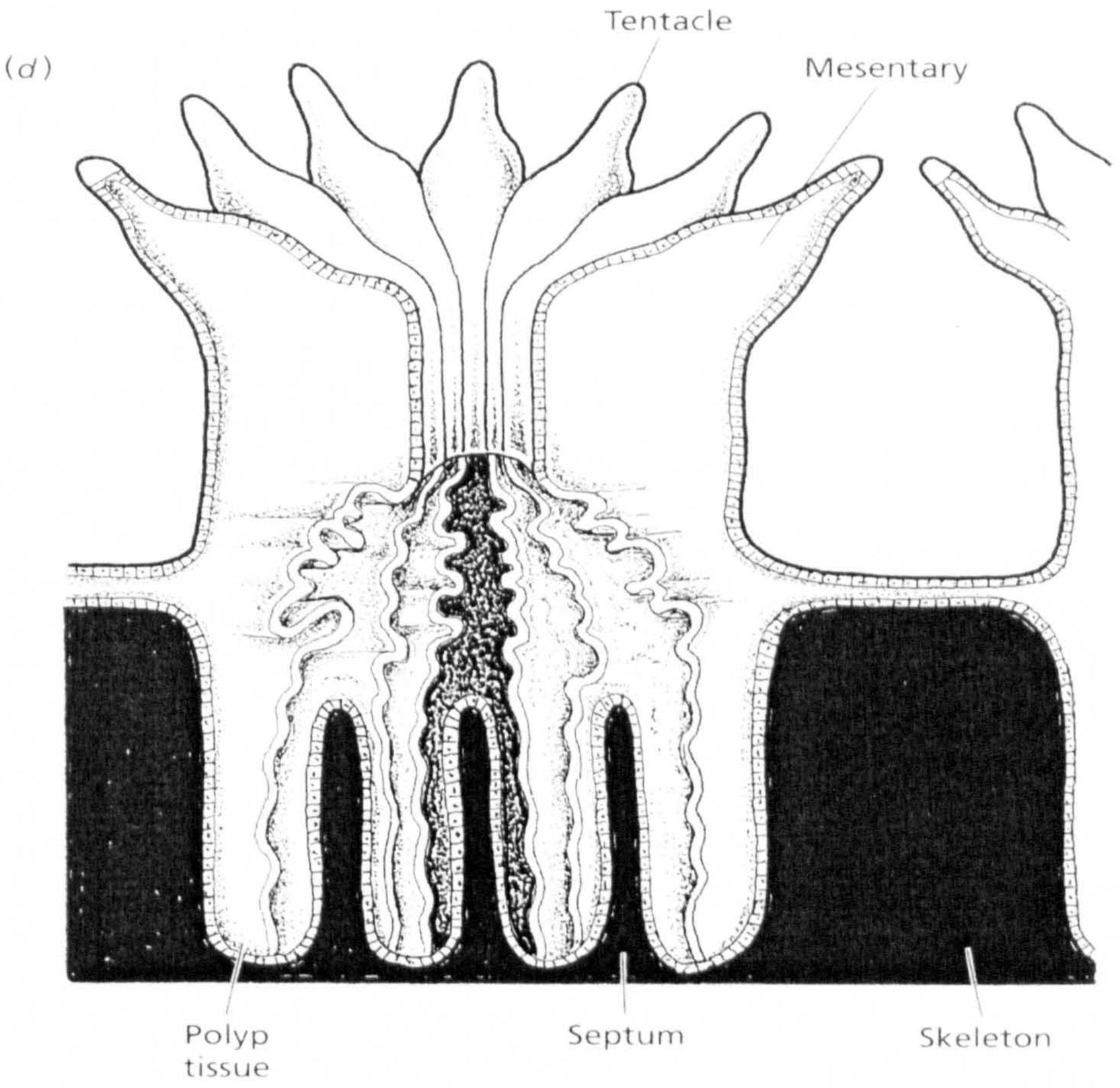


Figure 1.4 : Diagram showing the structure of hermatypic corals (from Moore 2001)

# Chapter 2 :

# Materials and methods

*1 - Organisms:*

***Emiliania huxleyi :***

Unicellular cultures of the coccolithophorid, *Emiliania huxleyi* (Lohmann) Hay & Mohler calcifying strain PCC.B11 were supplied by the Marine Biological Association Culture Collection, Plymouth. Cells were grown in 250 ml conical flasks containing 100 ml of Harrison's artificial seawater medium (Harrison *et al.* 1980), at pH 8.2 (appendix 1). Medium and glassware were autoclaved for 20 minutes at 120˚C. Cultures were grown at 15˚C, on a rotating shaker at 100 rpm, under a continuous downwelling photon flux density (PFD) of 120 μmol $m^{-2}$ $s^{-1}$, provided by white fluorescent lights.

Before the experiments, cells in mid-exponential growth phase (3 to 4 days after inoculation) were harvested by centrifugation for 10 minutes at 150 X g. Cells were resuspended in simplified medium from which $NaHCO_3^-$ was omitted (appendix 2), which was buffered with 25 mM bicine (N,N-bis[2-Hydroxyethyl]glycine) and the pH adjusted to 8.2.

**Hermatypic corals :**

Nubbins of *Porites porites* (Link) were collected in September 2000 from

3 m depth on a reef situated on the West Coast of Barbados. Nubbins were promptly returned to the wet-bench of the Bellairs Research Institute of McGill University, Holetown, Barbados.

Several colonies of *Acropora* sp. (Oken) of Indo-Pacific origin were supplied by the Tropical Marine Centre (Chorleywood, UK) between February and May 2001.

Further details concerning coral maintenance are provided in Chapter 6.

*2 - Oxygen production measurements:*

Photosynthesis consists of a series of photochemically catalysed oxidation-reduction reactions, which provide the necessary NADPH and ATP for the carbon fixation cycle. Because water is the first electron donor of the photosynthetic electron transport chain, a molecule of $O_2$ is produced for each $CO_2$ molecule fixed.

Oxygen evolution was monitored using Clark-type electrodes (Rank Brothers, Cambridge, UK and Hansatech, Norfolk, UK). The Clark-type electrode consists of a platinum disc functioning as a cathode embedded in a plastic insulator, and of a ring-shaped silver anode bathed in an electrolyte (KCl 3M). A gas-permeable Teflon membrane separates electrodes and reaction medium. This membrane allows the diffusion of $O_2$ molecules, but not of ions.

A magnetic stirring rod enables constant stirring of the reaction medium to prevent stratification occurring and to facilitate $O_2$ diffusion.

The Clark-type electrode works on the polarography principle, whereby when constant voltage (0.65 V) is applied across the electrodes, they become polarised and the following reactions take place :

$$O_2 + 4\,H^+ + 4\,e^- \longrightarrow 2\,H_2O \qquad \text{cathode}$$

$$4\,Ag \longrightarrow 4\,Ag^+ + 4\,e^- \qquad \text{anode}$$

Thus, as $O_2$ diffuses through the membrane into the electrolyte, it is immediately transformed into water molecules at the cathode. Silver is regenerated by the electrolyte according to the following equation :

$$Ag^+ + Cl^- \longrightarrow AgCl$$

The electrical signal produced by these reactions is proportional to the flux of $O_2$ (Delieu and Walker 1981). Because $O_2$ solubility, the electrode itself and the organism are all temperature sensitive, the whole apparatus (reaction medium, chamber and electrodes) is maintained at constant temperature by a water jacket, containing circulating water from a thermostatically controlled water-bath. Cool halogen light was provided by projectors.

Before the start of experiments, a DIC-free suspension of 1.5 to 2 x $10^6$ cells $ml^{-1}$ was incubated at 15 °C and at a PFD of 500 μmol $m^{-2}$ $s^{-1}$. This is the saturating PFD for photosynthesis and calcification in this alga (Nimer and Merrett 1992). Additions of bicarbonate were made through the capillary tube of the stopper. The rate of $O_2$ evolution was determined by slope analysis from the recorder trace of $[O_2]$ vs. time.

*3 - Photosystem II activity measurements :*

Photosynthesis was also directly measured by assessing photosystem II (PSII) activity using a Hansatech Fluorescence Monitoring System (FMS). This instrument measures chlorophyll fluorescence emissions. This is a non-intrusive method, which had originally been developed as a field tool to predict crop health.

This technique works on the principle that in plants, at room temperature, the reaction centre of PSII is the main source of variable fluorescence and that any change in fluorescence is related to a modification of the state of PSII.

Indeed, photons absorbed by chlorophyll molecules can trigger either photochemical or non-photochemical processes. The former uses the absorbed energy for photochemical reactions, hence constituting the first phase of photosynthesis. In contrast, non-photochemical processes dissipate energy and thus do not drive photosynthesis (Schreiber *et al.* 1994). In this case, energy is

either quenched as infrared radiation (heat) or red / far-red radiation (chlorophyll fluorescence). Increases in non-photochemical processes are correlated with a diminution in photosynthetic activity. Chlorophyll fluorescence emission is therefore a good indicator of the state of PSII.

The FMS provides two sorts of primary fluorescence parameters : dark- and light-adapted ones.

Fo, also called the fluorescence origin, characterises the minimum fluorescence yield, i.e. when the primary electron acceptor, $Q_A$, is fully oxidised (Schreiber *et al.* 1994). This fluorescence parameter is measured after dark adaptation. On the other hand, Fm, the maximum fluorescence yield, is gained by exposing dark-adapted tissue to a short, intense and saturating pulse of light. Thus, Fm corresponds to the fully reduced state of $Q_A$ (Schreiber *et al.* 1994). More details on the photochemistry and especially on the primary events of photosynthesis will be given in Chapter 4. The variable fluorescence, denoted Fv, can be expressed as followed :

$$Fv = Fm - Fo$$

Since Fv is the difference between Fm and Fo, it is an estimation of the maximum capacity for photochemical quenching. The most important dark-adapted fluorescence parameter is the ratio of variable to maximum fluorescence (Fv/Fm), which is directly proportional to the maximum quantum efficiency of PSII. Fv/Fm is biomass independent and is widely used as a measure of plant health.

PSII quantum efficiency (ϕPSII) is a light-adapted parameter and is the equivalent of Fv/Fm in the light. Fs, the steady state fluorescence yield, corresponds to Fo, and a saturating pulse of light is required to obtain Fm', the light-adapted equivalent of Fm. ΦPSII is calculated as follows :

$$\Phi PSII = (Fm' - Fs) / Fm'$$

The non-photochemical quenching parameters (qNP and NPQ) are computed using the equations below :

$$qNP = (Fm - Fm') / (Fm - Fo)$$

$$NPQ = (Fm - Fm') / Fm'$$

Cells of *E. huxleyi* were centrifuged for 10 minutes at 150 X g and resuspended in simplified artificial seawater medium (appendix 2). After DIC addition, 1 ml of cells was transferred to an Eppendorf vial. After 15 minutes of dark adaptation, the Eppendorf vial was fitted onto the FMS probe and measurements started. Figure 2.1 shows the conversion of actinic light emitted by the FMS probe into PFD when calibrated through a piece of Eppendorf vial.

Chapter 6 provides the details of the method used for measuring PSII quantum efficiency in *Acropora* sp..

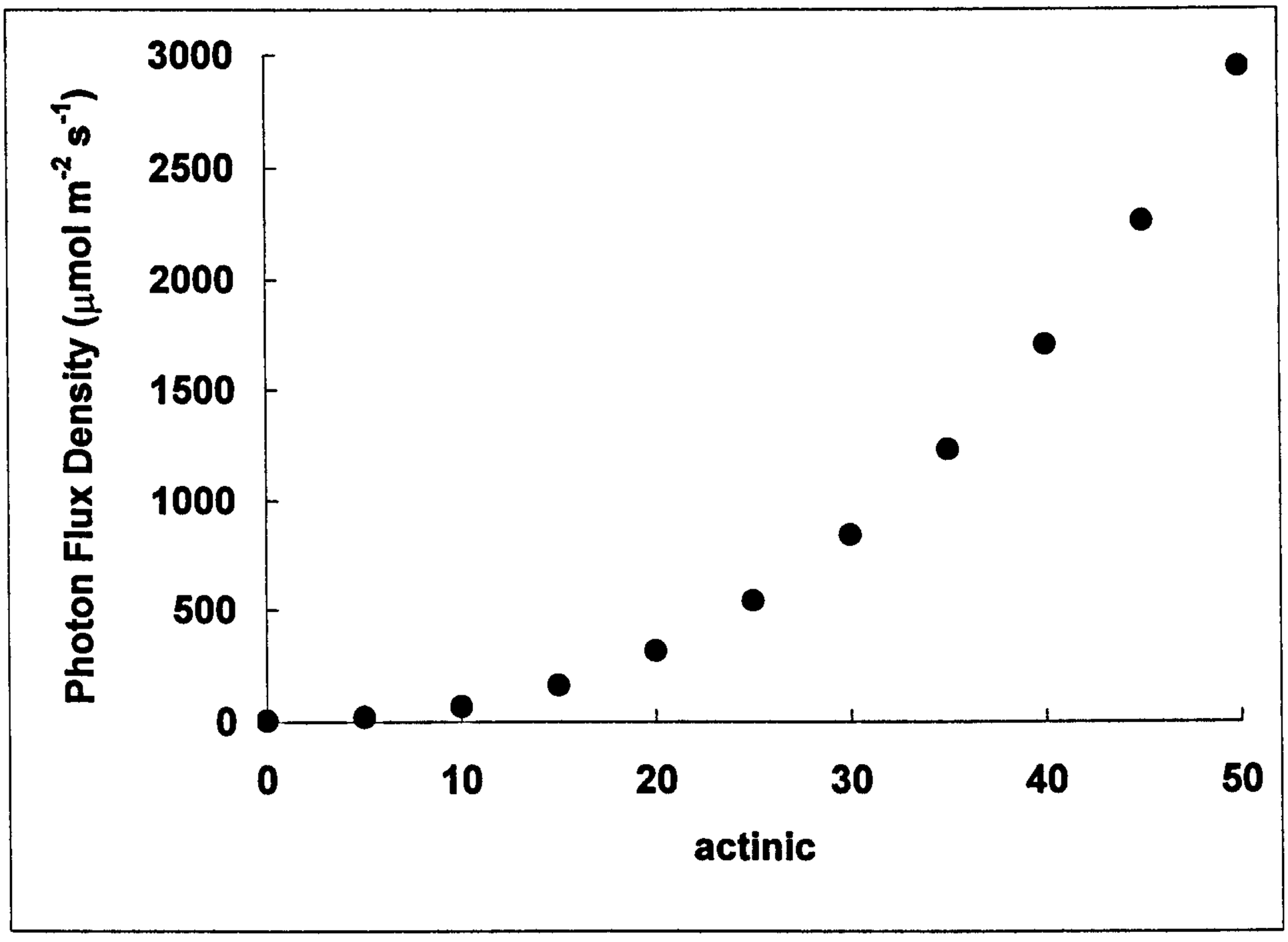


Figure 2.1 : The conversion of actinic light emitted by the FMS into PFD when calibrated through a piece of Eppendorf vial.

*4 - Radioactive carbon incorporation :*

Steemann-Nielsen (1952) was the first to use $^{14}C$ for the measurement of photosynthetic and calcification rates in algae In the present study, experiments were based on the filtration procedure described by Paasche (1963). The distinction between the $^{14}C$ used during photosynthesis for the production of organic compounds and the $^{14}C$ used during calcification is possible since the $CaCO_3$ produced is acid soluble.

DIC plus 5 or 6 μCi of $NaH^{14}CO_3$ (Nycomed Amersham, FA.3) were simultaneously injected into 20 ml of DIC-free suspension of $0.5 \times 10^6$ cells $ml^{-1}$. The total initial radioactivity of each flask, referred to as 'whole DPM', was assayed by withdrawing 20 μl of the solution ($V_{initial}$) and transferring it to a scintillation vial containing 5 ml of scintillation fluid (Ecoscint, National Diagnostic). Each flask was incubated on a shaker, at 15 °C and at a PFD of 500 μmol $m^{-2}$ $s^{-1}$. After 3 hours, 2 ml of the solution were filtered through a cellulose nitrate membrane filter with a pore size of 0.2 μm. Each filter was rinsed twice with 2 ml of filtered, unlabeled seawater. Six measurements were made for each treatment. Three filters were directly added to scintillation vials, each containing 5 ml of fluid. The remaining 3 filters were fumed with acid (HCl 1M) for 30s, before rinsing and placing in scintilliant. This procedure was carried out 4 times (with 3 pseudo-replicates for each measurement).

Radioactivity in the vials was measured using a Tri-carb 2200 CA liquid scintillation analyser. Because acid labile products originate only from calcification, the photosynthetic rates were computed from the DPM values of the

acid-fumed filters. The calcification rates were obtained by subtracting the acid-fumed DPM from the non-fumed ones.

The total carbon uptake was calculated from the specific activity of the radio-labelled $NaH^{14}CO_3$, using the following equations :

$$\mathbf{A} = \text{Total counts in each flasks} = \frac{V_{flask} \times \text{'whole DPM'}}{V_{initial}}$$

where $V_{flask}$ is the total volume of medium contained in each flask (µl), $V_{initial}$ is the volume of the sub-sample withdrawn from each flask before the incubation started (µl), and 'whole DPM' is the number of counts derived from the $V_{initial}$ sub-sample.

$$\mathbf{B} = \text{Total counts corresponding to 1 µmol of DIC} = \frac{\text{Specific activity} \times \mathbf{A}}{\text{'Total } ^{14}C \text{ injected'}}$$

where the specific activity is given in µCi µmol$^{-1}$ and 'Total $^{14}C$ injected' in µCi.

$$\textbf{Total carbon uptake} = \frac{\text{DPM} / \mathbf{B}}{\text{Cell number} \times \text{Time}} \quad \text{fmol cell}^{-1}\ \text{h}^{-1}$$

where the cell number is the number of cells contained in 2 ml of medium and the incubation time is expressed in hours.

Mrs B. Thake and Dr. Z. Weinberg-Flax assisted in some of the $^{14}C$ experiments and this help was gratefully acknowledged.

*5 - Scanning Electron Microscopy (SEM):*

Cells of *E. huxleyi* grown in media containing different calcium or magnesium concentrations were inoculated on at least 3 successive occasions in a freshly made medium before being harvested. Two methods were used to separate cells of *E. huxleyi* from the culture medium.

The first protocol enabled coccospheres to remain intact. Cells in their mid-exponential growth phase (2 ml) were added to 7 ml of a cold solution of 3 % (w/v) of glutaraldehyde in 0.05 M sodium cacodylate (pH 8.2). After 2 hours on a rotator at room temperature, the cells were filtered onto 0.45 μm cellulose nitrate membrane filters (Whatman) and washed with a cacodylate buffer (pH 8.2), following by rinsing with deionised water. After being left to dry overnight at room temperature, each filter was fixed onto a SEM stub and sputter-coated with a thin layer of gold. Samples were examined using a JEOL (JSM-35) Scanning Electron Microscope.

The film of the cells of *E. huxleyi* grown at different calcium concentrations was developed by Mr K. Pall, School of Biomedical Sciences, Queen Mary, University of London, UK.

These pictures were developed on glossy, grade 2 Kentmere Photographic paper (Kendal, UK), at 30 °C, in a AGFA PRO developing machine (type : 8657/200, F-Nr 2057).

The second protocol was much more intrusive, but yields organic material-free coccoliths. This procedure was used for the detailed analysis of the structure of the coccoliths of *E. huxleyi* grown under different calcium and magnesium concentrations. Cells in their mid-exponential growth phase were centrifuged for 10 minutes at 150 X g and resuspended for 4 minutes in 3 % bleach (Jasan supplies LTD, Harlow, UK). Cells were then centrifuged for 5 minutes at 150 X g and rinsed twice in deionised water. One drop of each sample was deposited onto a microscopic slide or a SEM stub for electron microscopy analysis. Samples were sputter-coated with a thin layer of gold and examined using either a JEOL (6300F) Scanning Electron Microscope or a Hitachi S-4500 FESEM.

*Other methods:*

pH was measured with a 420A Orion pH meter, calibrated with pH 7 and 9.2 buffers. The PFD was determined using a Hansatech light meter type QRT1. Cells counts were made with a Fuchs-Rosenthal haemocytometer (depth 0.2 mm, 1/16 $mm^2$). A minimum of 400 cells was counted for each sample.

# Chapter 3 :

# Bicarbonate transport mechanisms in *Emiliania huxleyi*.

Introduction :

Seawater contains about 2 mM DIC which consists of carbonate and bicarbonate ions and dissolved carbon dioxide. These three forms of carbon are present in equilibrium (Stumm and Morgan 1996, Beer 1994) and their relative proportions depend mainly on pH, but also on the salinity and temperature of the water (see Chapter 1 for details). Since seawater has a pH of 8.2, most of the carbon is in the form of bicarbonate (figure 1.1). Under these conditions, carbon dioxide represents less than 1% (11 μM at 20°C) of the total DIC concentration of seawater (Drechsler *et al.* 1993). $CO_2$ concentration is similar in air and seawater, but its diffusion rate in water is $10^4$ or more times slower than in air. Gutknecht *et al.* (1977) calculated the permeability of lipid membranes to $HCO_3^-$ and $CO_2$ and found values of $2 \times 10^{-7}$ and 0.2 cm $s^{-1}$ respectively. Bicarbonate diffuses more slowly across membranes because it is charged and is a much larger molecule. Miller and Colman (1980) compared the measured photosynthetic rates in *Coccochloris peniocystis* to the theoretical rate that could be supported solely by the spontaneous dehydration of $HCO_3^-$ to $CO_2$ in a closed system. They demonstrated that the measured rates of photosynthesis in this alga could only be supported by active transport of inorganic carbon from the medium. Beer (1994) has also shown that simple $CO_2$ diffusion is often insufficient to sustain the high level of photosynthesis observed in marine organisms. Consequently, many algae must posses a carbon concentrating mechanism (CCM) (Aizawa and Miyachi 1986, Badger *et al.* 1980).

Since bicarbonate is the most abundant form of DIC in seawater (93%), a bicarbonate rather than a $CO_2$ uptake mechanism would be more advantageous for algae. Several studies have confirmed that bicarbonate is the source of exogenous carbon for photosynthesis in many aquatic organisms (Maberly and Madsen 2002, Gao and Zou 2001, Buitenhuis *et al.* 1999, Beer 1994, Munoz and Merrett 1989, Dixon *et al.* 1987, Kerby and Raven 1985, Lucas and Berry 1985 Paasche 1964).

Bicarbonate can be transported across the plasmalemma by at least three distinct mechanisms. The first is by direct diffusion across the plasma membrane into the cell. $HCO_3^-$ is then converted into $CO_2$ by an internal carbonic anhydrase (CA), so that steady state flux of $CO_2$ from the plasmalemma to the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is maintained. Internal CA is found in most marine macroalgae (Giordano and Maberly 1989). But because of the very slow permeability of bicarbonate through unmodified lipid layers (Gutknecht *et al.* 1977), simple diffusion of $HCO_3^-$ through the plasma membrane cannot sustain the high requirement in carbon of *E. huxleyi*, since this alga needs carbon for calcification as well as photosynthesis.

An external or exofacial carbonic anhydrase can also transport carbon into the cell. This is a zinc-containing metalloenzyme, which performs the extracellular dehydroxylation of $HCO_3^-$ followed by the penetration of $CO_2$ into the cell. This seems an ubiquitous protein (Smith 1988), since it has been reported in a large number of microalgae (Iglesias-Rodriguez and Merret 1997, Sültemeyer *et al.* 1993, Badger and Price 1994, Aizawa and Miyachi 1986).

An anion exchange protein is the third mode of transport of bicarbonate across the plasmalemma. Several publications (Drechsler *et al.* 1993, Drechsler *et al.* 1994, Sharkia *et al.* 1994, Axelsson *et al.* 1995) have established the presence of a bicarbonate transport mechanism similar to the one in mammalian red blood cells in the plasmalemma of the green seaweed *Ulva* sp.. The anionic exchange protein AE1 facilitates bicarbonate diffusion across the external membrane by exchanging $Cl^-$ and $HCO_3^-$. Because this anion exchanger represents 25 % of the membrane protein of human red blood cells (Jennings 1984), it constitutes the most studied $Cl^-$ exchanger protein. The anion transport activity of this glycoprotein is located on the hydrophilic COOH-terminal domain (Kopito 1989). Disulfonic stilbenes are the most potent inhibitors of this carrier protein (Drechsler *et al.* 1993). The chemical probe 4-Acetamido-, 4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) is a commonly used inhibitor (Cabantchik and Greger 1992) since it is a covalent binding agent of the anion exchanger.

Earlier work by Weinberg-Flax (2000) in this laboratory established that in ambient seawater *E. huxleyi* possesses a protein that is functionally and structurally similar to the anion exchanger AE1 of human erythrocytes. Having found how bicarbonate is transported into *E. huxleyi*, Weinberg-Flax subsequently investigated the kinetics of photosynthesis with increasing DIC concentrations. Her finding agreed with the growing awareness that the ambient DIC concentration of seawater limits photosynthesis in many marine organisms (Mercado *et al.* 2001, Beer and Rehnberg 1997, Merrett *et al.* 1993), including *E. huxleyi* (Buitenhuis *et al.* 1999, Nielsen 1995, Nimer and Merrett 1993, Paasche 1964). A rather interesting pattern

emerged from her data. Her result, presented in figure 3.1, suggested that photosynthesis did not follow a rectangular hyperbola with increasing DIC concentrations, but was biphasic. Photosynthesis had different kinetics at low and high DIC concentrations, with a hiatus located at 1 mM DIC.

The aim of the present work was to see if the kinetics were truly biphasic and if so, to find the reasons for this unusual pattern. The relationships between photosynthesis, calcification and DIC concentration in *E. huxleyi* were therefore investigated.

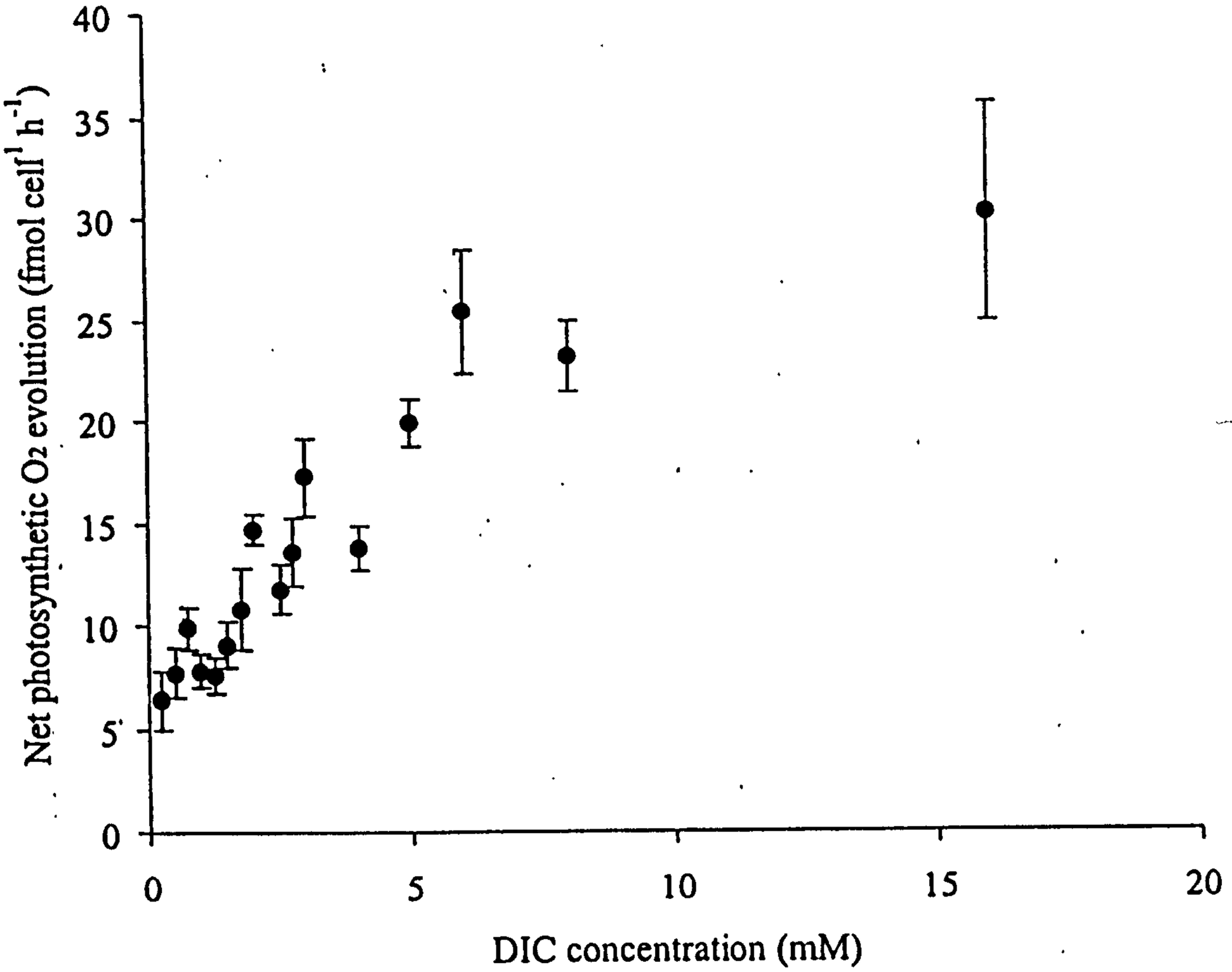


Figure 3.1 : The effect of different concentrations of DIC on $O_2$ production of a calcifying strain of *E. huxleyi* (from Weinberg-Flax 2000).

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## Materials and Methods :

Unicellular cultures of the coccolithophore, *Emiliania huxleyi* (Lohmann) Hay & Mohler (calcifying strain PCC.B11) were grown as described in Chapter 2. Non-calcifying, coccolith-less cells of this calcifying strain of *E. huxleyi* were obtained by growing the algae in calcium-free medium.

The methods employed for the measurements of $O_2$ evolution using Clark-type electrodes, of the induced fluorescence parameters provided by a Fluorescence Monitoring System and of $^{14}C$ incorporation using the filtration technique proposed by Steemann-Nielsen (1952) and modified by Paasche (1963) are also explained in detail in Chapter 2.

Three carbon transport inhibitors were used during this study :

- Acetazolamide (AZ), a membrane impermeable and specific inhibitor of carbonic anhydrase.
- Ethoxyzolamide (EZ), a membrane permeable and specific inhibitor of carbonic anhydrase.
- 4-Acetamido-, 4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS), a specific inhibitor of anion exchange protein.

Results :

*Rate of photosynthesis with different DIC concentrations :*

To test Weinberg-Flax's results using the $O_2$ electrodes that in *E. huxleyi* the kinetics of photosynthesis are indeed biphasic with increased DIC concentrations, the rate of photosynthetic $^{14}C$ incorporation into the cell was measured. Figure 3.2 shows that there is a 2.2 fold increase of the photosynthetic rate from 2 to 10 mM DIC, with values of 4.4 and 9.6 fmol $cell^{-1}$ $h^{-1}$ respectively. This technique confirms the $O_2$ results and shows that the kinetics of photosynthesis are biphasic with different DIC concentrations, with a hiatus located at 1 mM DIC.

*Rate of calcification with different DIC concentrations :*

Measurements of $^{14}C$ incorporation into the coccoliths were carried out to determine the kinetics of calcification with added DIC concentrations. Data presented in figure 3.3 demonstrate that calcification rates increase with adding DIC concentrations. Calcification also shows the same biphasic pattern as photosynthesis, with a hiatus again located at 1 mM DIC.

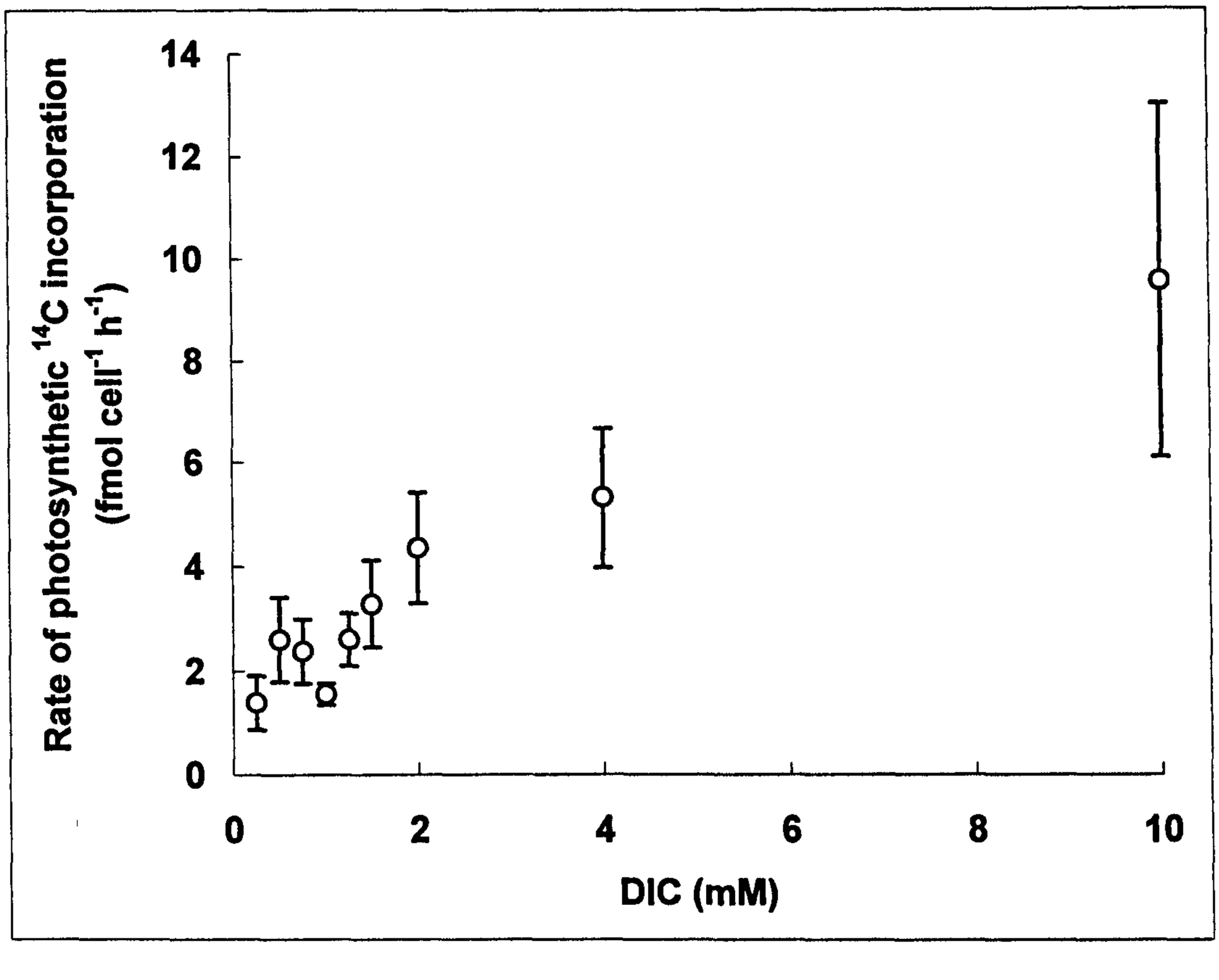



Figure 3.2 : The effect of different DIC concentrations on the rate of photosynthetic $^{14}C$ incorporation in *E. huxleyi*.

Data represent means ± SE, n = 4.

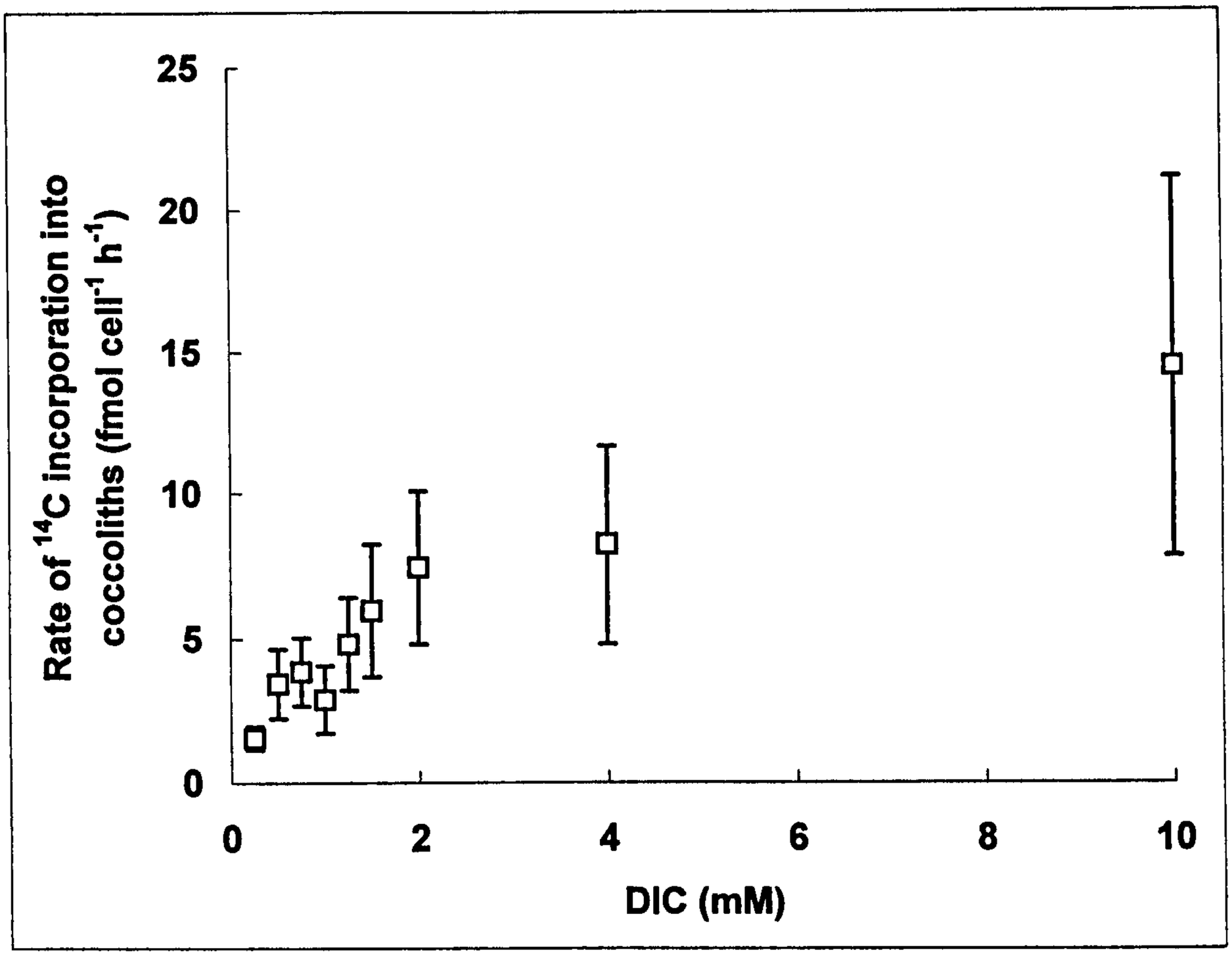



Figure 3.3 : The effect of different DIC concentrations on the rate of $^{14}C$ incorporation into coccoliths in *E. huxleyi*.

Data represent means ± SE, n=4.

*Time course of the rate of $^{14}C$ incorporation during photosynthesis and calcification :*

Measurements of $^{14}C$ incorporation by photosynthesis and calcification were performed after incubation for different lengths of time in an $O_2$ electrode chamber. Figure 3.4 shows that the rates of photosynthesis and calcification decrease rapidly with time. This is probably due to the heavy stirring; hence subsequent experiments were always carried out under gentle stirring.

*Rate of $O_2$ production with different DIC concentrations in non-calcifying cells of E. huxleyi :*

Non-calcifying cell were obtained by growing the calcifying strain of *E. huxleyi* (PCC.B11) in calcium-free medium. Plate 3.1 is a micrograph of a cell of the calcifying strain of *E. huxleyi* grown at 9.1 mM calcium and illustrates that the coccosphere has a large number of coccoliths. Plate 3.2 shows a coccolith-less cell of the calcifying strain of *E. huxleyi* grown in calcium-free medium (no coccoliths).

The rate of $O_2$ production with different DIC concentrations was determined in coccolith-less *E. huxleyi* to test if calcification affects the biphasic kinetics of photosynthesis. Coccolith-less cells were incubated in the $O_2$ chamber in calcium-free medium to prevent calcification to occur. Figure 3.5 shows the rate of $O_2$ production with added DIC concentrations in non-calcifying cells of *E. huxleyi*. This graph again shows the characteristic biphasic pattern

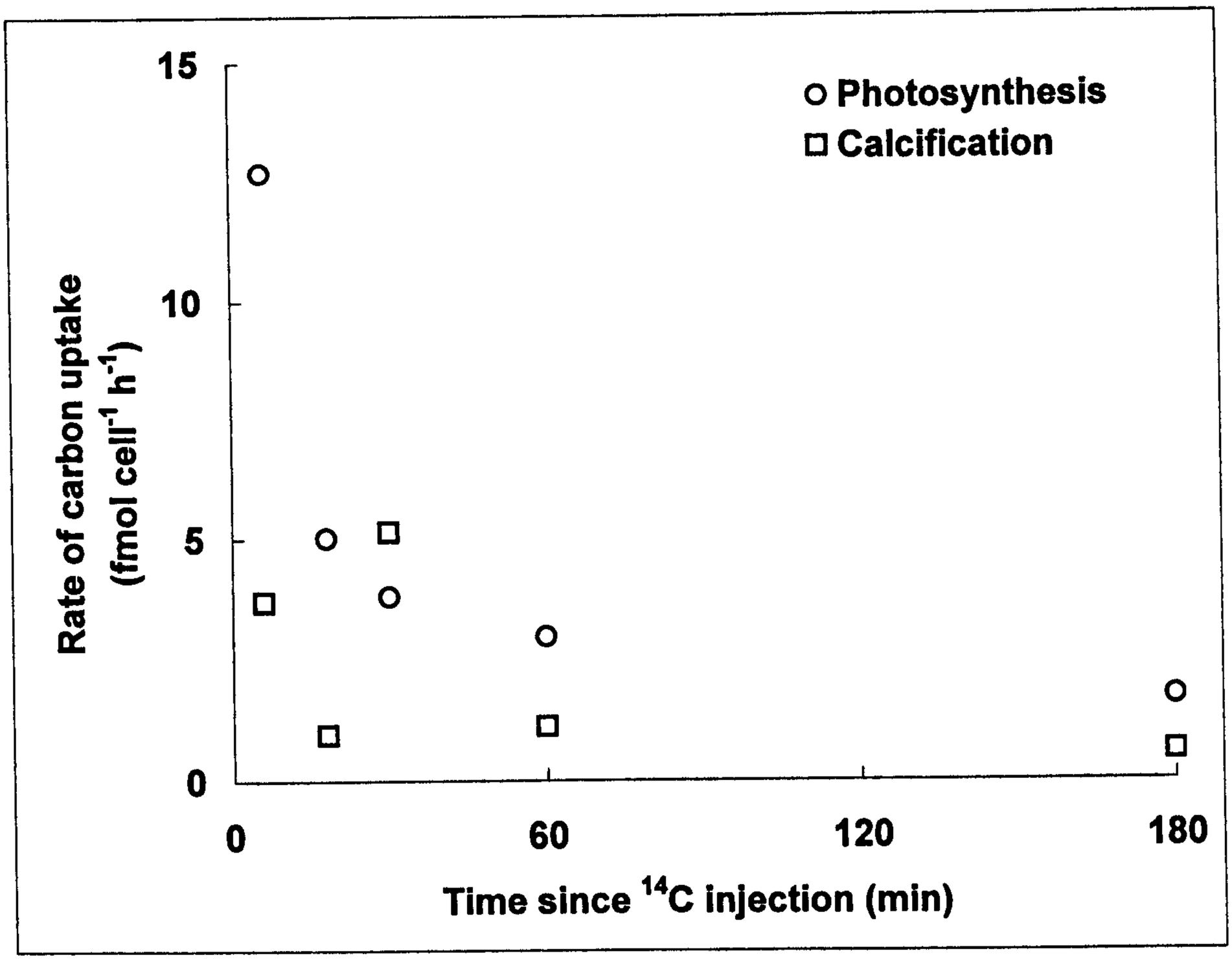



Figure 3.4 : Time course of the rate of $^{14}C$ incorporation during photosynthesis and calcification. Cells were incubated in the chamber of a Clark-type $O_2$ electrode.

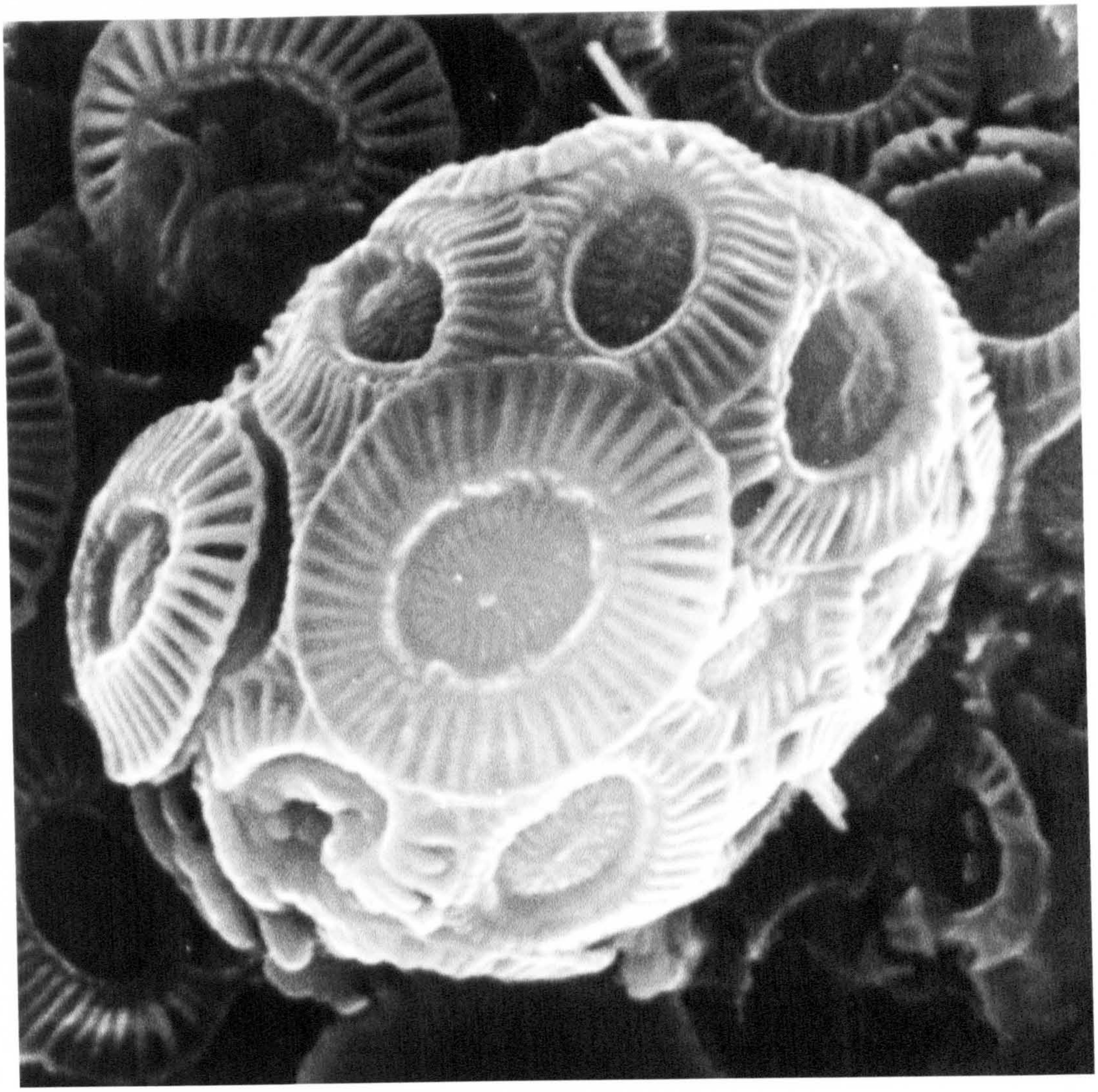


Plate 3.1 : SEM photograph of a coccolith-bearing cell of *E. huxleyi* grown in a ambient seawater. Bars = 5 μM.

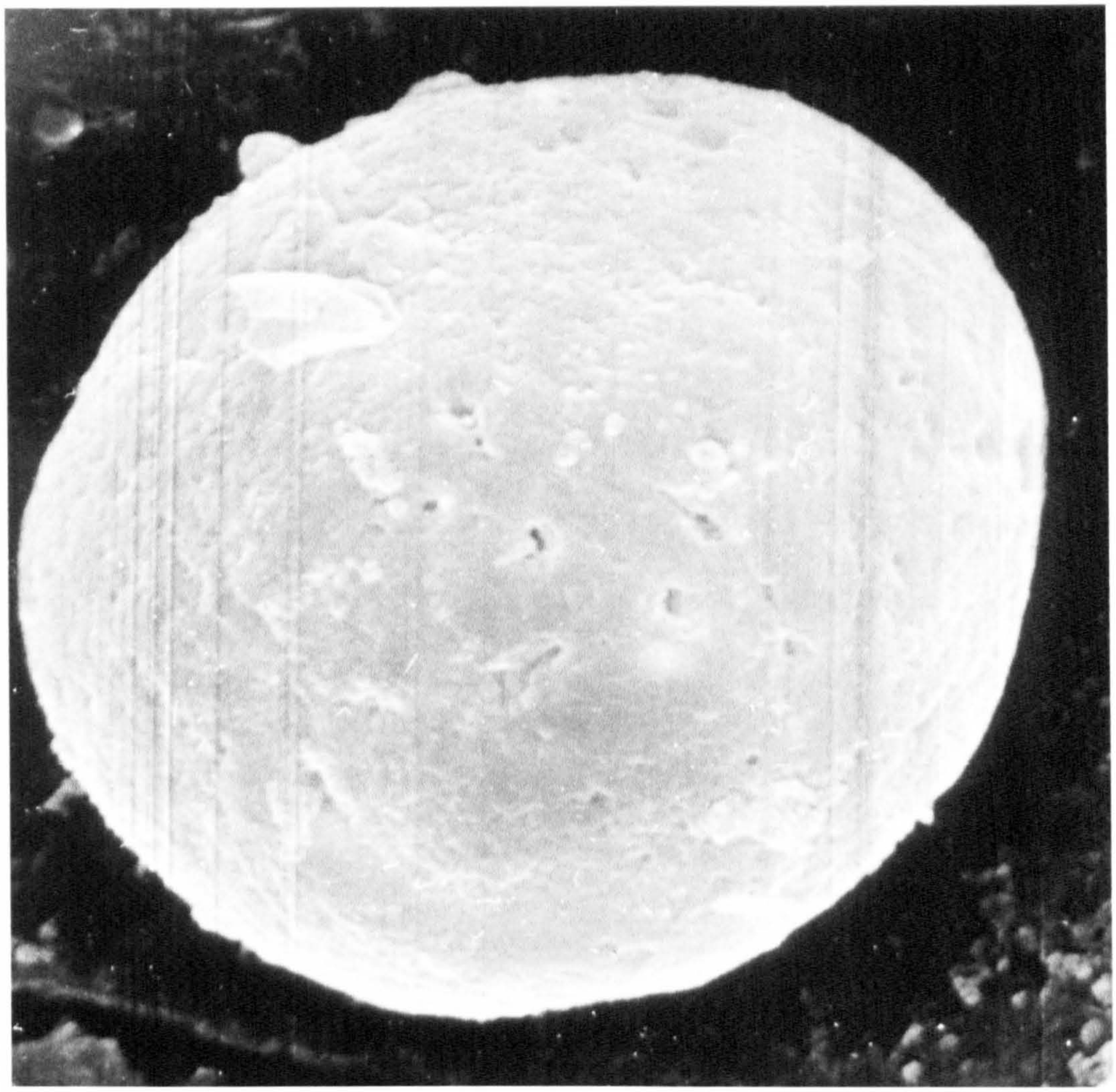


Plate 3.2 : SEM photograph of a coccolith-less cell of *E. huxleyi* grown in calcium-free seawater medium. Bars = 5 μM.

of photosynthesis, with a hiatus located at 1 mM DIC. Thus, even in the coccolith-less cells, where no calcification is occurring, the kinetics of photosynthesis are biphasic with respect to DIC concentration.

*Comparison of photosynthetic rate of calcifying and non-calcifying cells of E. huxleyi with complete coccosphere :*

Non-calcifying cells of *E. huxleyi* with complete coccosphere were obtained by incubating coccolith-bearing cells for 3 hours in calcium-free medium. Figure 3.6 demonstrates that photosynthetic $^{14}C$ incorporation is equal in calcifying and non-calcifying cells of a calcifying strain of *E. huxleyi*.

*Identification of an anion exchange protein at 2mM DIC :*

Figure 3.7 shows the effect of 0.75 mM SITS on the photosynthetic $^{14}C$ incorporation in calcifying cells of *E. huxleyi* grown and incubated at 2 mM DIC. SITS inhibits the photosynthetic rate by 63 %.

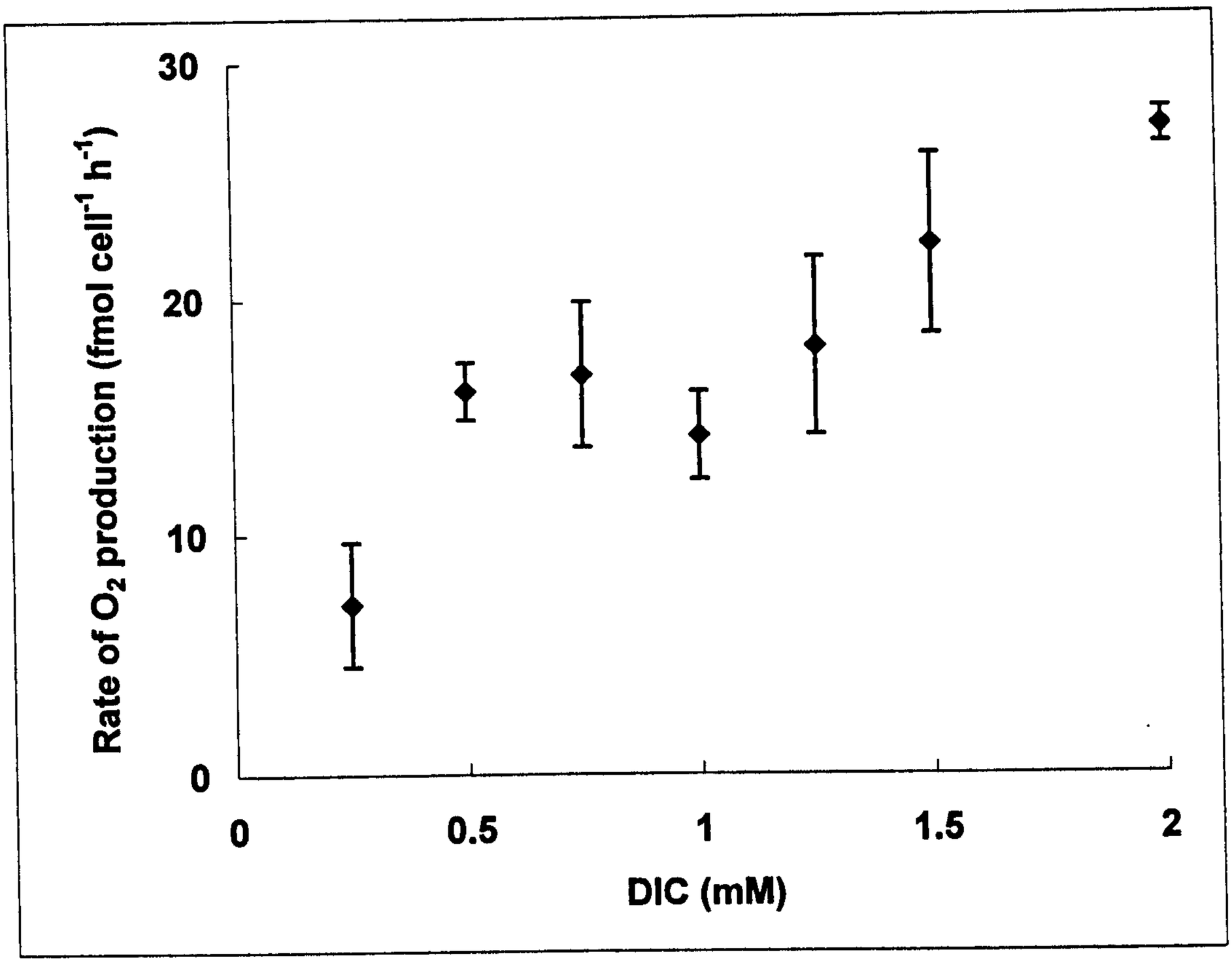



Figure 3.5 : The effect of different concentrations of DIC on the rate of $O_2$ production in coccolith-less cells of a calcifying strain of *E. huxleyi*. Cells were grown and incubated in calcium-free seawater.

Data represent means ± SE, n = 3-5.

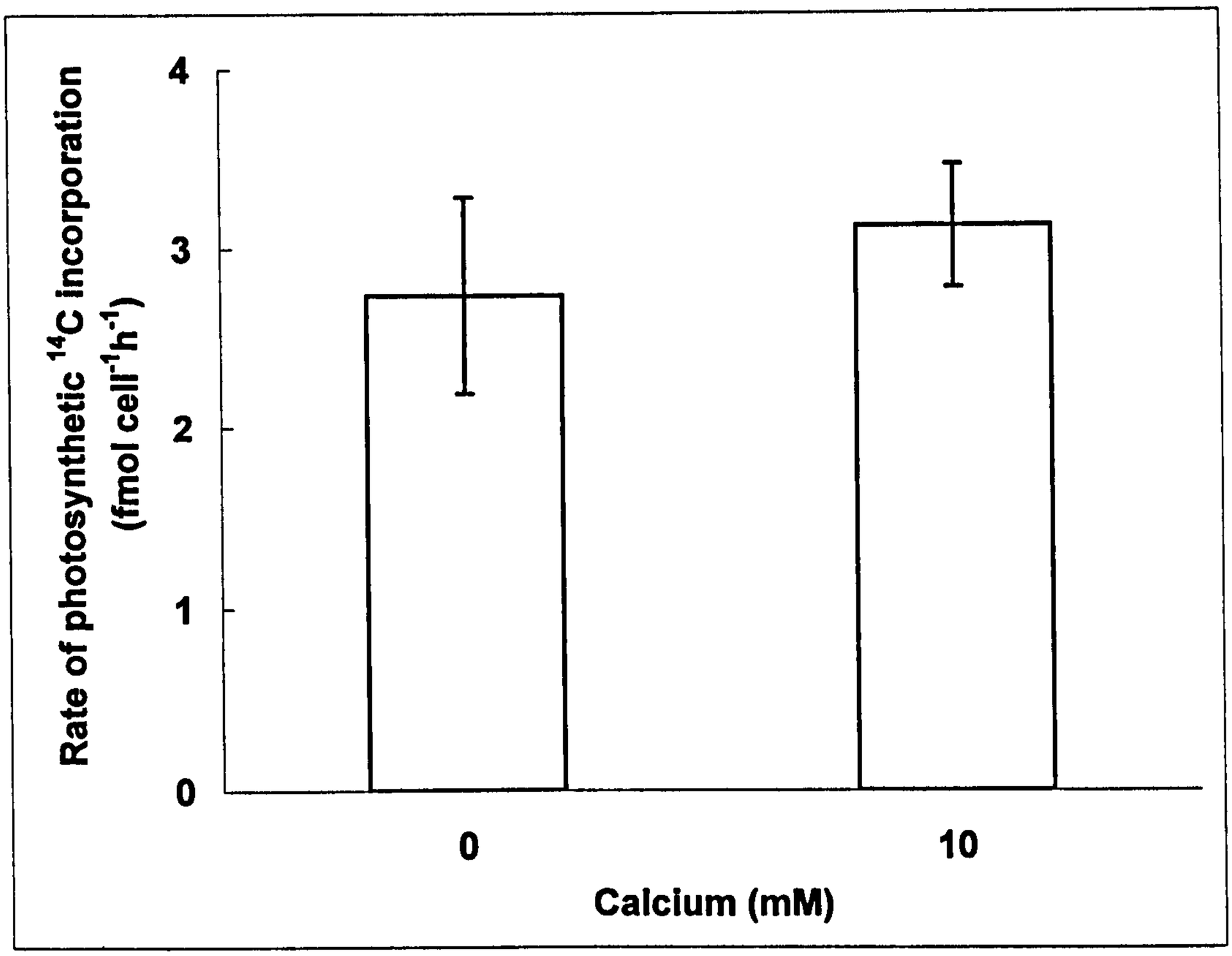



Figure 3.6 : The effect of calcium on photosynthetic $^{14}C$ incorporation of calcifying and non-calcifying cells of a calcifying strain of *E. huxleyi*. Cells were grown in normal seawater and incubated in either normal or calcium-free medium. Data represent means ± SE, n = 4.

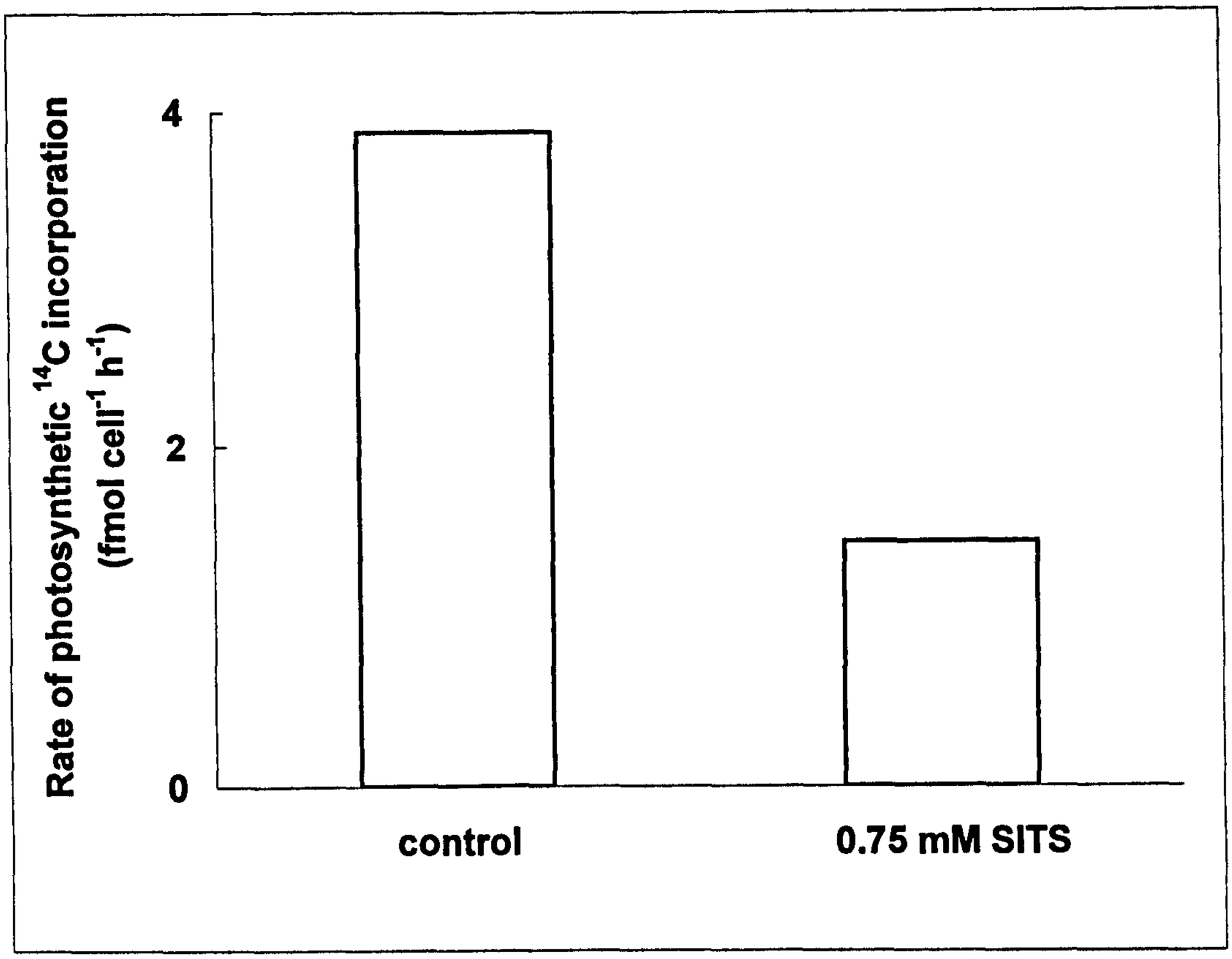



Figure 3.7 : The effect of SITS on photosynthetic $^{14}C$ incorporation in calcifying cells of *E. huxleyi*, grown and incubated at 2 mM DIC.

*Identification of an anion exchange protein in coccolith-less cells :*

Figure 3.8 shows that 0.75 mM SITS also inhibits the $O_2$ production rates of coccolith-less cells of *E.huxleyi*. So, these cells have not lost their bicarbonate transporting ability.

Cells were grown and incubated at 2 mM DIC.

*Identification of an anion exchange protein in coccolith-bearing cells grown and incubated at 0.5 mM DIC :*

Figure 3.9 shows that, at 0.5 mM DIC, net $O_2$ production is reduced to zero with the addition of 0.75 mM of SITS.

Figure 3.10 presents the percentage inhibition of photosynthesis at 0.5 mM with low SITS concentrations (from 0.05 to 0.75 mM). The graph is a dose response curve, with 3, 16 and 74 % inhibition of photosynthesis by 0.05, 0.1 and 0.25 mM SITS respectively. This result shows that in *E. huxleyi*, at 0.5 mM DIC, inhibition of photosynthesis by SITS is not the consequence of some chemical alteration of the anion exchanger, but of specific affinity binding of the inhibitor to the active site of the plasmalemma-bound protein. Although double reciprocal plots of DIC dose responses with or without inhibitor addition is the practice commonly used to detect competitive inhibition, it was not necessary to do so at low DIC concentrations because SITS is a well-known competitive inhibitor. In addition, SITS competitive inhibition was checked by Weinberg-Flax (2000) using the same strain of *E. huxleyi* at and above 2 mM DIC.

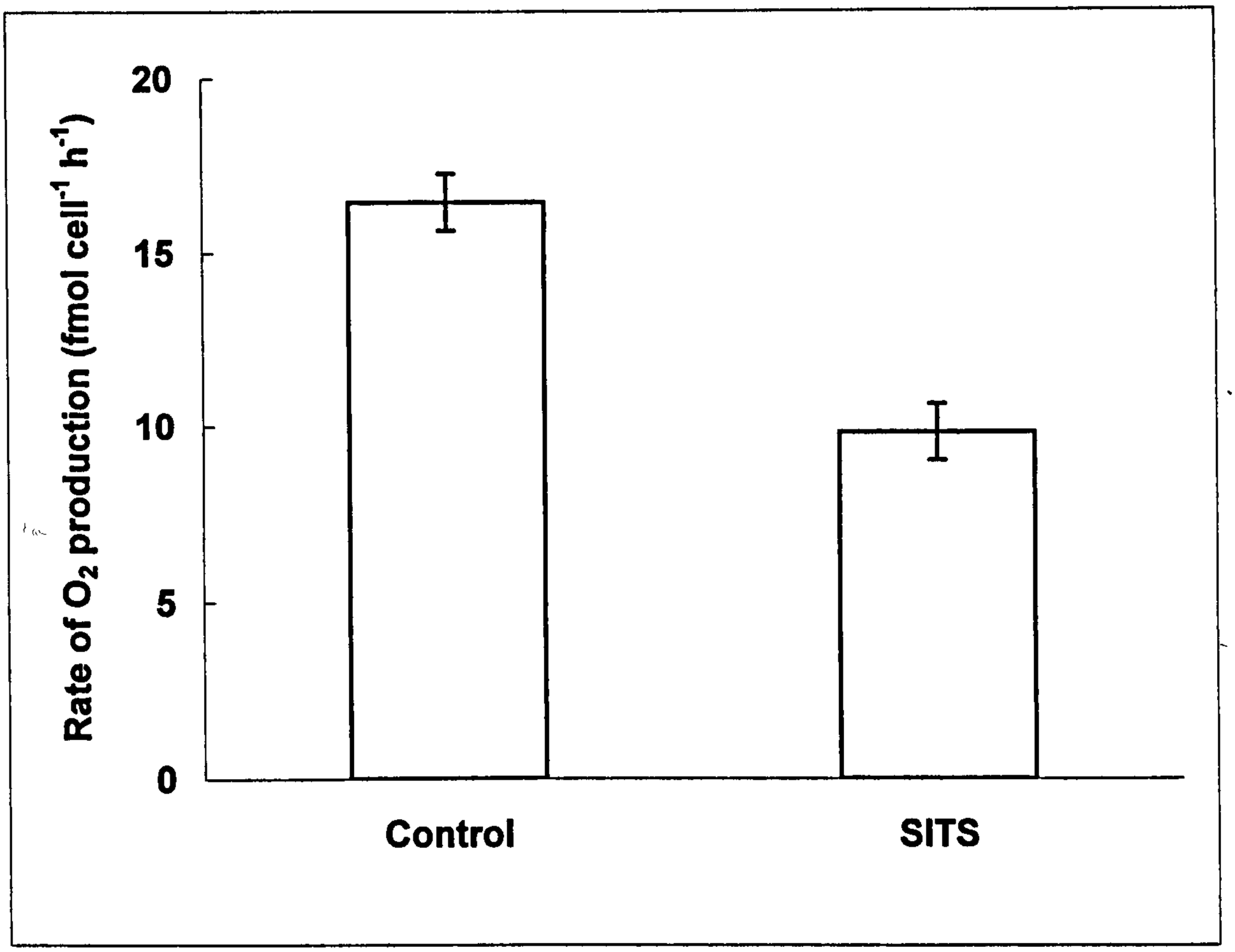



Figure 3.8 : The effect of 0.75 mM SITS on the $O_2$ production rate of coccolith-less cells of *E.huxleyi*. Data represent means ± SE, n = 7

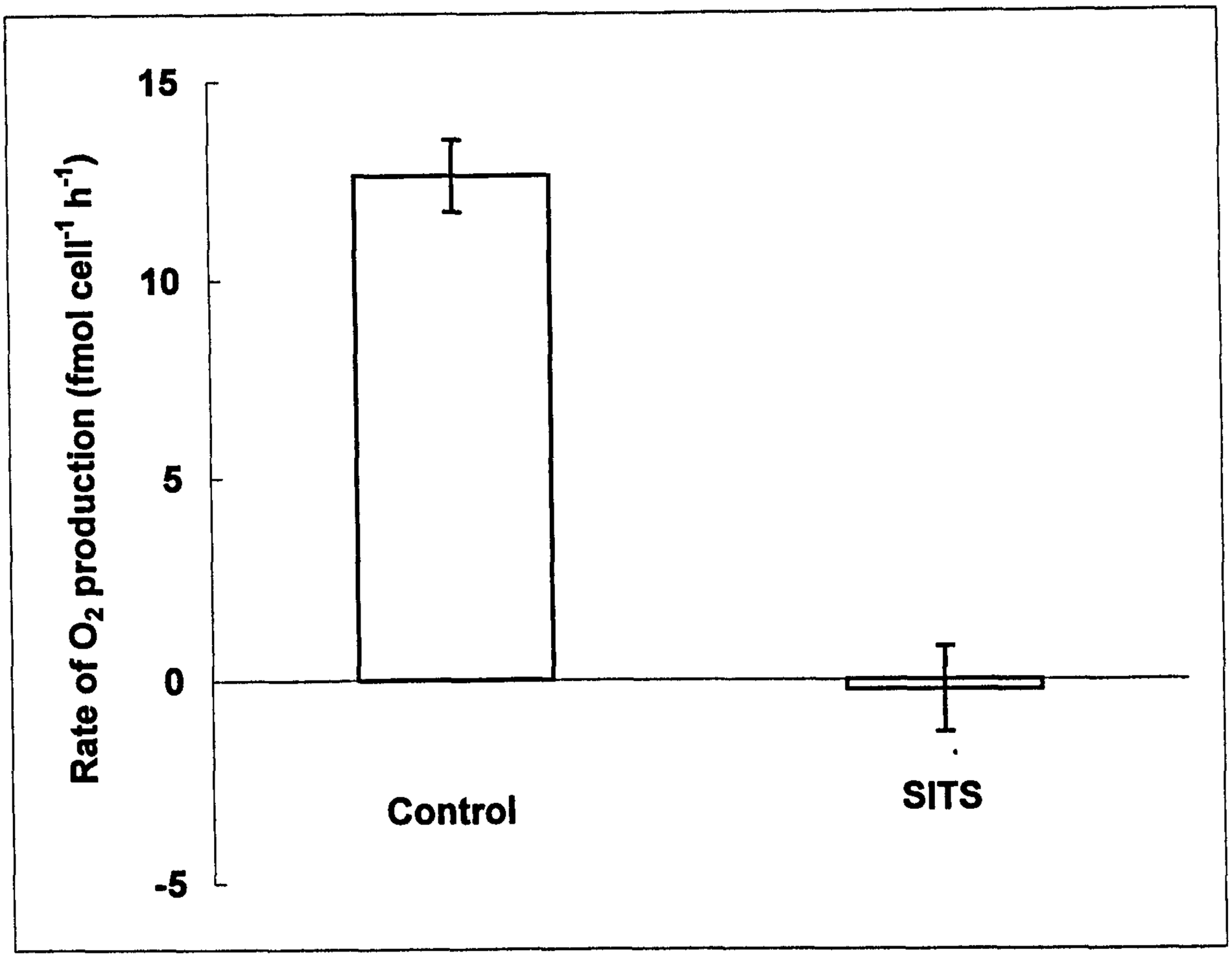



Figure 3.9 : The effect of 0.75 mM SITS on the $O_2$ production rate of a calcifying strain of *E. huxleyi* grown and incubated at 0.5 mM DIC.

Data represent means ± SE, n = 11.

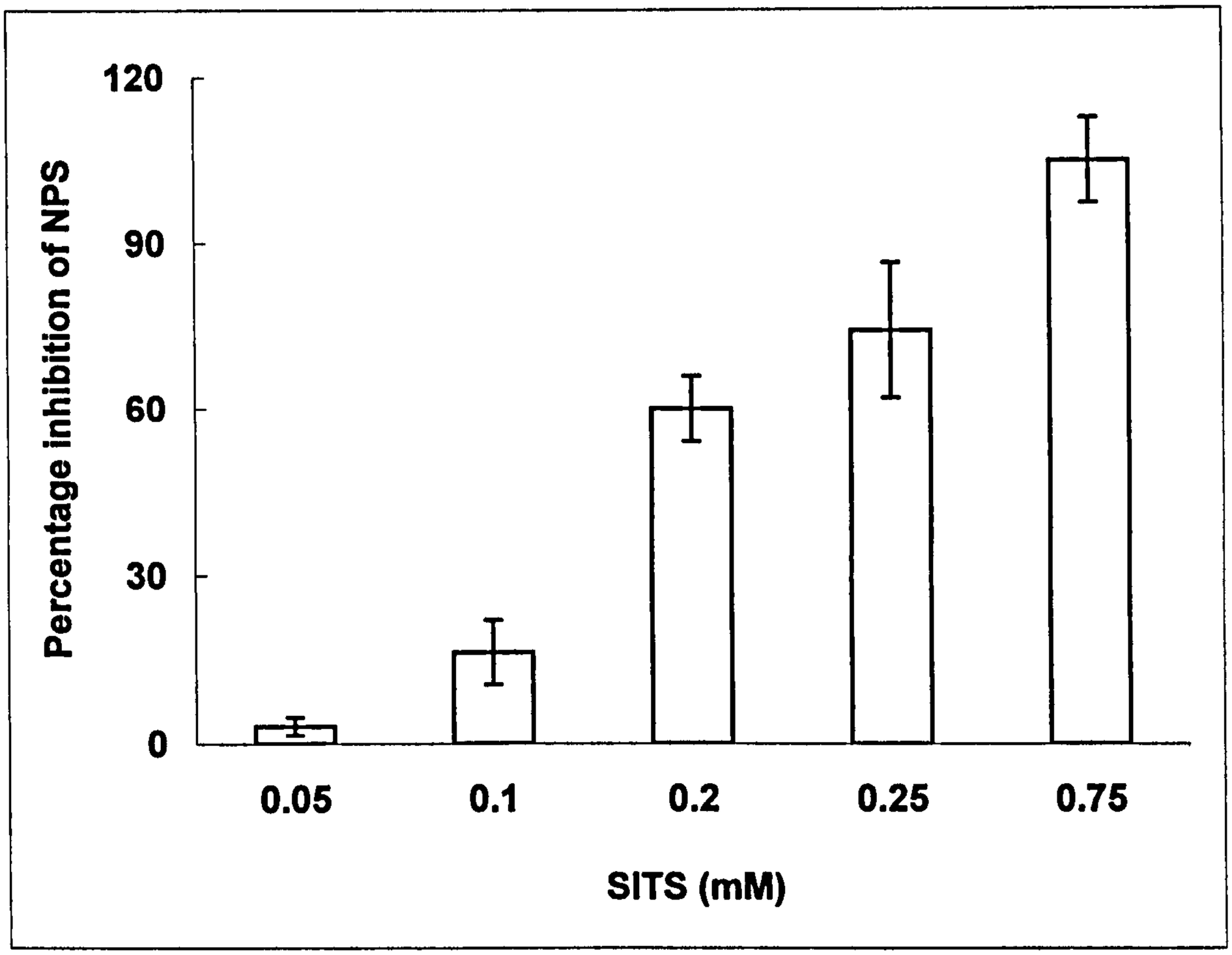



Figure 3.10 : Dose response of $O_2$ production rate and SITS in calcifying cells of *E. huxleyi* grown and incubated at 0.5 mM DIC.

Data represent means ± SE, n = 9-11.

*Identification of an external carbonic anhydrase :*

External CA activity was assayed in a batch culture of *E. huxleyi* in mid-exponential growth phase at a low DIC concentration. The cells were grown and resuspended at 0.5 mM DIC. AZ, a membrane impermeable specific inhibitor of external CA, was used. AZ does not dissolve in distilled water and was therefore prepared in three different solvents : ethanol, DMSO and distilled water. In the last of these, the pH was raised to 11 for dissolution and then lowered back to 8.2 (Beer and Rehnberg 1997).

Figure 3.11 shows the effect of 100 μM AZ dissolved in ethanol on photosynthesis at 0.5 and 2 mM DIC. Photosynthesis is inhibited by 36.8 % in the AZ treatment, whereas it is only reduced by 6.9 % with the addition of 50 μL of ethanol. At 2 mM DIC, the percentage of inhibition of NPS is very low, with 15.3 and 13.4 % for AZ and ethanol treatments respectively. T-tests between AZ and ethanol treatments give p values of 0.02 and 0.59 at 0.5 and 2 mM DIC respectively.

The effect of 100 μM of AZ dissolved in DMSO on NPS is presented in figure 3.12. Again, at 0.5 mM DIC, there is a stronger inhibition of NPS in the AZ than ethanol treatment, with values of 56.6 and 28.3 % respectively. T-tests indicate that those percentages are significantly different, with a p value of 0.004. In contrast, at 2 mM DIC, the percentage inhibition of NPS by AZ and DMSO are not statistically different, with a p value of 0.71.

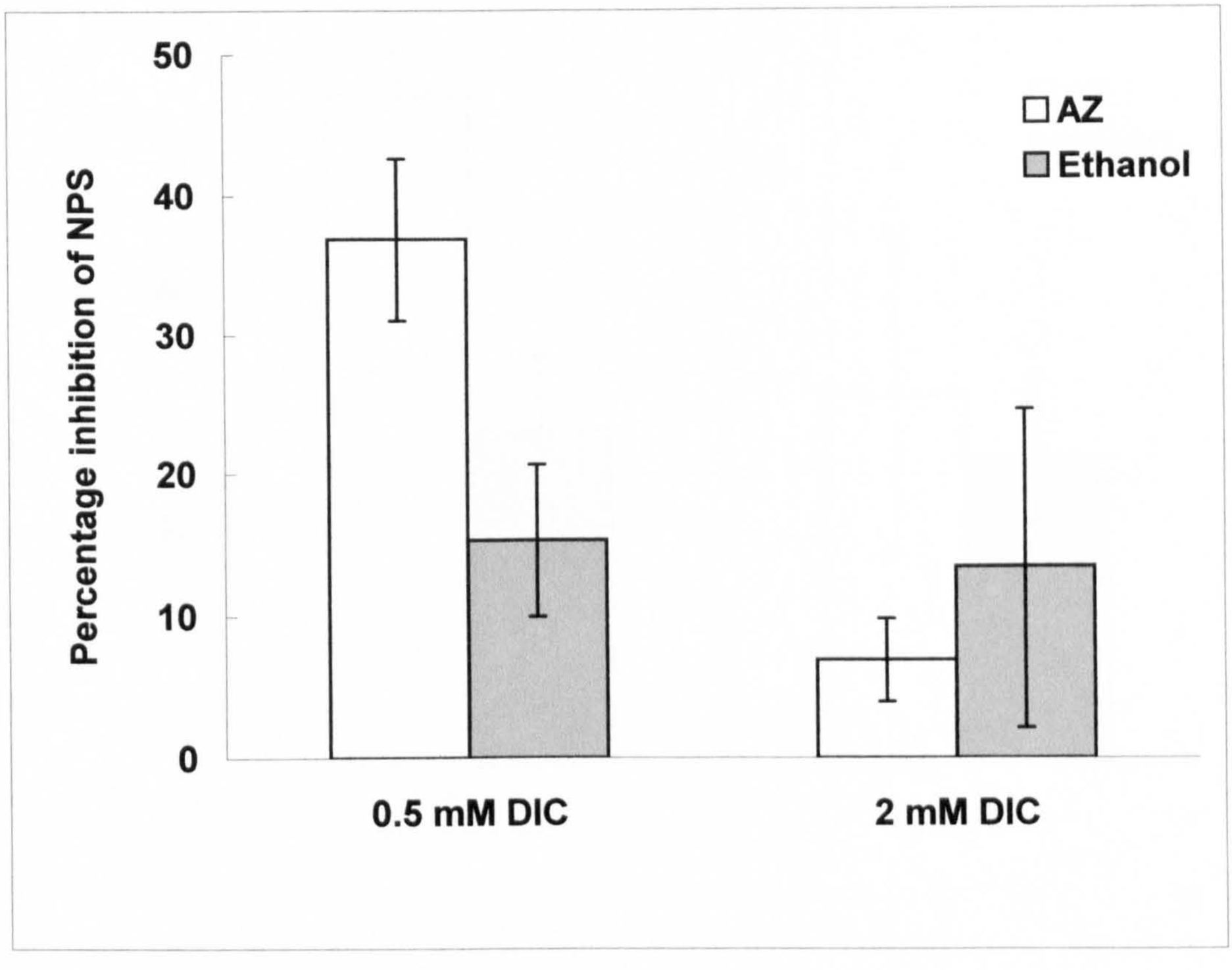



Figure 3.11 : Percentage inhibition of net photosynthesis by 100 μM AZ dissolved in ethanol in calcifying cells of *E. huxleyi* grown and incubated at 0.5 and 2 mM DIC. Data represent means ± SE, n = 6 and 5 at 0.5 and 2 mM DIC respectively.

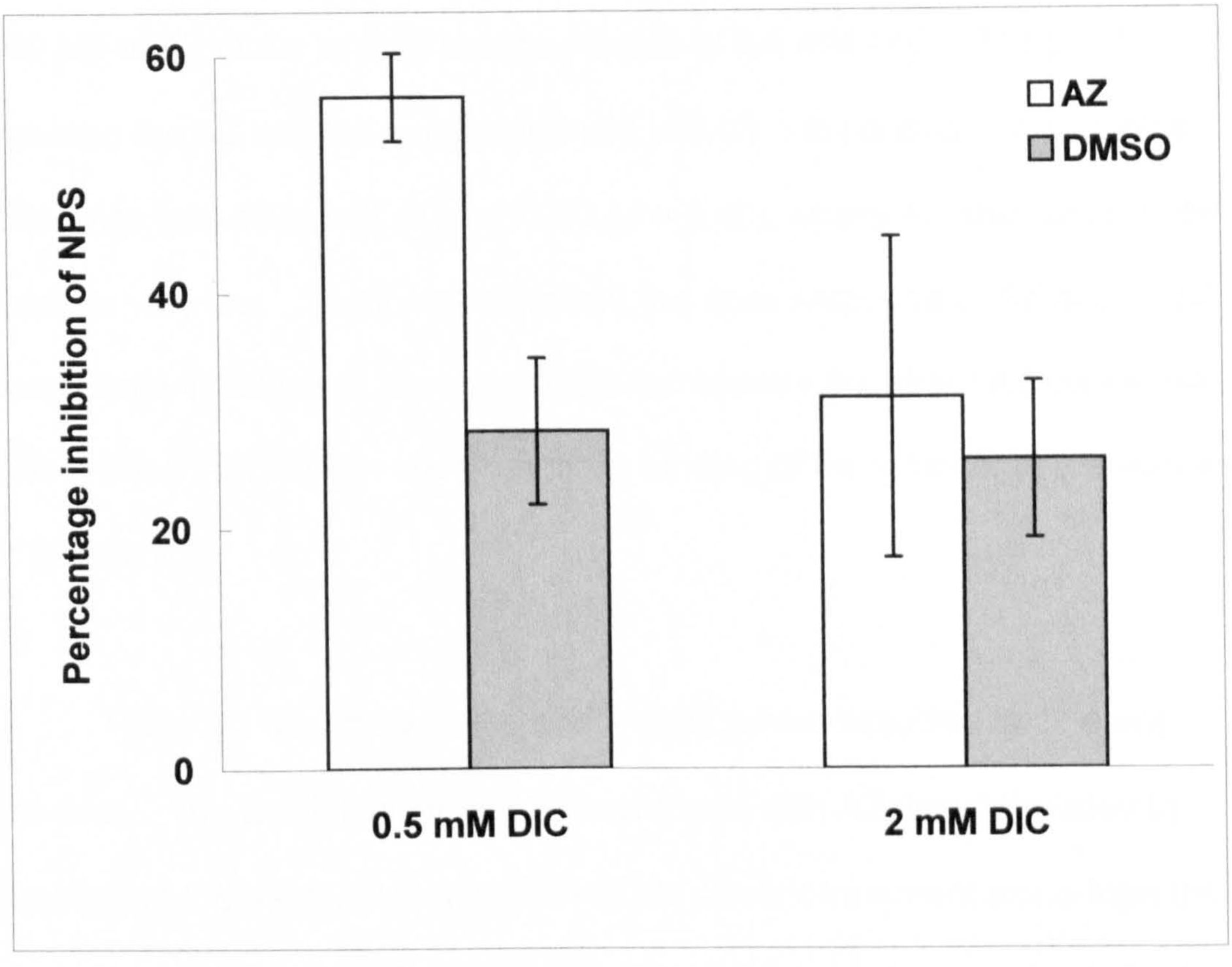



Figure 3.12 : Percentage inhibition of net photosynthesis by 100 μM AZ dissolved in DMSO in calcifying cells of *E. huxleyi* grown and incubated at 0.5 and 2 mM DIC. Data represent means ± SE, n = 4-5.

Finally, AZ was prepared in distilled water, whose pH was raised to 11 to allow dissolution and then lowered back to 8.2. Figure 3.13 shows again that 100 μM of AZ alone inhibits photosynthesis at 0.5 mM DIC. The p value between the AZ and the water treatment is 0.03. In contrast, no statistical difference was observed at 2 mM DIC (p = 0.98), where AZ and water inhibition remains very low. The insert represent the dose response of AZ at 0.5 mM. The percentage inhibition of photosynthesis increases with added AZ concentrations. This implies that there is specific affinity binding of the inhibitor to the active site of the enzyme.

Table 3.1 summarises the effect of AZ when dissolved in different solvents. The real inhibition of photosynthesis with AZ was calculated by subtracting the percentage inhibition of the solvent treatment alone from the AZ plus solvent treatment. Real percentage inhibition of photosynthesis by AZ was similar for all three experiments.

Figure 3.14 shows the percentage inhibition of NPS with 50 μM AZ and EZ at 0.5 mM DIC. The membrane permeable inhibitor of CA, EZ, has a much bigger effect than AZ, the membrane impermeable inhibitor. Only 11 % of NPS is inhibited by 50 μM AZ, while the same concentration of EZ reduces photosynthesis by 53 %. EZ is inhibiting both external and internal CA. The large difference in the percentage of inhibition with the two inhibitors confirms that AZ has not traversed the membrane and is only inhibiting external CA.

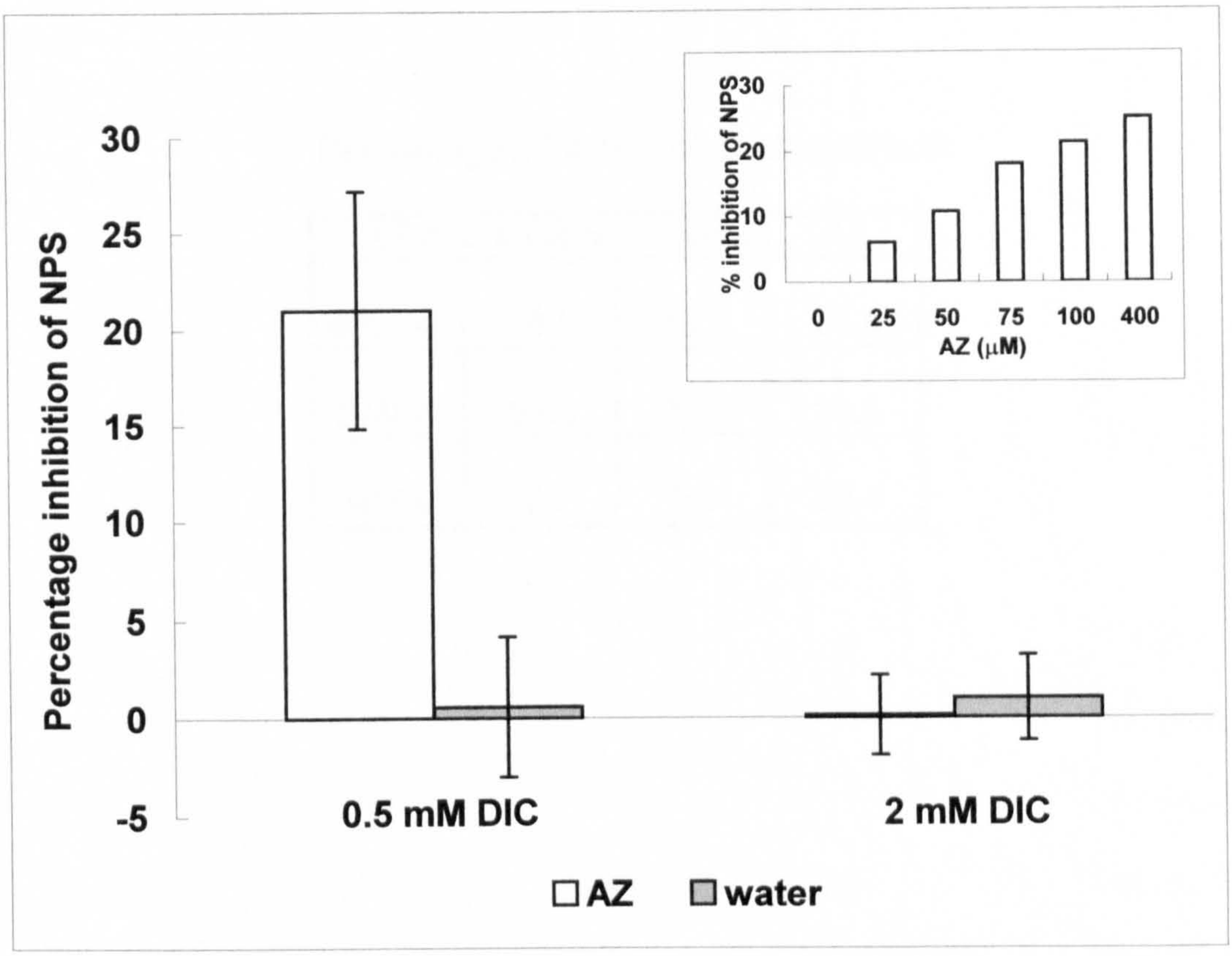



Figure 3.13 : Percentage inhibition of net photosynthesis by 100 μM AZ dissolved in distilled water in calcifying cells of *E. huxleyi* grown and incubated at 0.5 and 2 mM DIC. AZ was dissolved in distilled water by increasing the pH to 11 and then lowered back to 8.2 for experiment. Data represent means ± SE, n = 8-11. Insert : AZ dose response at 0.5 mM DIC.

Percentage inhibition of photosynthesis

| AZ and solvent | | solvent | **AZ** |
|---|---|---|---|
| ethanol | 36.8 | 15.2 | **21.6** |
| DMSO | 56.6 | 28.3 | **28.3** |
| water | 21 | 0.6 | **20.4** |

Table 3.1 : Table summarising the percentage inhibition of net photosynthesis by 100 μM AZ dissolved in the different solvents (ethanol, DMSO and water of pH 11) in *E. huxleyi*.

Cells were grown and incubated at 0.5 mM DIC.

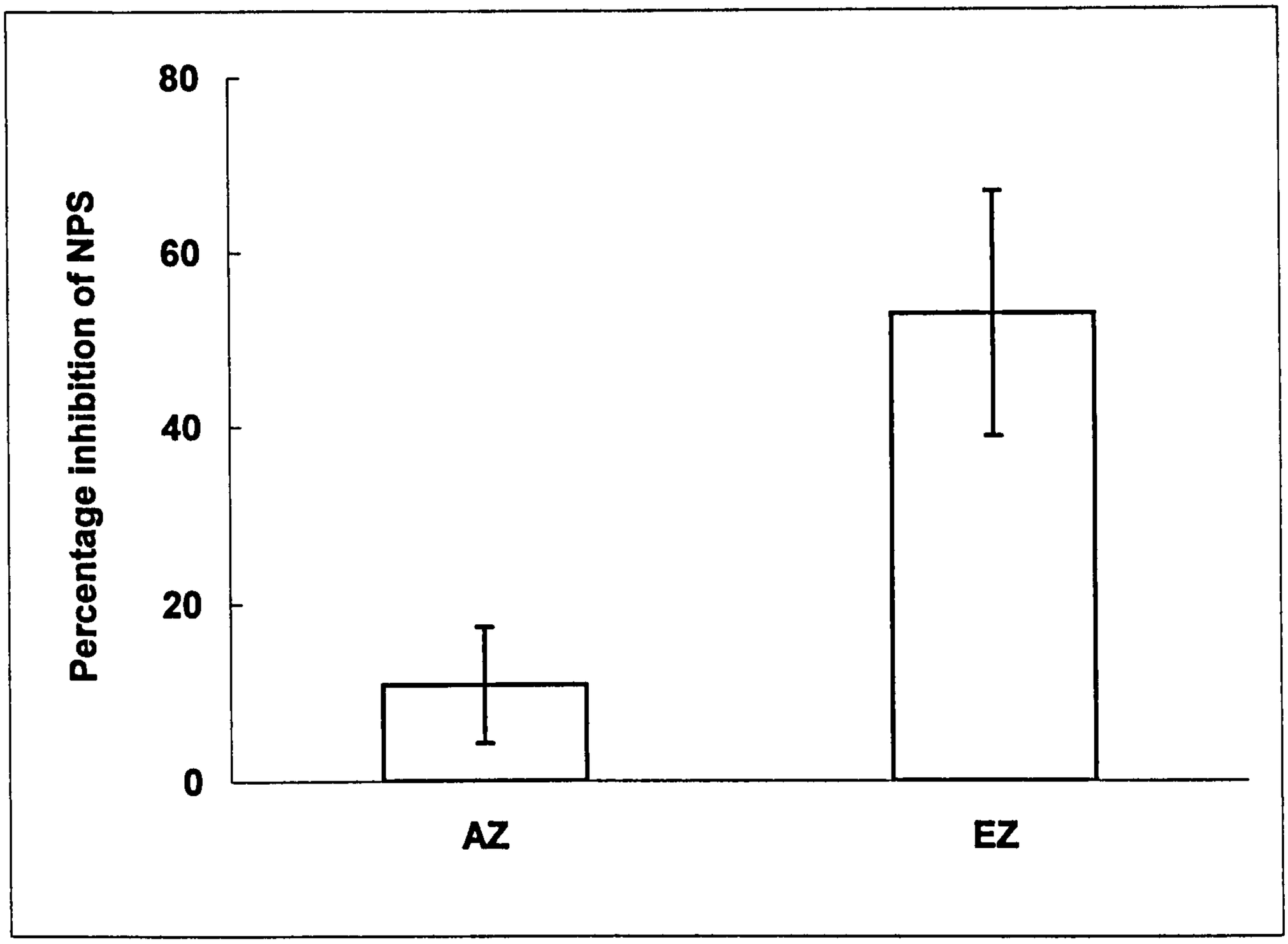



Figure 3.14 : Percentage inhibition of net photosynthesis by 50 μM AZ and EZ in calcifying cells of *E. huxleyi* grown and incubated at 0.5 mM DIC. AZ was dissolved in distilled water by increasing the pH to 11 and then lowering it back to 8.2 for experiment. Data represent means ± SE, n = 3 and 11 for EZ and AZ treatment respectively.

*Effect of different inhibitors on photosystem II activity :*

In addition to $^{14}C$ incorporation and $O_2$ evolution, measurements of PSII activity made with a Fluorescence Monitoring System were also employed in this study (see Chapter 2 for more details). Figure 3.15 summarises the effect of different inhibitors on the quantum efficiency of PS II ($\phi$PSII) in *E. huxleyi*. 0.75 mM SITS, 100 $\mu$M AZ and 100 $\mu$M EZ were used. At 0.5 mM DIC, all three inhibitors lower $\phi$PSII. At 2 mM DIC only SITS and EZ decrease $\phi$PSII. This method confirms the data obtained with the $O_2$ electrodes : anion exchange protein and internal CA are active at all DIC concentrations, but external CA is only mediating carbon across the membrane at low DIC concentrations.

*Relationship between the chloride ion and DIC :*

Figure 3.16 presents the photosynthetic rate of *E. huxleyi* at different DIC concentrations in normal seawater and in water enriched with 25 % choline chloride (ChlCl) or sodium chloride (NaCl). At 0.5 mM DIC, photosynthesis is reduced in chloride-enhanced media. The control treatment (i.e. normal seawater) gives a rate of $O_2$ production of 17 fmol cell$^{-1}$ h$^{-1}$, whereas the addition of 25 % ChlCl or NaCl to the medium causes a diminution of this rate to 12 fmol cell$^{-1}$ h$^{-1}$. The inhibition of photosynthesis by chloride ions can be overcome by increasing the DIC concentration.

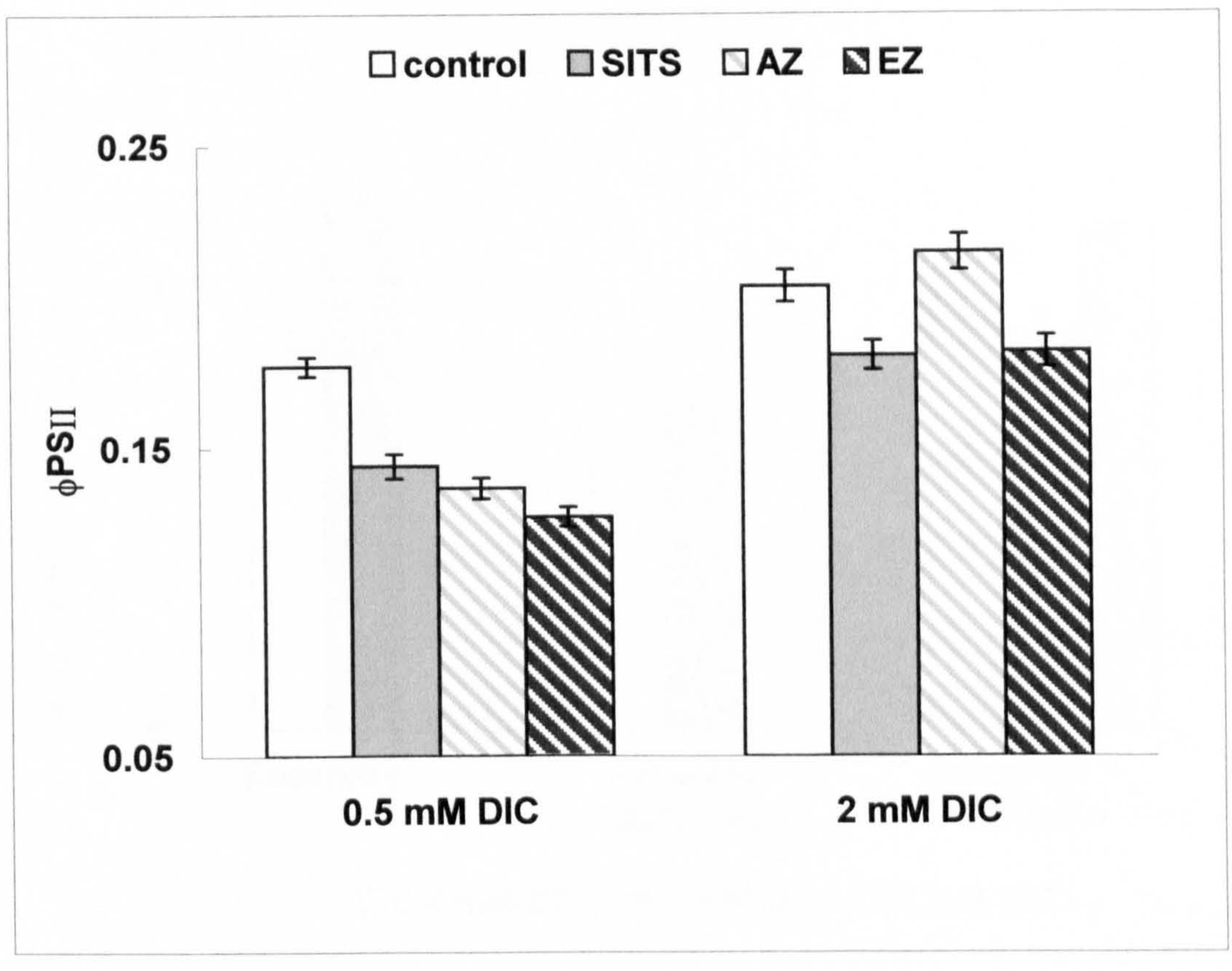



Figure 3.15 : The effect of 100 μM AZ, 100 μM EZ and 0.75 mM SITS on photosystem II activity of calcifying cells of *E. huxleyi* grown and incubated at 0.5 and 2 mM DIC. Data represent means ± SE, n = 4.

Saturating actinic light was used in this experiment.

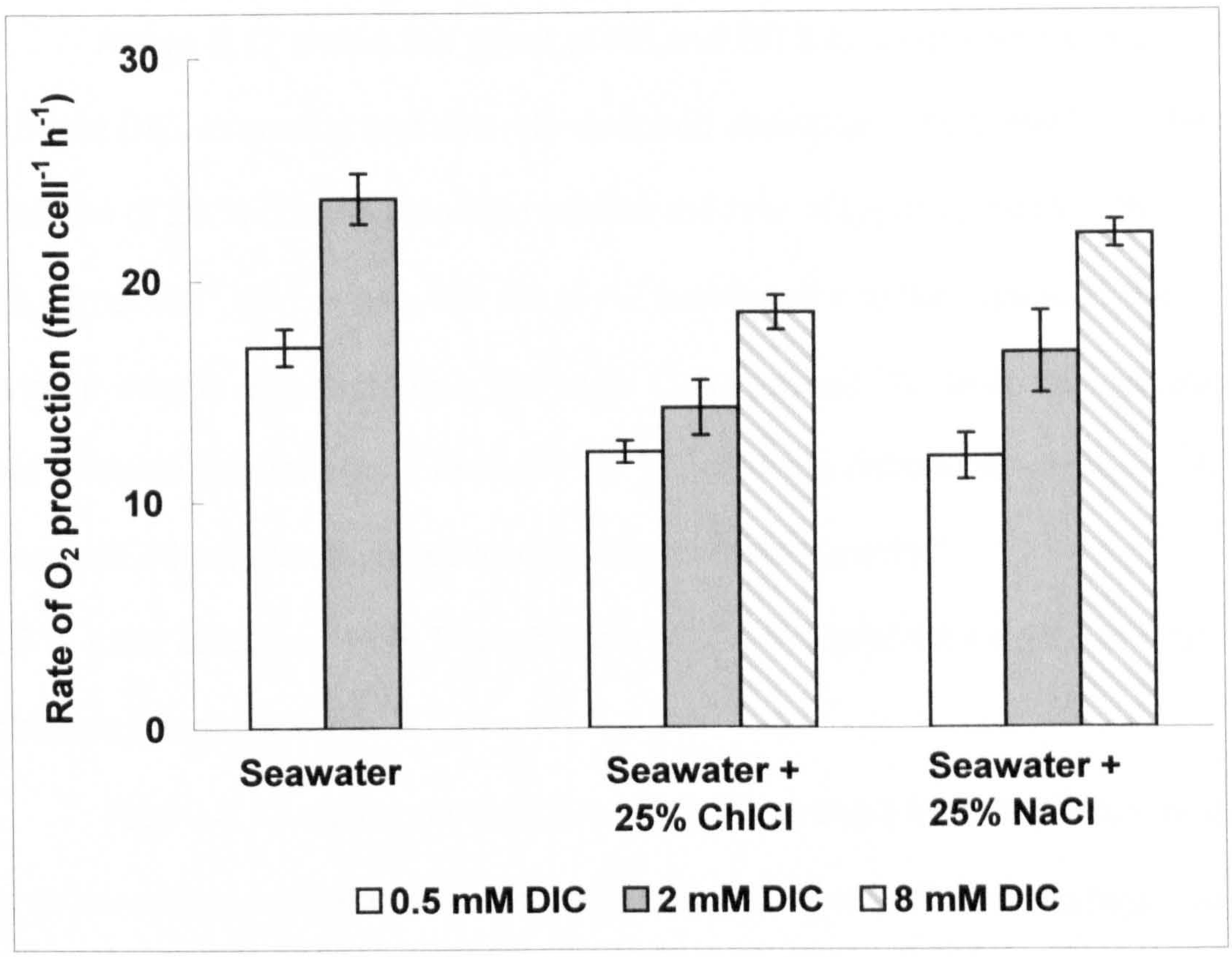



Figure 3.16 : The combined effect of chloride ion and DIC on the of $O_2$ production rate of calcifying cells of *E. huxleyi* grown at 0.5 mM DIC. Cells were incubated in normal seawater and seawater enriched with 25 % of choline chloride or sodium chloride at different DIC concentrations.

Data represent means ± SE, n = 6.

*Interaction between chloride and bicarbonate transport mechanisms :*

Figure 3.17 shows the effect of AZ and SITS on photosynthesis at 0.5 mM DIC, in normal and chloride-enriched seawater. As previously, the addition of 25 % choline chloride reduces the rate of $O_2$ production, from 17.5 to 12.6 fmol $cell^{-1}$ $h^{-1}$. When 100 μM of AZ is injected into the chamber, the photosynthetic rate decreases further to 13.6 fmol $cell^{-1}$ $h^{-1}$ in normal seawater, but remains constant (11.2 fmol $cell^{-1}$ $h^{-1}$) in chloride-enhanced medium. AZ does not inhibit photosynthesis when the medium contains 25 % extra chloride. In *E. huxleyi*, external CA is therefore inactive at high chloride concentrations.

Figure 3.17 also show that 200 μM SITS strongly inhibits photosynthesis in both normal and chloride-enriched seawater. NPS even becomes negative with the addition of 1 mM SITS. Hence, the anion exchanger is still active in the medium with high chloride concentration.

This result is confirmed in figure 3.18, which presents the percentage inhibition of NPS by SITS, at 0.5 mM DIC, in normal and 50 % ChlCl enriched seawater. The effect of 200 μM SITS on photosynthesis is similar in both media, with 59.6 and 55.1 % inhibition in normal and chloride-enhanced seawater respectively. Thus, high chloride concentrations do not affect the activity of the anion exchanger at 0.5 mM DIC in *E. huxleyi*.

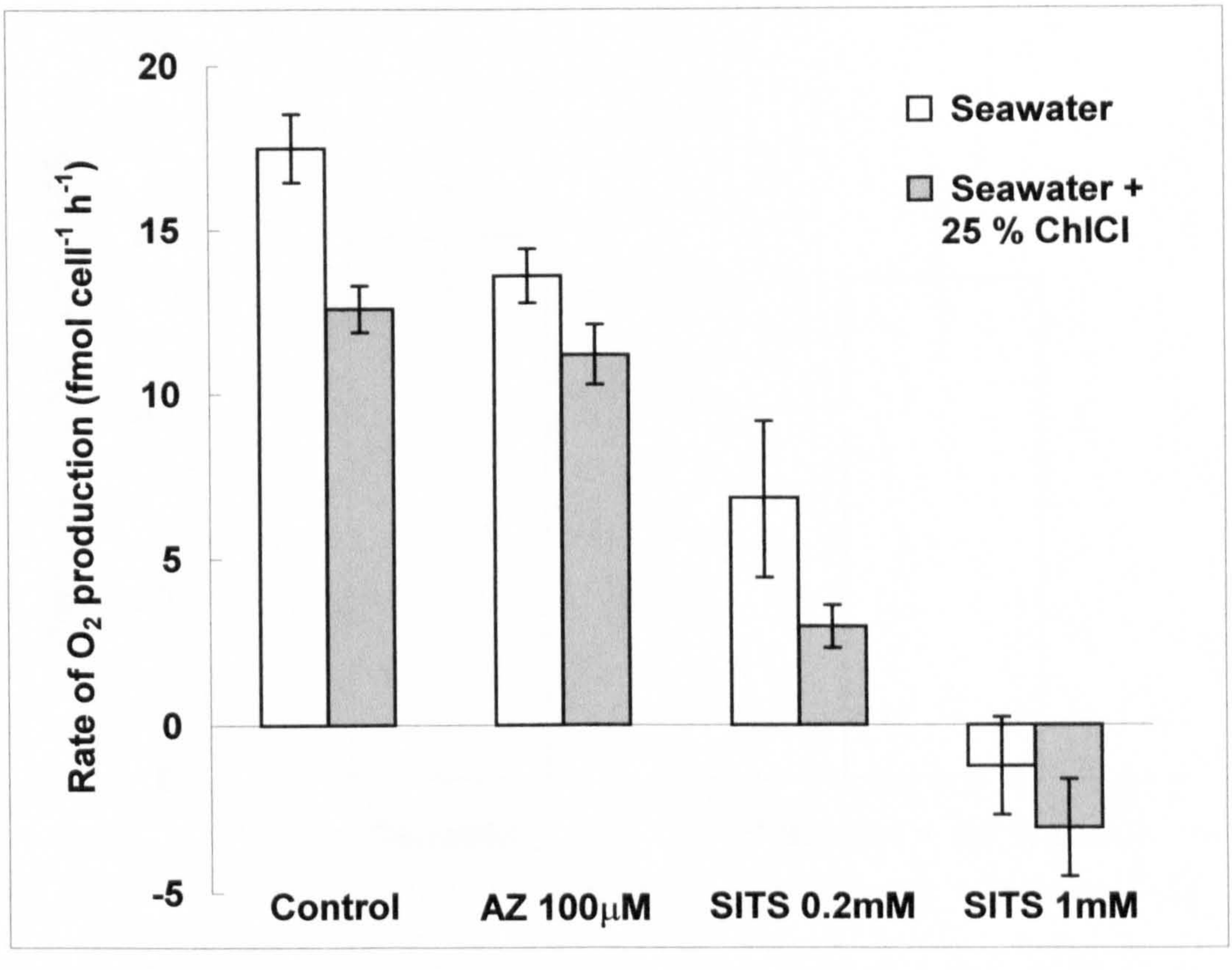



Figure 3.17 : The effect of AZ and SITS on the $O_2$ production rate of calcifying cells of *E. huxleyi* grown and incubated at 0.5 mM DIC, in normal and 25 % choline chloride enriched seawater. Data represent means ± SE, n = 5-6.

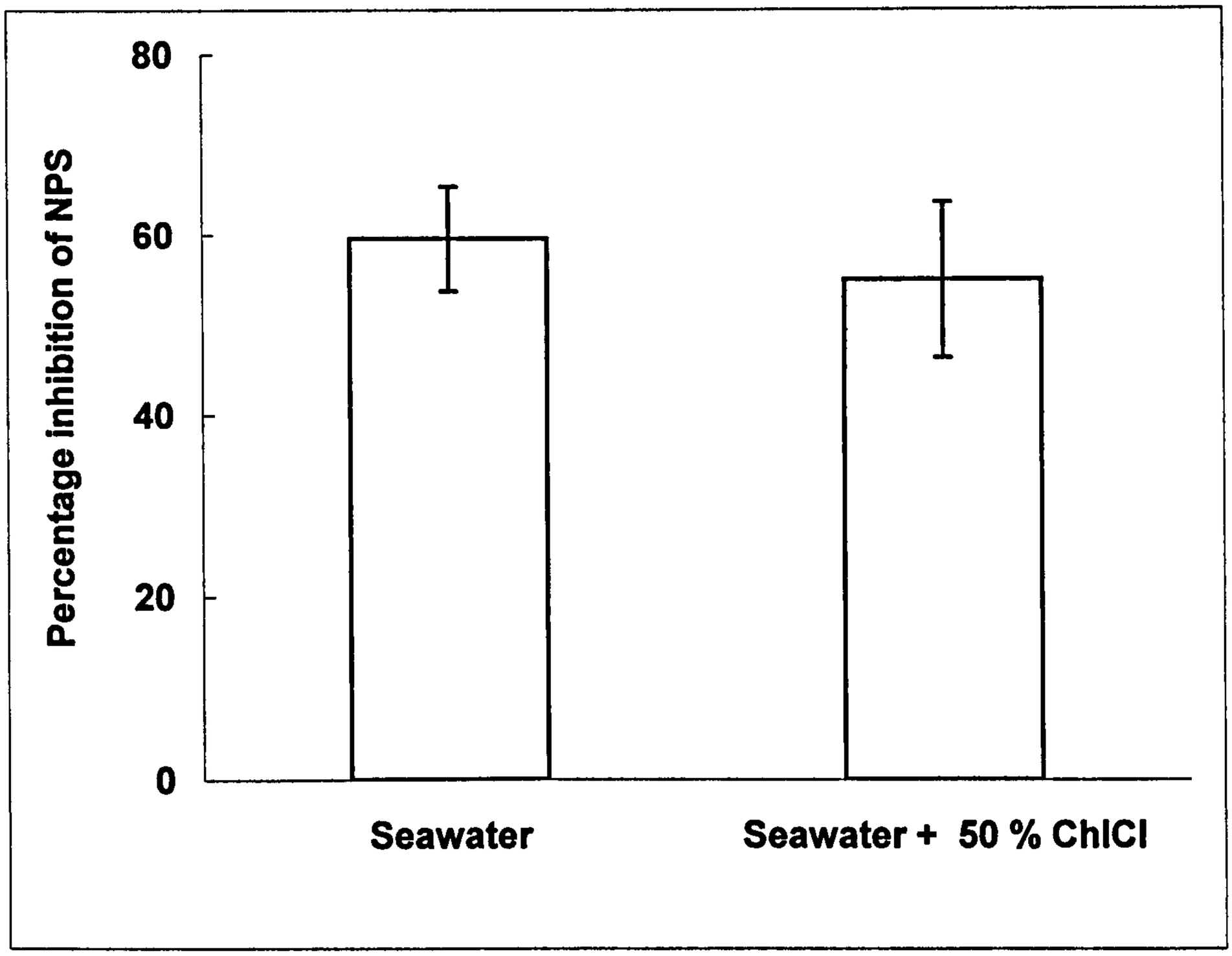



Figure 3.18 : The effect of 200 μM SITS on the $O_2$ production rate of calcifying cells of *E. huxleyi* grown and incubated at 0.5 mM DIC, in normal and 50 % choline chloride enriched seawater. Data represent means ± SE, n = 5-7.

*Comparison of the photosynthetic rate of cells grown at different DIC concentrations :*

Figure 3.19 shows that the $O_2$ production rate of *E. huxleyi* incubated at 0.5 mM DIC is higher when cells are grown at 0.5 rather than at 2 mM DIC, with values of 18.2 and 9.3 fmol $cell^{-1}$ $h^{-1}$ respectively.

*Identification of anion exchange protein in stationary growth phase cells :*

Cells of *E. huxleyi*, grown at 0.5 mM DIC, were 6 days old and therefore in the stationary growth phase when they were tested for anion exchange protein activity at 0.5 mM DIC. Figure 3.20 shows that photosynthesis is strongly inhibited by the addition of 200 μM SITS, with values of 8.5 and 3.3 fmol $cell^{-1}$ $h^{-1}$ for control and SITS treatments respectively.

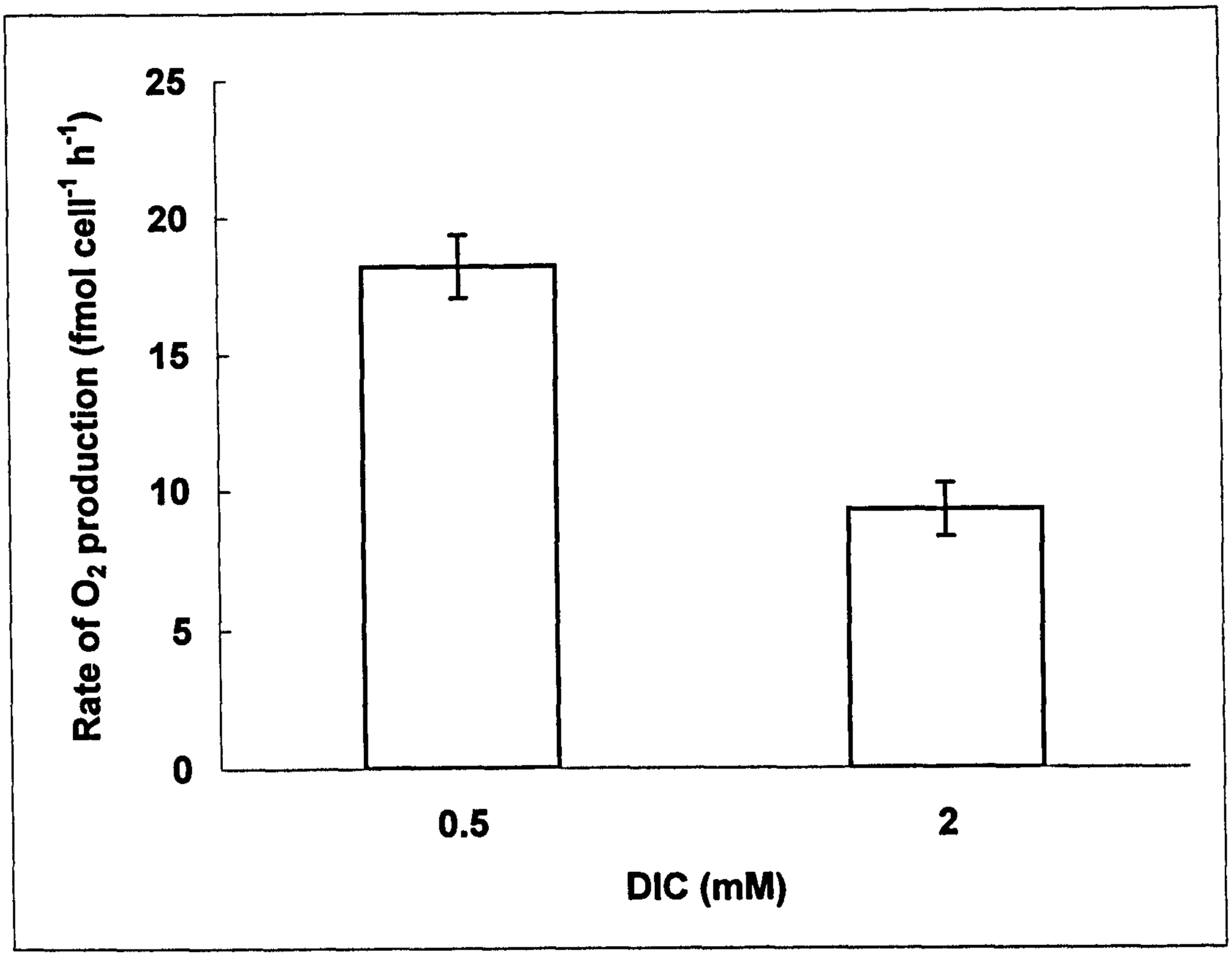



Figure 3.19 : The effect of different concentrations of DIC in the growth medium on the $O_2$ production rate of *E. huxleyi* incubated at 0.5 mM DIC. Data represent means ± SE, n= 11.

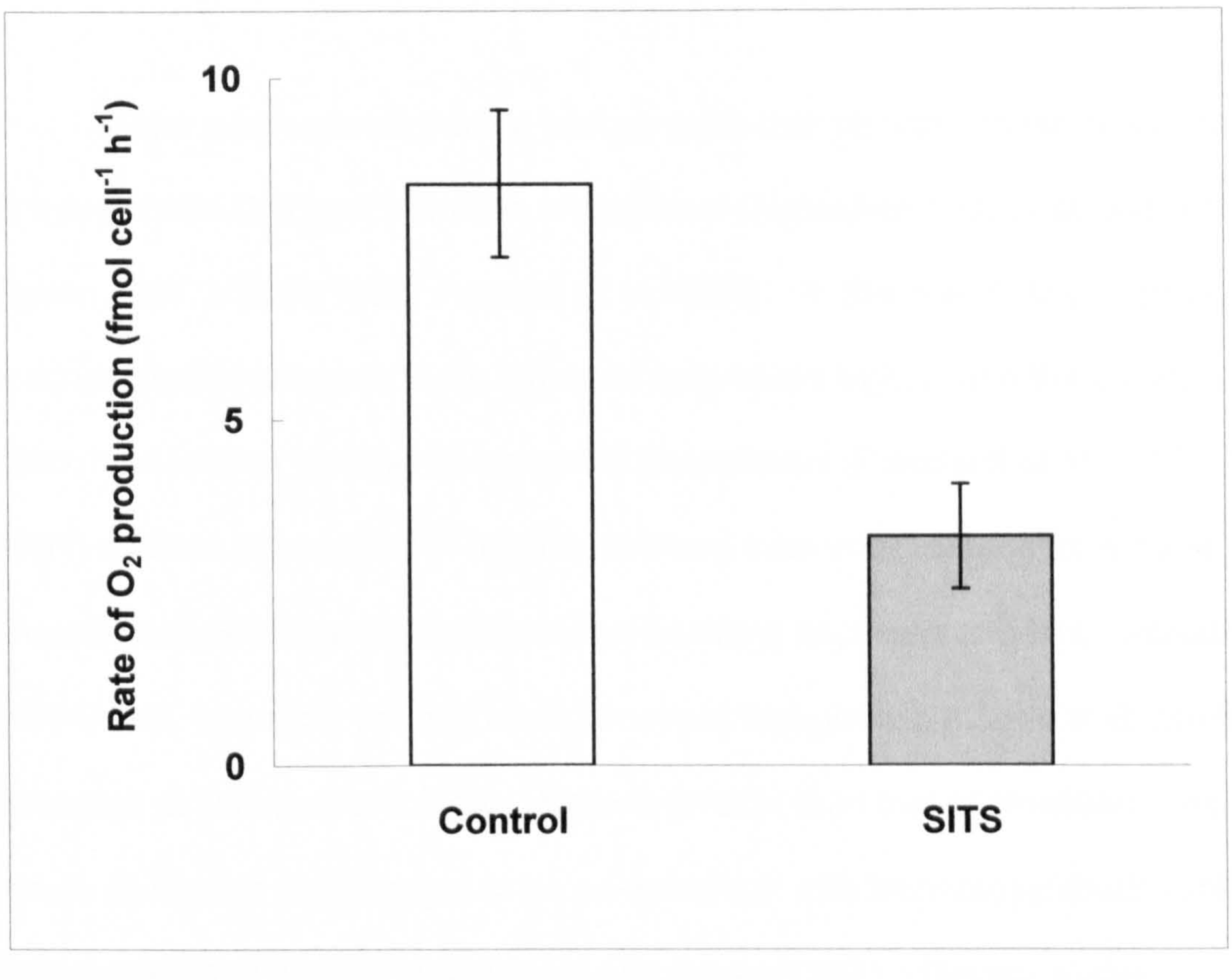



Figure 3.20 : The effect of 200 μM SITS on the rate of $O_2$ evolution of stationary growth phase cells of *E. huxleyi* grown and incubated at 0.5 mM DIC. Data represent means ± SE, n= 5.

Discussion :

Major textbooks on marine biology state that photosynthesis is saturated at the ambient DIC concentration of seawater (Nybakken 1988, Falkowski and Raven 1997, Valiela 1985, Parsons *et al.* 1984). In the ocean, DIC is present at a concentration of two to three orders of magnitude higher than the commonly cited main limiting factors, nitrogen and phosphorus (Riebesell *et al.* 1993, Raven 1993) and five to six order of magnitude more than iron. Hence, only these essential nutrients have been considered limiting to photosynthesis. Paasche (1964) was, however, the first to demonstrate that photosynthesis in *E. huxleyi* saturates at DIC concentrations 15 times greater than that of seawater. As a result, *E. huxleyi* was thought to be an exception with its photosynthetic rate unsaturated at 2 mM DIC (Falkowski and Raven 1997). Decades after Paasche's work, Holbrook *et al.* (1988) show that the photosynthetic rates of five macroalgae, collected from Andros Island (Bahamas), were not saturated at 2.5 mM DIC. Their finding was the trigger of the growing awareness of the limiting effect of the ambient DIC concentration of seawater on the photosynthetic rate of marine organisms (Mercado *et al.* 2001, Beer and Rehnberg 1997). The present study has confirmed that the photosynthetic rate of the calcifying strain of *E. huxleyi* PCC.B11 is not saturated at 2 mM DIC.

In addition to simple substrate-limitation effect on photosynthesis, DIC has also a more profound effect on the photosynthetic kinetics of *E. huxleyi*. Paasche (1964) suspected that the kinetics of photosynthesis with DIC were unorthodox in *E.huxleyi*, but regarded it as a function of his specific strain. Sekino et al. (1996) working with a South Pacific strain of *E.huxleyi* noted that in order to transform their data into a double reciprocal plot, a linear model could only be fitted if twin slopes were used and thus suggested the kinetics of photosynthesis to be biphasic. By measuring the $O_2$ production rate of *E. huxleyi* over a close range of DIC concentrations, Weinberg-Flax (2000) also suspected that the photosynthetic kinetics were biphasic and this seemed clear without transformation of the data. In the present study, measurements of the rate of $^{14}C$ incorporation into acid stable compounds confirm that the kinetics of photosynthesis are biphasic with increasing DIC concentrations. Similarly to Weinberg-Flax's data, photosynthesis shows a change in its kinetics at 1 mM DIC.

This sudden shift in the kinetics of photosynthesis in *E. huxleyi* could be caused by calcification. Indeed, the crystallisation of calcium carbonate during calcification is accompanied by the liberation of one molecule of $CO_2$ (equation 1.4). Theoretically, Rubisco could then incorporate this molecule of $CO_2$ into the Calvin cycle. If calcification did not begin until 1 mM DIC, the change in the kinetics of photosynthesis could then be explained by the sudden increase of $CO_2$ from calcification.

Unfortunately, this rather neat idea has now been shown to be wrong. Calcification is not only taking place at DIC concentrations lower than 1 mM, but its kinetics also have the same characteristic biphasic pattern with increasing DIC concentrations. It could be argued that points at 1 mM DIC simply look like outliners. Two different technique did however produce the same result, so this pattern can not be the consequence of a methodological error due to the technique employed. In addition, since 2 mM DIC is still limiting to photosynthesis, there is no obvious reason why photosynthesis and calcification should decrease with increasing DIC concentrations.

In this study, calcification and photosynthetic rates were both determined using measurements of the $^{14}C$ incorporation into the cell. This technique was adapted for *E. huxleyi* by Paasche (1963). When the rates of photosynthetic $^{14}C$ incorporation from the present work were compared with the $O_2$ production rates measured by Weinberg-Flax (2000), a discrepancy was found. $O_2$ evolution rates were 3 times higher than the rates of photosynthetic $^{14}C$ incorporation. These should theoretically be equal, since one molecule of water split during the light phase of photosynthesis should result in the fixation of one molecule of $CO_2$ by Rubisco during the dark phase of photosynthesis. To find out if the difference in rates was caused by an error in methodology or if it was real, $^{14}C$ experiments were performed inside an $O_2$ electrode chamber, leading to simultaneous $^{14}C$ and $O_2$ measurements. The obvious difference in the protocol of the two techniques is the incubation time, 30 minutes with $O_2$ electrodes and 3 hours for measurements of $^{14}C$ incorporation. Time course measurements show an exponential decrease of the rates of both calcification and photosynthetic $^{14}C$

incorporation. These rates are therefore not linear with time. On the other hand, $O_2$ evolution rates remain constant over 1 hour (data not shown). Thus, comparisons of the rates of photosynthetic $^{14}C$ incorporation and $O_2$ evolution should not be done. This idea is supported by Paasche (1964) who affirms that "comparisons (*of $^{14}C$ method*) with methods in which oxygen production is measured are of limited values unless the photosynthetic quotient is accurately known". It is also possible that after injection, $^{14}C$ binds to some unidentified molecules, but that this unspecific binding stops after a while. Experiments were thus performed for 3 hours in order to prevent such effects.

Many authors consider that calcification and photosynthesis are linked based on the stoichiometry between these two processes (see Paasche 2001 for review). Since the rates of $^{14}C$ incorporation are not linear with time, one should be cautious about using the C/P ratio for concluding on the possible relationship between photosynthesis and calcification. Comparison of photosynthetic rates of calcifying and non-calcifying cells seems to be a more appropriated approach.

Thus, to test further the assumption that calcification does not cause the biphasic pattern, cells of *E. huxleyi* were grown and incubated in calcium-free medium. Electron microscopy confirmed that no calcification happens in this medium. Previous experiments on calcification in *E. huxleyi* have used non-calcifying strains (Sikes *et al.* 1980, Paasche 1964) or coccoliths removal with acid treatment (Sekino *et al.* 1996). However, it is difficult to draw any general conclusions from experiments done on different strains of *E. huxleyi* because of large variability between strains (Elzenga *et al.* 2000, Young and Westbroek

1991). On the other hand, when acid treatment is performed, a calcifying strain of *E. huxleyi* can be used, since the acid only removes the plates located in the coccosphere without actually stopping the calcification process.

In the present study, a calcifying strain of *E. huxleyi* was grown in calcium-free medium. This is a non-aggressive procedure that can be used with a calcifying strain in order to stop calcification. Coccolith-less cells that were produced that way were not N-cells since they conserved their ability to calcify. In fact, when these were resuspended in normal medium (i.e. containing $CaCl_2$) they again produced coccoliths. In calcium-free medium or after being replaced in normal seawater their growth rate remained the same as that of plated cells (1.51-1.57 divisions $d^{-1}$). In addition, while N-cells and C-cells differ in their carbon transport mechanism (Paasche 2001), in this study, as in coccolith-bearing cells, the photosynthetic rate of coccolith-less cells was sensitive to SITS addition, thus suggesting that the anion exchanger remains active after inoculation in calcium-free medium. This suggests that no genetic mutation has occurred.

In calcium-free seawater, the kinetics of photosynthesis of coccolith-less cells with DIC remain biphasic, with a hiatus again at 1 mM DIC. This is further confirmation that the biphasic pattern of photosynthesis is not the consequence of calcification, but of two 'reactions' with different affinities and rates.

Calcification and photosynthesis both use DIC, but calcification needs bicarbonate (equation 1.4), while photosynthesis requires carbon dioxide (equation 1.2). Many authors state that photosynthesis is enhanced by the production of $CO_2$ and protons from calcification (Buitenhuis *et al.* 1999, Nimer *et al.* 1996, McConnaughey 1994, Sikes *et al.* 1980). They argue that *E. huxleyi* takes up bicarbonate, which is solely used for the formation of coccoliths. The molecule of $CO_2$, released during mineralisation is then fixed by Rubisco. According to this model, calcification is an intermediate process that allows external bicarbonate to become available in the form of $CO_2$ for photosynthesis. The protons generated during calcification can also reduce the cytosolic pH and thus enhancing the dehydration of bicarbonate to $CO_2$.

Previous studies, which measured photosynthesis at different calcium concentrations, did not give an irrefutable answer on whether or not calcification enhances photosynthesis in *E. huxleyi*. When measuring the photosynthetic rate of a calcifying strain of *E. huxleyi* over a range of calcium concentrations (0 to 6.7 mM), Paasche (1964) found that photosynthesis remains constant, with the exception of a decrease at concentrations below 1 mM. He related this diminution in photosynthesis to the possibility that cells are shocked by low calcium concentrations. Since Paasche measured photosynthesis only at low calcium concentrations, no inference can be made on the possible supply of $CO_2$ from calcification to photosynthesis in *E. huxleyi* in ambient seawater. His results do, however, suggest that calcification does not enhance photosynthesis. Using a much larger range of calcium concentrations (0-15 mM), Nimer *et al.* (1996) measured an increase in the photosynthetic rate of *E. huxleyi* with rising

concentrations of calcium in the medium. Because photosynthesis and calcification were both saturated at 10 mM, they concluded that these two processes were equally dependant on the calcium concentration of the medium. The PFD used in their experiment was 50 μmol $m^{-2}$ $s^{-1}$, and light was thus not saturated for photosynthesis (Nielsen 1995). With this experimental design, the effect of different calcium concentrations on photosynthesis cannot be truly observed, because the rate of photosynthesis may be influenced by the combined effect of the low PFD and the calcium concentration of the medium.

More recently Buitenhuis *et al.* (1999) have also suggested that at low DIC concentrations, *E. huxleyi* photosynthesis relies on diffused $CO_2$, whereas at higher DIC concentrations photosynthesis depends on $CO_2$ and protons generated by calcification. They measured the calcification and photosynthetic rate of *E. huxleyi* as a function of the different chemical species of DIC. They concluded that bicarbonate is the direct source of carbon for calcification but not for photosynthesis. One should be cautious about their conclusions, since the same pattern of argument leading to the conclusion that external bicarbonate is directly used for calcification can be applied for photosynthesis. They also automatically assumed that photosynthesis and calcification are dependent on each other because of their proportional increase, but both processes could depend on a common third factor.

Data presented in this thesis clearly show that in ambient seawater and at saturating light, calcification does not supply photosynthesis with $CO_2$. Photosynthetic rates, measured as $^{14}C$ incorporation into acid stable compounds, are indeed similar in calcifying and non-calcifying cells of *E. huxleyi*. These measurements were made after 3 hours incubation in normal or calcium-free seawater. Since on average one coccolith is formed every hour (Paasche 1968), many molecules of $CO_2$ were liberated by calcification during this incubation period and were potentially available to Rubisco. Measurements of $^{14}C$ incorporation into the coccoliths have confirmed that coccolith-bearing cells actively calcify in normal seawater, whereas calcification stopped completely when incubated in calcium-free seawater. If $CO_2$ was supplied to Rubisco via calcification, the lack of calcification in cells incubated in calcium-free medium would cause a diminution of the photosynthetic $^{14}C$ incorporation rate as compared with the photosynthetic rate of cells in normal seawater. Since this is not the case, it can be concluded that, in ambient seawater and at saturating light for calcification and photosynthesis, calcification does not enhance photosynthesis. This was confirmed by $O_2$ measurements, as the photosynthetic rate of coccolith-bearing cells was not faster than that of coccolith-less cells. This study therefore resolves the disagreement in the literature in favour of Paasche.

Given that calcification does not provide Rubisco with $CO_2$, inorganic carbon for photosynthesis has to be drawn directly from the medium. The fact that $CO_2$ is the substrate for Rubisco does not automatically imply that it is the form of DIC that enters the cell. Indeed, internal CA activity has been detected

in *E. huxleyi* (Nimer *et al.* 1995, Nimer *et al.* 1994). Hence, this coccolithophore possesses the necessary machinery to convert bicarbonate into carbon dioxide to provide to Rubisco. Bicarbonate could then potentially be the exogenous source of carbon for both calcification and photosynthesis.

Several studies have confirmed this idea that in *E. huxleyi* photosynthesis is supported by $CO_2$ originating from $HCO_3^-$ flux into the cell (Nimer and Merrett 1992, Paasche 1964). Nevertheless, for many years, in ambient seawater, no carbon uptake mechanism had been detected in this coccolithophore, so that although bicarbonate was well-established as a source of carbon for photosynthesis, its precise mechanisms of transport into the cell remained unclear (Paasche 2001). In fact, there are many reports that this alga lacks external CA activity in ambient seawater (Sikes and Wheeler 1982, Merrett 1991, Nimer *et al.* 1994, Sekino and Shiraiwa 1994). This enzyme was however detected in this coccolithophore in ambient seawater by Nimer and Merrett (1993), but only when the culture was 11 days old, which means that cells were already in the stationary phase of growth.

Nimer *et al.* (1996) and Dong (1992) have provided evidence that a number of other macroalgae have an anion exchanger that mediates carbon across the plasmalemma by exchanging bicarbonate and chloride ions. Despite the lack of CA activity in cells of a high calcifying strain of *E.huxleyi* in the exponential growth phase, Nimer *et al.* (1996) recorded a halving of the DIC concentration of the medium in which those were incubated. This dramatic uptake remained unexplained since the addition of 0.5 mM DIDS

(4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid), a specific inhibitor of anion exchanger did not reduce the photosynthetic rate of these cells. Thake (personal communication 1999) was the first to demonstrate the presence of a SITS sensitive mechanism of bicarbonate uptake in cells of *E. huxleyi* in both exponential growth phase and ambient seawater. Weinberg-Flax (2000) repeated Thake's experiment and provided strong evidence that *E. huxleyi* possesses an anion exchanger on its plasmamembrane, which is functionally and structurally similar to the one in the human red blood cells. The discrepancy between this result and that of Nimer *et al.* (1996) may arise from methodological differences, since for $O_2$ measurement cells are exposed to DIDS for only a few minutes (15-20), while the pH drift experiment of Nimer *et al.* (1996) was carried out for 12 hours. DIDS and SITS are labelled as light sensitive (SIGMA, Poole, UK) and exposure to bright light for 5 hours completely inactivates this chemical (B. Thake, personal communication, 2002), so it is possible that prolonged exposure under continuous light even at a PFD of 50 $\mu$mol $m^{-2}$ $s^{-1}$ inactivated the inhibitor during the experiment of Nimer *et al.* (1996).

In this study, measurements of the rate of photosynthetic $^{14}C$ incorporation with and without SITS, a specific inhibitor of the anion exchange protein AE1, have confirmed that this glycoprotein is active in *E. huxleyi* at the ambient seawater concentration of 2 mM DIC.

The change in the kinetics of photosynthesis and calcification observed at 1 mM DIC could be caused by the induction of this protein at this DIC concentration. This is however not the case, since the rate of $O_2$ production is

also inhibited by SITS at 0.5 mM DIC. So, the anion exchanger transports bicarbonate into cells of *E. huxleyi* at both low and high concentrations of DIC. Hence, the activity of the anion exchange protein cannot alone explain the change of kinetics observed at 1 mM DIC in the photosynthetic and calcification rates. The only possible explanation is that another process / mechanism is induced or stops when there is 1mM DIC in the medium.

As mentioned previously, Nimer and Merrett (1993) and Nimer *et al.* (1994) detected external CA activity in old cultures of *E. huxleyi*. In those conditions, all the cells were in the stationary growth phase and all the DIC present in the medium was depleted (Dong *et al.* 1993). The question of whether it is the growth phase of the alga or the DIC concentration in the medium that causes the external CA to be active in the 11 days old culture and not in the 4 days old one (Sekino and Shiraiwa 1994, Merrett 1991 and Sikes and Wheeler 1982) was resolved by Nimer *et al.* (1997). They found extracellular CA activity in cells of *E. huxleyi* in the exponential growth phase, when incubated in limited $CO_2$ and $HCO_3^-$ conditions. Unfortunately, in their experiment, cells were not incubated at the ambient pH of seawater of 8.2 but at 8.7, and light was not saturated for photosynthesis since a PFD of 50 $\mu$mol $m^{-2}$ $s^{-1}$ was used. In the present study, artificial seawater was buffered to pH 8.2 and a PFD of 500 $\mu$mol $m^{-2}$ $s^{-1}$ was used to demonstrate external CA activity, at 0.5 mM DIC, in cells of *E. huxleyi* in their mid-exponential growth phase. So, data from this thesis ascertain that the DIC concentration of the medium and not the age of the culture controls external CA activity in *E.huxleyi*.

This finding is in agreement with the widely accepted view that CA activity is induced by low $CO_2$ concentrations (Elzenga *et al.* 2000, Kaplan *et al.* 1998, Satoh *et al.* 1998, Nimer *et al.* 1997, John-McKay and Colman 1997, Sasaki *et al.* 1997, Nimer *et al.* 1996, Matsuda and Colman 1995, Suzuki *et al.* 1994, Tsuzuki and Miyachi 1989). Raven and Newman (1994) affirm that a low $CO_2$ environment does not simply induce but derepress the external CA activity of many photosynthetic aquatic organisms. Iglesias-Rodriguez and Merrett (1997) could not detect external CA activity in *Phaeodactylum tricornutum* when the DIC concentration was 2 mM, but could do so when the medium contained 1 mM DIC.

This ability to vary the carbon transport mechanisms with changing environmental conditions has also been observed in *Ulva lactua* (Axelson *et al.* 1995). These authors have described the physiological state of the macroalga as being either a 'CA state' when photosynthesis is fully inhibited by AZ at pH 8.2 or an 'AE state' when photosynthesis is strongly inhibited by DIDS addition at a pH of 9.4. Their work demonstrates that a 'CA state' alga incubated at a pH of 9.4 gradually loses external CA activity and in compensation gains an active anion exchanger. The opposite is true for an 'AE state' alga when put in seawater of pH 8.2.

*E. huxleyi* too changes its carbon uptake mechanisms according to the external conditions. At 2 mM DIC, the anion exchanger is the only bicarbonate transport mechanism in *E. huxleyi*, but at 0.5 mM DIC both anion exchange protein and external CA mediate carbon across the membrane. When the DIC concentration of the medium is low, two active bicarbonate transport mechanisms

are necessary to supply to both photosynthesis and calcification. As the DIC concentration increases, the anion exchanger becomes sufficient to support the carbon need of the alga, and the external CA activity is subsequently suppressed. The necessity for an active external CA was described by Sültemeyer (1998). While reviewing studies showing evidence of external CA activity in a large range of algae, he concluded that 'external CA, while not important for maintaining photosynthesis, is important for maximum $CO_2$ fixation under low $C_i$ concentration by ensuring efficient utilisation of external $C_i$'.

This physiological capacity of *E. huxleyi* to response to environmental changes can be visualised by the biphasic nature of the kinetic of photosynthesis and calcification with adding DIC concentrations. At low DIC concentrations external CA and anion exchanger are both transporting bicarbonate across the plasmalemma of *E. huxleyi*. This association creates a high affinity / low rate system. At 1 mM DIC the external CA is totally inhibited and only the $HCO_3^-$ / $Cl^-$ exchanger provides carbon to the cell. This switch off of one of the bicarbonate transport mechanism causes a change in the kinetics of photosynthetic and calcification rates. The bicarbonate mediated system is then characterised by low affinity / high rate kinetics.

Furthermore, the data presented demonstrate clearly that there is a relationship between DIC, chloride concentrations and external CA activity. At 0.5 mM DIC, photosynthesis is indeed inhibited by a 25 % increase in the chloride concentration of the medium, with either sodium chloride or choline chloride addition. This inhibition of photosynthesis by chloride can be overcome

by adding DIC to the seawater. Chloride and DIC concentrations are then closely linked and have opposite effects on photosynthesis.

Moreover, at 0.5 mM DIC, 100 μM AZ does not further inhibit photosynthesis when an increased chloride concentration is already reducing the photosynthetic rate. In contrast, in the control solution, (i.e. normal seawater), AZ reduces the photosynthetic rate. Thus, external CA activity ceases when there is a high chloride concentration in the medium.

Somewhat surprisingly, increased chloride concentrations have no effect on the activity of the anion exchanger, as the percentage inhibition of photosynthesis with SITS remains constant with or without added chloride.

In summary, at 0.5 mM DIC, DIC and chloride concentrations have opposite effects on photosynthesis and high chloride concentrations do not affect the activity of the anion exchanger, but strongly inhibit external CA.

From these data, a model of the carbon transport mechanisms in *E. huxleyi* at different DIC concentrations can be conceived. At low DIC concentrations, both an anion exchanger and an external CA transport DIC into *E. huxleyi*. The external CA does so by performing extracellular dehydration of $H_2CO_3$ followed by the penetration of $CO_2$ into the cell, whereas the anion exchanger takes up bicarbonate into the cell in exchange for a chloride ion. The system is then characterised by its high affinity and low rate.

As the DIC concentration of the medium rises, the exchange rate of bicarbonate and chloride ions increases. This can be observed in figure 3.2, where the photosynthetic rate is 1.7 times higher at 2 than 0.5 mM DIC. More carbon is fixed during photosynthesis because more bicarbonate enters the cell. As more bicarbonate ions are taken up by the glycoprotein, more chloride ions are extruded. Several studies have shown that CA activity is inhibited by high chloride ion concentration. Stemler (1986) reported that CA was activated at low chloride concentrations in higher plants. Dionisio-sese and Miyachi (1992) have also investigated the effect of NaCl on CA activity in several marine microalgae. They found that the effect of NaCl varied between the species and in some microalgae CA activity was inhibited by high NaCl concentrations, while in others NaCl had no effect. In salt substitution experiments they found that chloride is the ion that inhibits CA activity. However, *Pleurochrysis sp.*, the one coccolithophore they studied exhibited a chloride stimulation of CA activity.

At 1 mM DIC, because of the high rate of bicarbonate transport across the membrane, the chloride concentration in the periplasmic space may become high enough to inhibit external CA activity. Although it is probable that the increment of the bulk phase chloride concentration is unlikely to be high, small differences may strongly affect external CA since several recent reports have shown that CA and anion exchanger can be closely associated (Sterling *et al.* 2001, Reithmeier 2001, Vince *et al.* 2000, Vince and Reithmeier 2000). In fact, Sterling *et al.* (2001) showed that in red blood cells these two proteins are linked within a metabolon (i.e. 'a weakly associated complex of sequential metabolic enzymes') and that this association allows maximal bicarbonate transport. If such a metabolon is present in *E.huxleyi*, small changes in chloride concentrations due

to increased activity of the anion exchanger could be enough to inhibit the associated CA. Whatever the exact mechanism, it is clear that over 1 mM DIC the anion exchanger remains the only mechanism to transport DIC across the membrane and the system is characterised by its low affinity and high rate.

Nimer and Merrett (1996) have also observed this change in affinity and rate of photosynthesis with increasing DIC concentrations in *E. huxleyi*. They showed that as opposed to cells in the exponential growth phase, where photosynthetic rate is not saturated at 2 mM DIC, $V_{max}$ is reached at 0.8 mM DIC in cells in the stationary phase of growth. As previously mentioned, external CA activity was recorded in such cells (Nimer and Merrett 1993). Data from this thesis show that these also posses an active anion exchanger. So, cells of *E. huxleyi* in the stationary growth phase have both active external CA and anion exchanger to transport carbon across the plasmalemma. Conditions in the stationary growth phase are therefore similar to the one of cells in the exponential growth phase incubated at 0.5 mM DIC. These considerations explain why Nimer and Merrett (1996) found that such a low DIC concentration saturates photosynthesis. In cells in the stationary phase of growth the association of the two carbon transport creates again a high affinity / low rate system.

In the present work, the induction of the external CA at low DIC concentrations was rapid, and took less than 15-20 minutes. During experiments in which photosynthesis and calcification rates were measured at different DIC concentrations, cells of *E. huxleyi* were grown in medium containing 2 mM DIC. Cells were resuspended in synthetic $HCO_3^-$-free seawater for 15-20

minutes before DIC was added to the medium to start the experiment. Such a short period of incubation in low DIC concentration is sufficient to induce external CA activity in *E. huxleyi*. Similarly, Sültemeyer *et al.* (1998) found a fast induction time in *Synechococcus sp.* A 10 minute exposure to low DIC concentration was indeed sufficient to increase the photosynthetic affinity of the cyanobacterium for DIC. The signal transduction process by which DIC is transported into cells remains poorly understood (Matsuda and Colman 1995). The rapid induction suggests post-translational regulation rather than *de novo* synthesis of the protein from DNA (Sültemeyer 1998).

Furthermore, data presented in this report show that the photosynthetic rate of *E. huxleyi* incubated at low DIC concentration is almost 2 times higher when the culture was grown at 0.5 rather than at 2 mM DIC. This discrepancy may be the consequence of two regulatory pathways leading to a fast or a slow induction of external CA activity. This would explain the difficulty in detecting external CA activity in cells grown at 2 mM and incubated at 0.5 mM DIC (data not shown). During this experiment, this inhibitor technique may not be sensitive enough to detect the newly active enzyme. Cells grown at 0.5 mM DIC may have synthesised more external CA, which would explain why the same technique then becomes efficient at detecting external CA activity.

Considerations such as this suggest a two-step induction of external CA when *E. huxleyi* is exposed to low DIC concentrations : a fast induction with post-translational regulation and a slow induction with *de novo* synthesis of the enzyme. A similar two-step regulation of external CA activity was also observed

in *Synechococcus sp.* in order to reach the complete high affinity state of photosynthesis (Sültemeyer *et al.* 1998).

Data presented in this report provide some basic information for an understanding of DIC transport mechanisms in *E. huxleyi* and of their effect on the calcification and photosynthetic rates of this alga. The coccolithophorid possesses two carbon transport mechanisms, an anion exchanger active at all DIC concentrations and an external CA, which is induced by low DIC concentrations and repressed by high chloride ion concentrations. Further investigations remain necessary to isolate the genes coding for external CA and to determine the details of the induction and repression of this enzyme in *E. huxleyi.*

# Chapter 4 :

## Bicarbonate effects on photochemistry in *Emiliania huxleyi*.

Introduction :

Oxygenic photosynthesis appeared 3.5 billion years ago (Schopf 1993, Awramik 1992) with the formation in cyanobacteria of the Z scheme, in which photosystem I (PSI) and photosystem II (PSII) reaction centre cores are connected in series (Falkowski and Raven 1997, Blankenship 1992). The 'inner core' of PSII is highly conserved in all $O_2$ producing photosynthetic organisms (Christen *et al.* 1999, Larkum and Howe 1997), but some differences exist in the nature of the regulating sub-units (Christen *et al.* 1999). The PSII reaction centre is the key component of the so-called 'light reaction', where the electronic energy of photon excitation is transformed into electrochemical energy (NADPH and ATP). The first phase of oxygenic photosynthesis can therefore be described as a series of photochemically catalysed oxidation-reduction reactions. In the second phase, called the dark reaction, Rubisco uses the photosynthetically produced molecules of NADPH and ATP to fix inorganic carbon. These reactions, by which $CO_2$ molecules are converted into carbohydrates, form the Calvin cycle.

Only photosynthetically active radiation, in the violet to red part of the visible spectrum (400 - 700 nm), possesses enough energy to be absorbed by algal pigments and trigger photosynthetic reactions (Prezelin and Boczar 1986). Algal pigments are made of chlorophylls (chl.), phycobilins and carotenoids. Since the reaction centres of PSI and PSII contain chl. a exclusively, all the other pigments and some additional forms of chl. a constitute the Light Harvesting

Complex (LHC), also called antenna or accessory pigments. Although chl. a is the only pigment that is absolutely required for photosynthesis, the LHC plays an essential role in increasing the spectral range of light energy absorbed by each alga. Carotenoids (β-carotene, 19'-oxyhexanoyl-fucoxanthin, diatoxanthin, diadinoxathin) are the main accessory pigments of *E. huxleyi* (Brand 1994). This coccolithophore possesses a single 'giant' chloroplast, which as shown in plate 4.1 is apposed to the plasmalemma and circles almost the entirety of the cell (Pienaar 1994).

The photosynthetic units (LHC and reaction centres of PSI and PSII) are organised across the thylakoid membrane, within the chloroplast. Figure 4.1 displays the photosynthetic electron transport chain (ETC) of eukaryotes as published in Schreiber *et al.* (1994). PSII is characterised by its phototrap denoted $P_{680}$; its electron ($e^-$) donor, the manganese-containing water splitting enzyme complex; and its primary $e^-$ acceptor, a plastoquinone (PQ). $P_{700}$ is the term used for the phototrap of PSI, while a plastocyanin (copper-containing protein) and ferredoxins A and B (iron and sulfur rich protein) are its $e^-$ donor and acceptor respectively.

Figure 4.2 shows the structure of chl. a. This molecule is made of a porphyrin ring, which contains in its centre, a chelated magnesium ion connected at four sites to pyrrole nitrogen atoms, to which electron ($e^-$) donors can attach (Prezelin and Boczar 1986). Upon excitation, the redistribution of the pi-electrons of the porphyrin ring forces the molecule to reach a higher excited electronic state, as represented in figure 4.3. This high excited state leads to

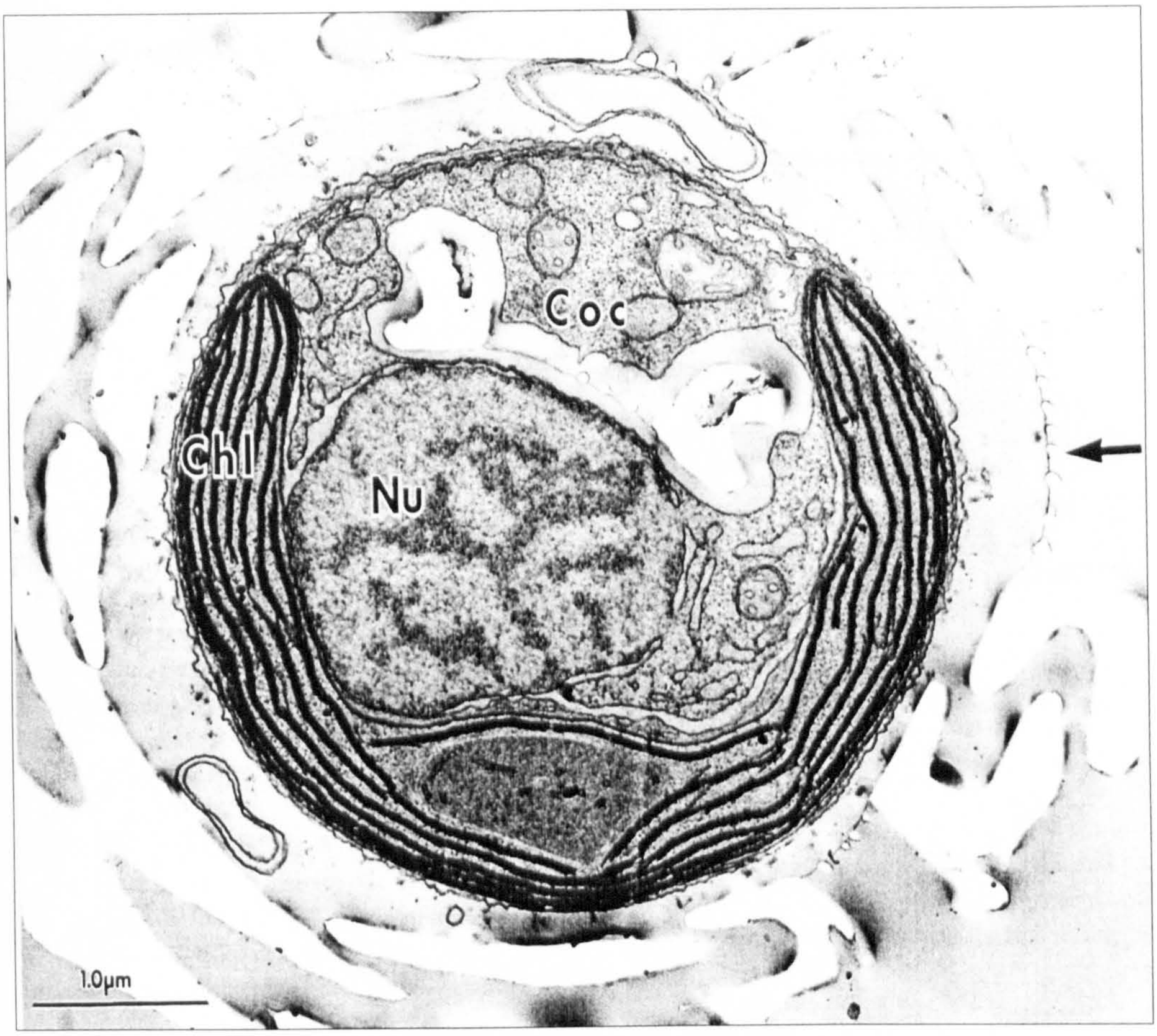



Plate 4.1 : A cross section in a cell of *E. huxleyi* using transmission electron microscopy (from Pienaar 1994).

Abbreviations : Chl = chloroplast, Nu = nucleus, Coc = coccolith vesicle.

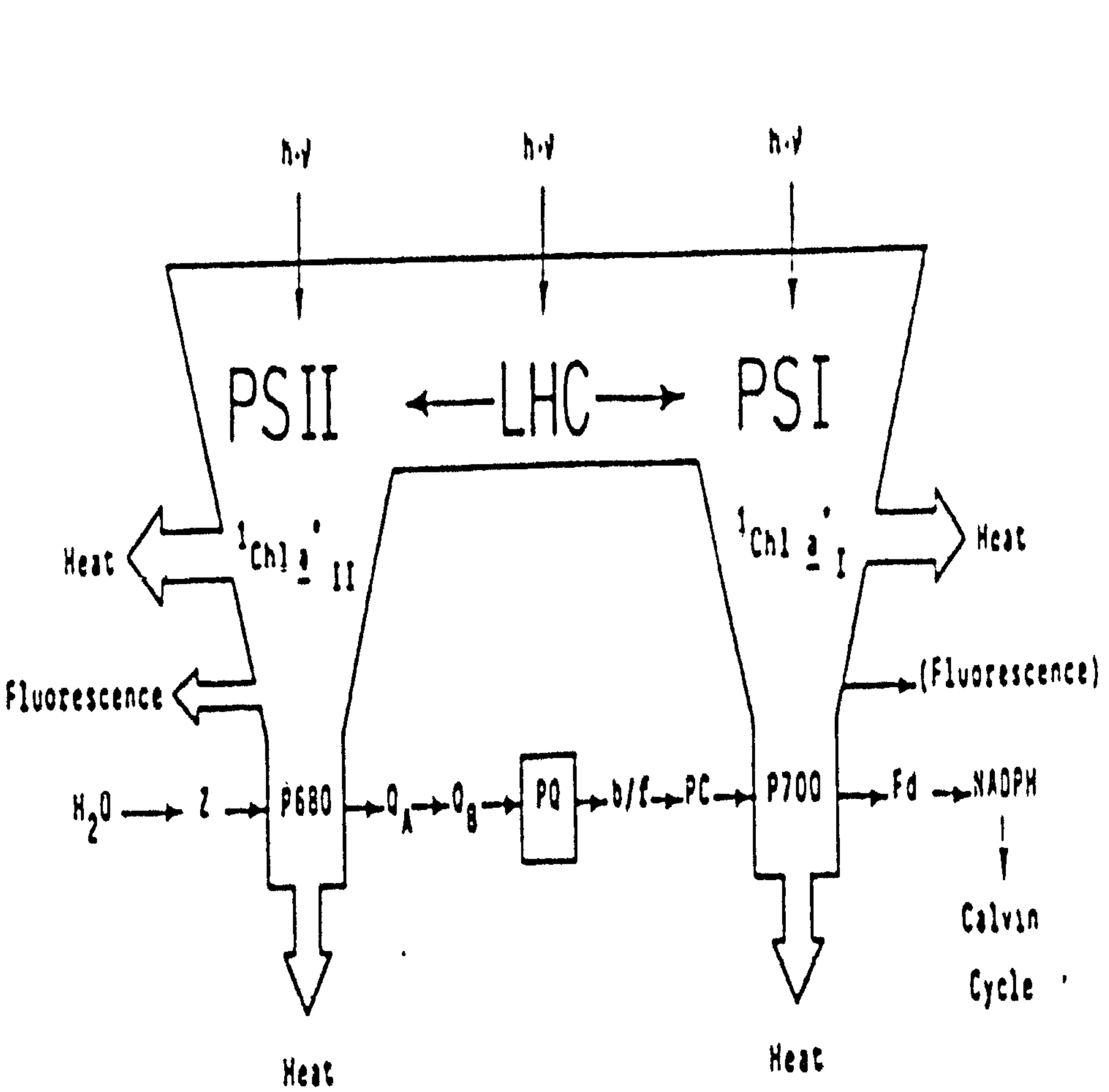



Figure 4.1 : Diagram of the photosynthetic electron transport chain of oxygen evolving eukaryotic organisms (from Schreiber *et al.* 1994).

Abbreviations : LHC = light harvesting complex, PSII = photosystem II, PSI = photosystem I, Chl = chlorophyll, PQ = plastoquinone, Q = quinone, b / f = cytochrome complex $b_6$ / f, PC = plastocyanin, Fd = ferredoxin.

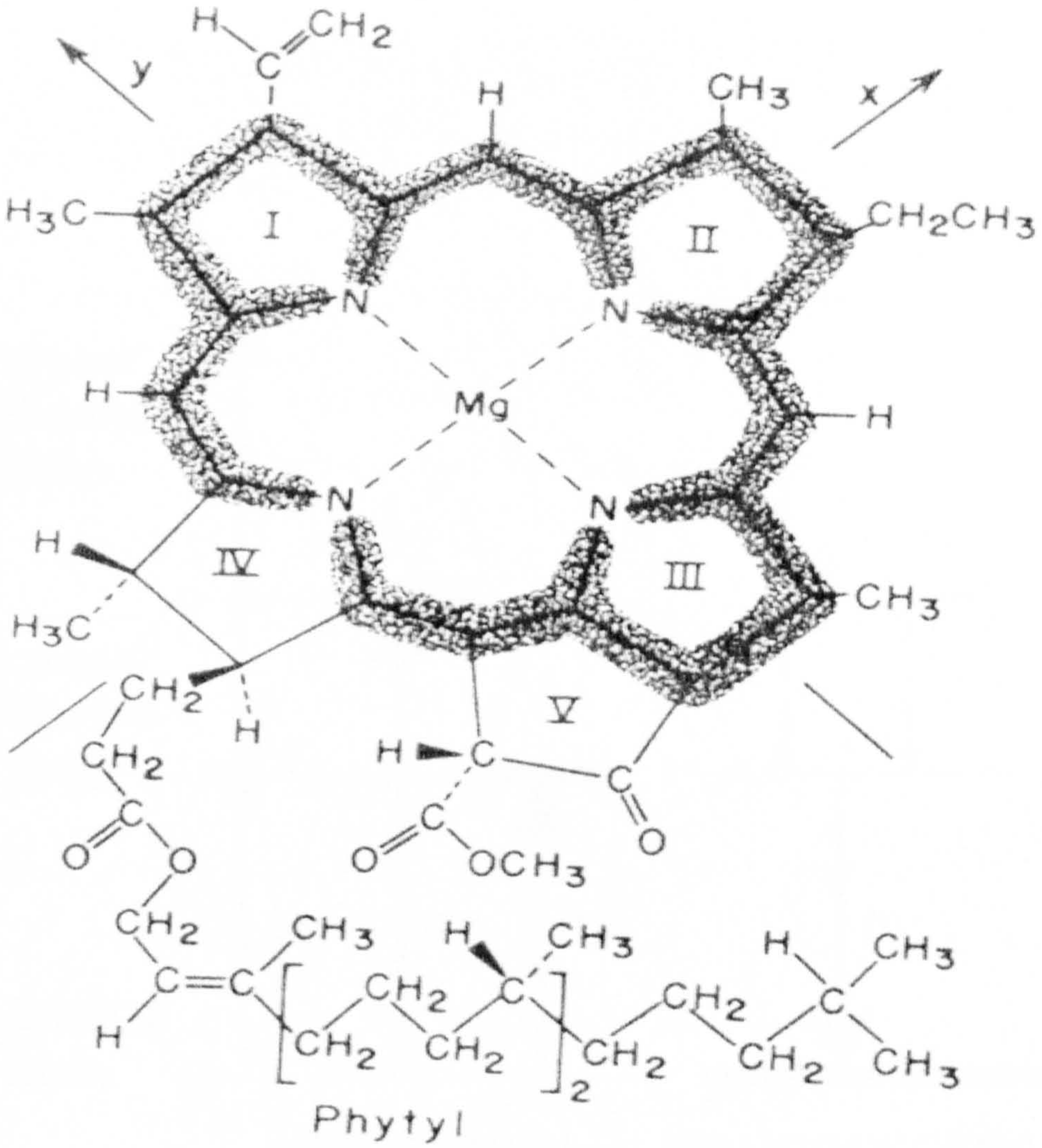



Figure 4.2 : The molecular structure of chlorophyll a (from Prezelin and Boczar 1986). The shading area indicates the porphyrin-electrons.

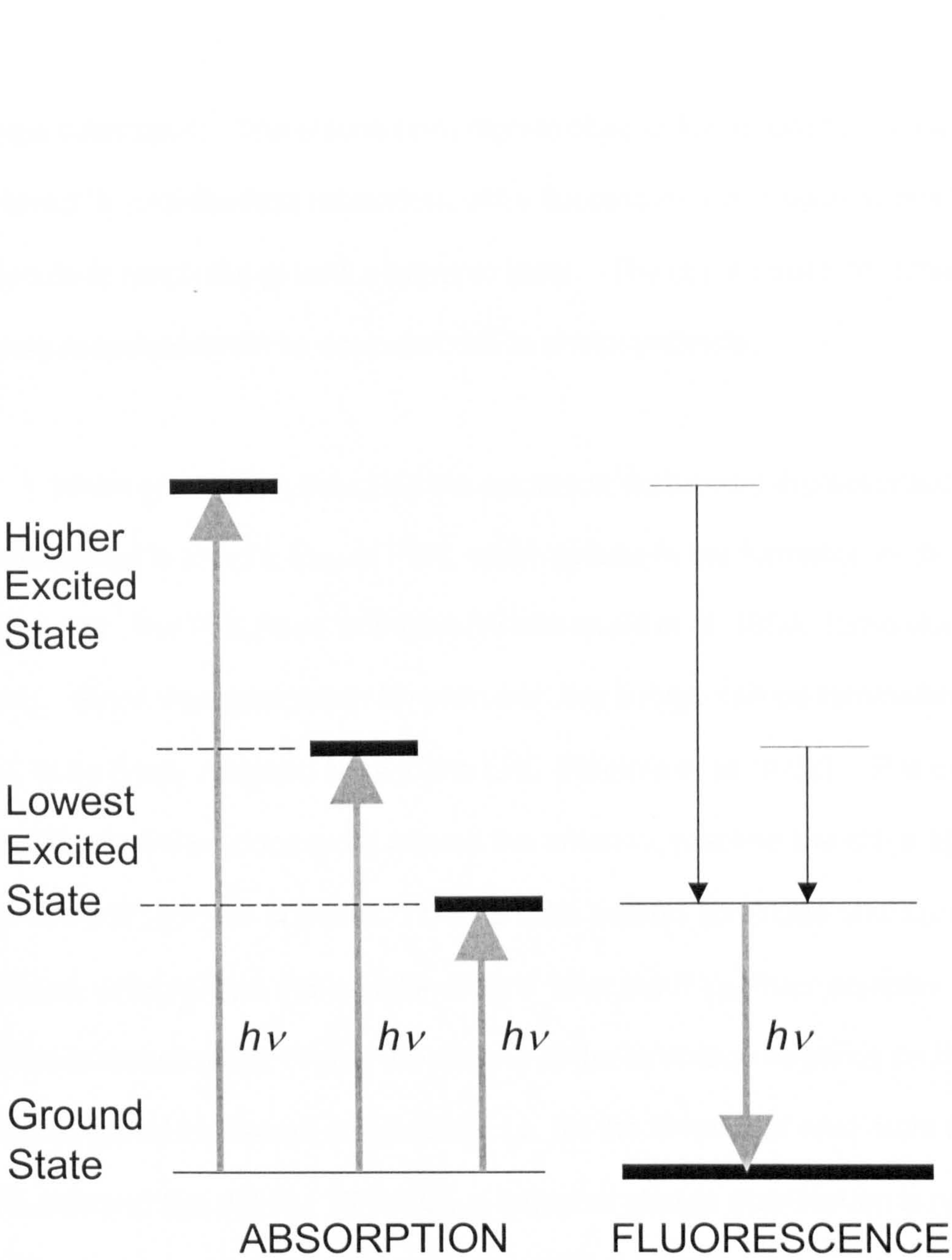



Figure 4.3 : The energy level diagram of chlorophyll a showing spectral transitions (adapted from Prezelin and Boczar 1986). On the right, the thin arrows show radiationless relaxation, while shaded arrows indicate fluorescence emission.

charge separation. Transitions from high-excited to low-excited states are achieved by radiationless relaxation, while fluorescence emission allows the molecule to reach the ground electronic state. The chl. a structure is therefore closely associated with its essential role in photosynthesis.

When photons hit the LHC, the excitation derived by the absorbed quanta is transferred to the chl. $P_{680}$ of PSII, which results in the formation of the primary radical pair : the $P^{+}_{680}Pheo^{-}$ complex (Wasielewski *et al.* 1989, Jankowiak *et al.* 1989). Since this association is reversible, the energy can be retransferred to $P_{680}$, to be finally released back in the LHC (Roelofs *et al.* 1992). The excitation, which has travelled once more around the antenna, reaches the chl. a again to reform the $P^{+}_{680}Pheo^{-}$ complex. This circular pattern continues until $Q_A$ is oxidised, which allows the transfer of an $e^{-}$ from the $P^{+}_{680}Pheo^{-}$ complex to $Q_A$, therefore reducing $Q_A$. Thus, the lifetime of the excitation depends on the time that is required for charge stabilisation, i.e. for the transfer of an $e^{-}$ from $Pheo^{-}$ to $Q_A$ (Laisk and Oja 2000). When $Q_A$ is reduced, charge stabilisation is not possible, and the excitation travels in the antenna. The light reaction is controlled by the oxidised / reduced state of $Q_A$.

The primary photochemical event occurring in PSII is the charge separation between the $e^{-}$ donor, $P_{680}$ and the $e^{-}$ acceptor, pheophytin (Yamagishi and Katoh 1985). Although this reaction is limited by the oxidised / reduced state of $Q_A$, it is mostly controlled by the $CO_2$ fixation rate of Rubisco. Indeed, excess light, which is characterised by a surplus of chemical-bond energy and reductant production, results from an imbalance between the excitation arrival

rate in the reaction centre (i.e. the rate of $Q_A$ reoxidation) and the rate of ATP and NADPH molecules utilisation by Rubisco (Laisk and Oja 2000). When $Q_A$ is oxidised, the excitation has a lifetime of 300 – 400 ps, whereas it reaches 1 – 2 ns in the reduced state (Laisk and Oja 2000). Thus, the reduced state of $Q_A$ corresponds to a longer period of high-energy state of PSII. This high excitation pressure (Gray *et al.* 1996) creates great danger for the photosynthetic apparatus. Chlorophylls bearing long-living excitation are strong reducers and can therefore transfer $e^-$ to lipids or other components in an uncontrolled manner. Plants have developed non-photochemical quenching ($q_N$) mechanisms to funnel this excess energy to prevent photosynthetic damage.

Different forms of non-photochemical quenching exist. Laisk and Oja (2000) described $q_N$ as being a 'succession of different processes developing in time'. The antenna localised (Horton *et al.* 1994, Crofts and Yerkes 1994), energy-dependent quenching mechanism, denoted $q_E$, is the fastest of all $q_N$ processes. $q_E$ is induced by the acidification of the thylakoid's lumen and depends on the presence of antheraxanthin and zeaxanthin (Horton *et al.* 1994, Krause and Weis 1991). $q_E$ is not activated by the pH gradient itself. Moreover, $q_E$ controls the reversible stage of $q_N$. Hence, $q_E$ maintains the excitation lifetime constant, independently of the presence or absence of excess light. $q_E$ provides photoprotection (Osmond *et al.* 1999) without causing any changes in the number of active PSII or in their turn over rates. So, $q_E$, the antenna-based photoprotective mechanism, does not damage PSII.

When $q_E$ activity becomes insufficient to quench long-lived excess energy, photoinhibition mechanisms are initiated (Horton *et al.* 1994). Photoinhibition processes, also called photoinactivation (Osmond *et al.* 1999), can be irreversible. In fact, although they protect the photosynthetic apparatus in high light conditions, they remains active even after the diminution of the photon flux density, hence causing unnecessary quantum loss (Osmond *et al.* 1999). The slow induction non-photochemical quenching mechanism called $q_I$ represents the reversible stage of the photoinhibition process. Since relaxation requires hours to days, $q_I$ causes the reduction in the number of active PSII and in their turnover rates, thus damaging the reaction centres. The exact mechanism of photoinhibition remains unclear (Laisk and Oja 2000). Nevertheless, whether the damage caused by $q_I$ originates from the donor or acceptor side of PSII, it is clear that photoinhibition leads to the degradation of the $D_1$ protein in the PSII core centre. Although the protein $D_1$ can be repaired, this is a slow process (Aros *et al.* 1993), which necessitates the transportation of the particular PSII into a different region of the thylakoid.

The non-photosynthetic quenching mechanism of transition type, called $q_T$, remains poorly quantified and understood. This process causes a detached part of PSII antenna to moves to a different region of the thylakoid (Walters and Horton 1991). $q_T$ equilibrates the excitation rates of PSI and PSII at low absorbed quantum flux densities, before $q_E$ activation, and thus, $q_T$ does not result in any PSII damage.

Although a lot is still unknown about non-photochemical quenching mechanisms, plants have clearly developed many methods to adapt to the fact the ultimately, in high light, the photochemistry is limited by the rate of $CO_2$ fixation by Rubisco. So, at high photon flux densities, the dark reaction limits the light reaction. In such conditions, the carbon concentration is the only factor limiting photosynthesis.

In an article entitled 'Optima and limiting factors', Blackman (1905), adapting Liebig's 'law of the minimum' (Rabinowitch 1951), postulated that the photosynthetic rate was only limited by one factor at a time. This author illustrated this notion by producing a family of curves (figure 4.4) with separated saturation plateaux and identical initial slopes. These 'Blackman-type' curves imply that at high PFD, the carbon concentration is the limiting factor of photosynthesis, whereas light is the only limiting factor at low PFD. He states that 'for each intensity of light falling on a leaf there is a different optimum amount of carbon dioxide'. Other studies have shown that the photosynthetic rates of many plants were limited by a single factor at a time (Maberly 1985).

In contrast, Bose (1924) disagreed with the theory of single limiting factor. To support his argument, he produced the set of curves showed in figure 4.5. The 'Bose-type' curves have separated saturation plateaux and distinct initial slopes. According to this model, at high PFD, as in the Blackman's model, photosynthesis is limited only by carbon concentrations, but as opposed to the Blackman's model, at low PFD both light and carbon limit photosynthesis. This implies that at low PFD, although Rubisco has an ample supply of carbon in

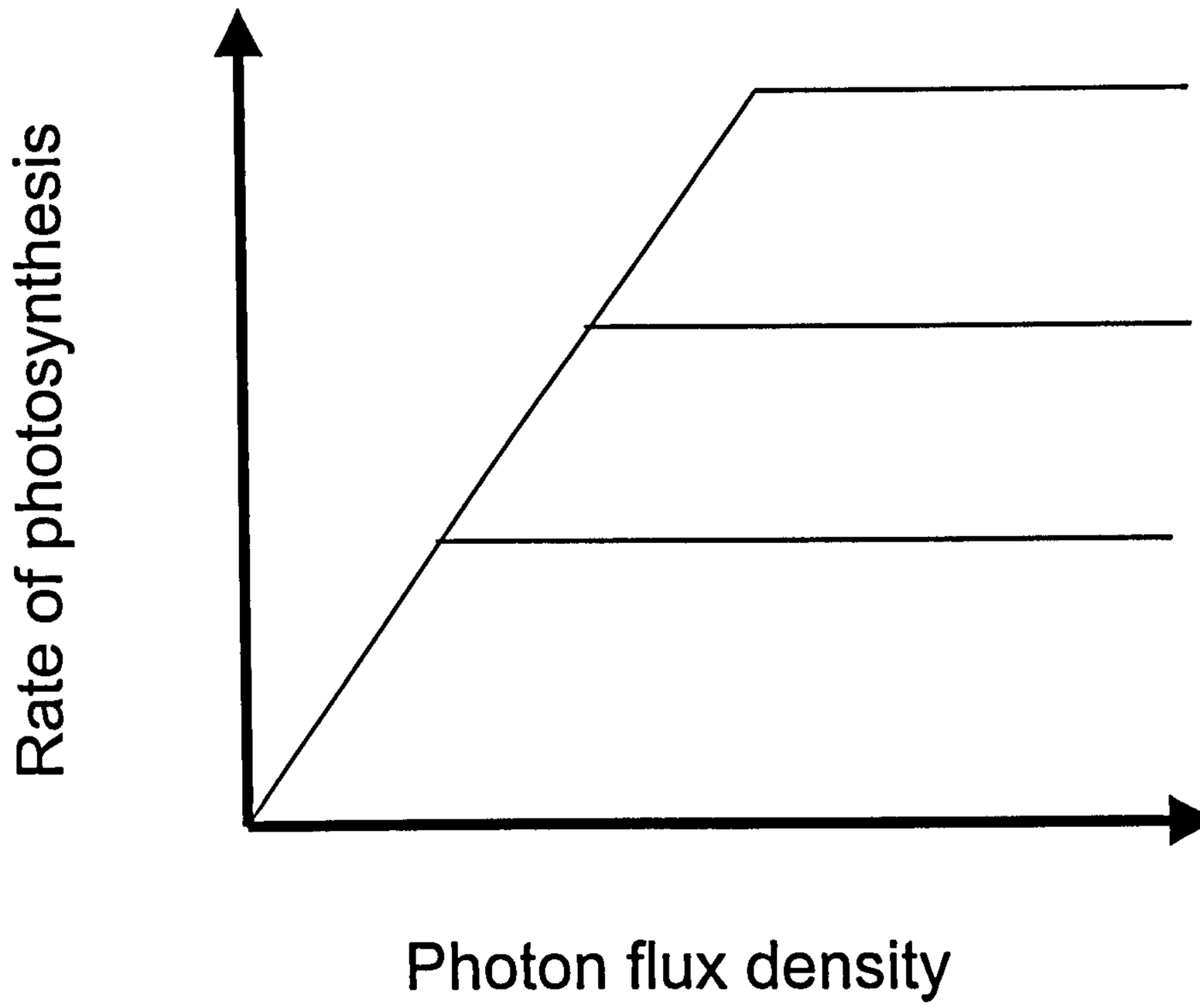



Figure 4.4 : Diagram representing the family of curves produced by Blackman (1905), showing that only one factor limits photosynthesis at a time. Reproduced from Rabinowitch (1951).

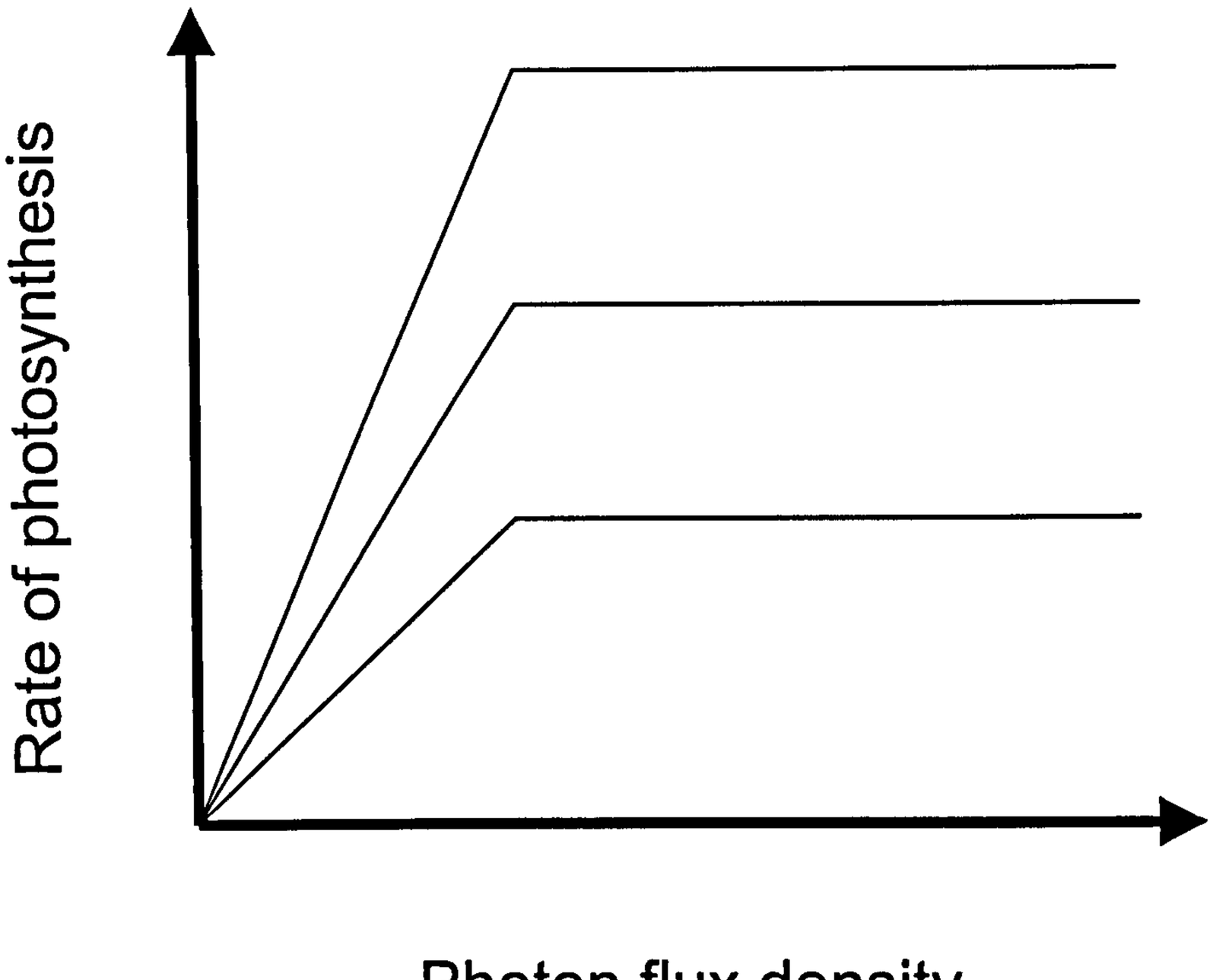



Figure 4.5 : Diagram representing the family of curves produced by Bose (1924), showing that two factors can limit photosynthesis at one time.

Reproduced from Rabinowitch (1951).

comparison to the amount of NADPH and ATP produced, the photosynthetic rate is somehow enhanced by increased DIC concentrations. In this model, the light reaction itself is stimulated by carbon addition.

At saturating light, the photosynthetic rate of *E. huxleyi* is stimulated by increased DIC concentrations (Weinberg-Flax 2000, and Chapter 3 of this thesis). The aim of this work was to determine if the photosynthetic rate of *E. huxleyi* behaves according to the Blackman or the Bose model, by providing biochemical and physiological evidence. Oxygen evolution and induced fluorescence measurements were therefore carried out in whole cells of *E. huxleyi*, at limiting and saturating PFD, and at different DIC concentrations. The FMS was used in addition to $O_2$ measurements, because it provides useful information on non-photochemical quenching.

## Materials and Methods :

Unicellular cultures of the coccolithophore, *Emiliania huxleyi* (Lohmann) Hay and Mohler (calcifying strain PCC.B11) were grown as described in Chapter 2. The strain PCC.B11 was isolated from Norway.

The methods employed for the measurements of photosynthetic $O_2$ evolution using a Clark-type electrode (Rank Brothers) and of the induced fluorescence parameters provided by a Fluorescence Monitoring System (FMS) are also explained in detail in Chapter 2.

In the experiments where the effect of increased PFD on PSII activity was determined in *E. huxleyi* using a FMS, the following script was used :

gain : 50

mod : 2

log : 1

wait : 10

FvFm : 2.5,85,0.7

wait : 30

**act : 5**

wait : 30

ΦPSII : 2.5, 85, 0.7

wait : 30

} Sequence a

Repetition of the sequence a for the actinic values of 10,15,20,25,30 and 35

ΦPSIIR : 2.5, 85, 0.7

The Photosynthesis – Irradiance data gained using the above script showed that actinic values of 15 and 30 were limiting and saturating light respectively. The calibration (through a piece of Eppendorf vial) gave PFD values of 161 and 848 μmol $m^{-2}$ $s^{-1}$ respectively (see Chapter 2 for more details). Experiments were conducted on *E. huxleyi* using the FMS and the following script to determine PSII activity and non-photochemical quenching at either 15 or 30 actinic values.

gain : 50

mod : 2

log :1

wait : 10

FvFm : 2.5,85,0.7

wait : 10

**act : 15 or 30**

wait : 5

ΦPSII : 2.5, 85, 0.7

wait : 60

ΦPSIIR : 2.5, 85, 0.7

Results :

*PI curves :*

Figure 4.6 shows that the $O_2$ production rate of *E. huxleyi* cells, grown and incubated at 2 mM DIC, saturates at a PFD of 300 μmol $m^{-2}$ $s^{-1}$. Whether PI curves were established by measuring the rate of $O_2$ production with sequential addition of quanta (from low to high light) or with sequential decrease of quanta (from high to low light), the PFD value at which photosynthesis saturates remains constant. Hence, the PFD value, at which photosynthesis saturates, does not depend on the light history of the cell, although the two methods yield differences in rates of $O_2$ production. In fact, $O_2$ production is higher around 100 μmol $m^{-2}$ $s^{-1}$ with sequential addition of light quanta. So, care was taken in subsequent experiments to avoid photodamage.

Figure 4.7 shows the effect of different PFD on the quantum efficiency of PSII (ϕPSII) in *E. huxleyi* grown and incubated at 2 mM DIC. PSII activity saturates at a PFD of 545 μmol $m^{-2}$ $s^{-1}$, which corresponds to an actinic light of 25. In subsequent experiments, actinic light values of 15 (161 μmol $m^{-2}$ $s^{-1}$) and 30 (848 μmol $m^{-2}$ $s^{-1}$) were used as limiting and saturating light respectively.

Dark respiration rates measured as $O_2$ consumption in the dark seem extremely high given that Nielsen (1995) reported that they are communly 10-20% of maximum photosynthetic evolution. It is possible that during this experiment, cells were not too healthy.

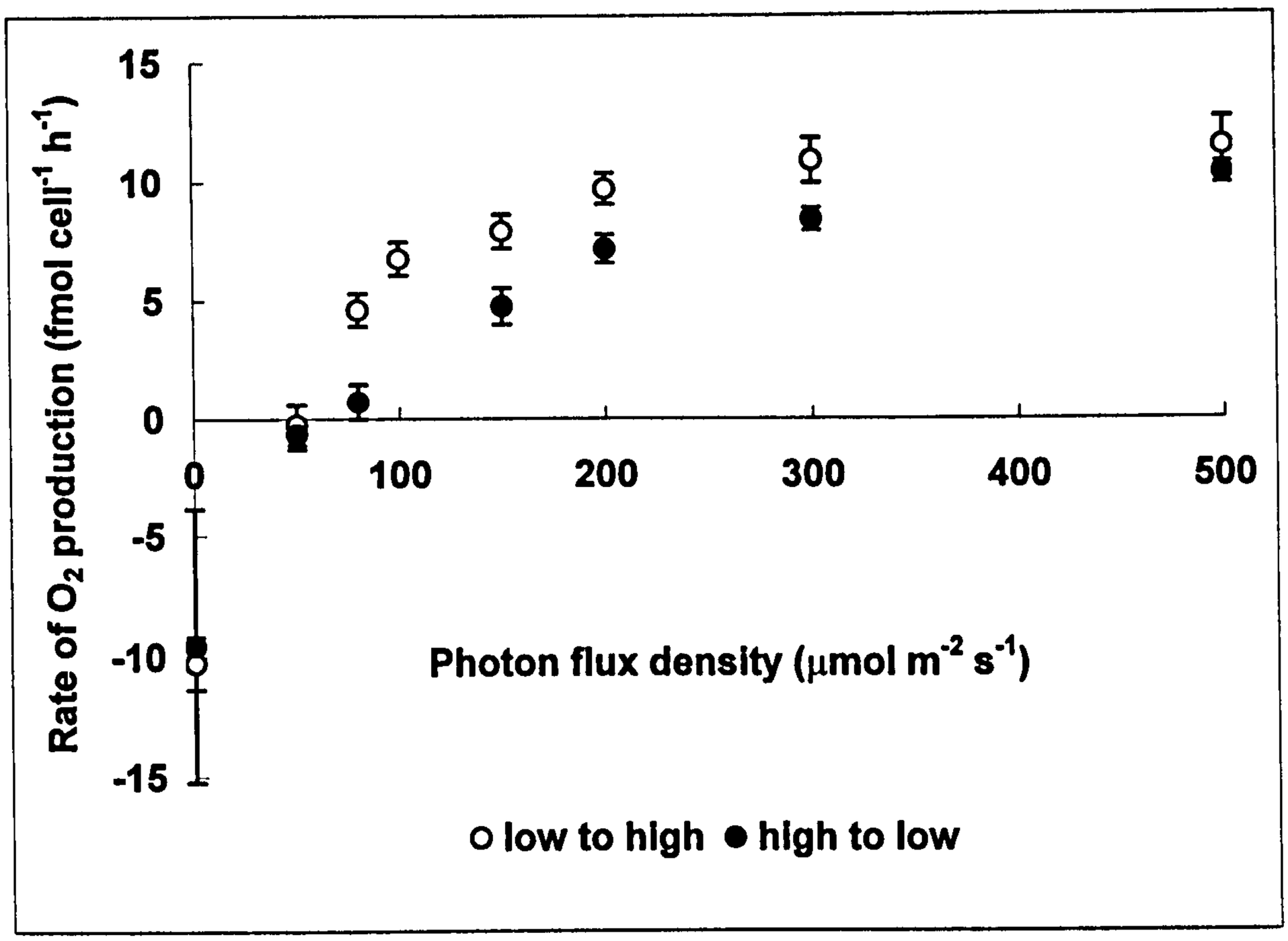



Figure 4.6 : The effect of different PFD on the rate of $O_2$ production of *E. huxleyi*, grown and incubated at 2 mM DIC. Measurements were performed by exposing the cells to either low to high or high to low PFD.

Data represent means ± SE, n = 5-7.

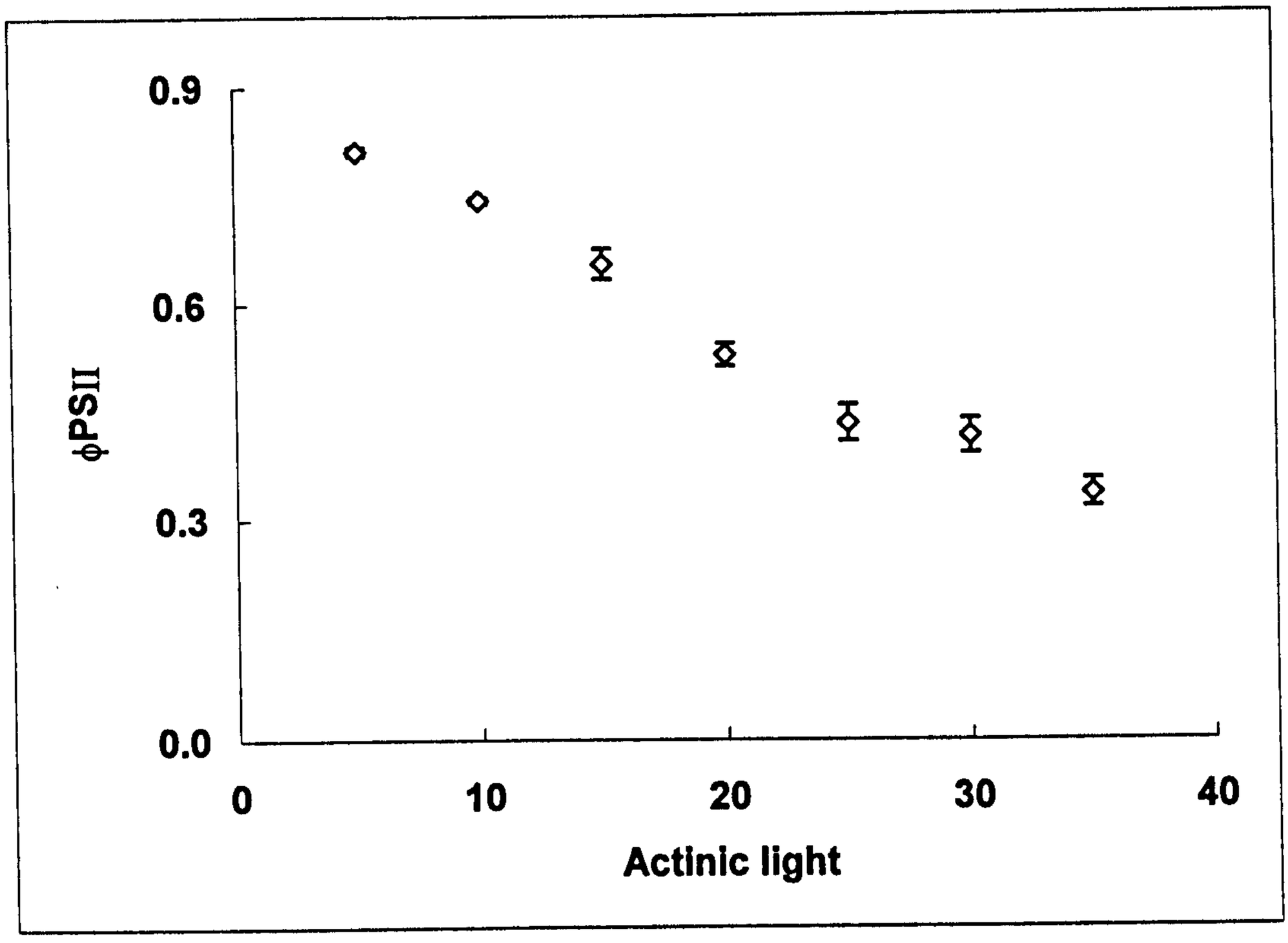



Figure 4.7 : The effect of different PFD on the quantum efficiency of PSII of *E. huxleyi*, grown and incubated at 2 mM DIC. Measurements were performed by exposing the cells to increasing PFD.

Data represent means ± SE, n = 3.

*The effect of light and carbon on the rate of $O_2$ production :*

Figure 4.8 shows that the rate of $O_2$ production is higher at 2 than 0.5 mM DIC whether the cells are incubated at the limiting or saturating PFD of 80 or 500 μmol $m^{-2}$ $s^{-1}$. These cells were grown at 2 mM DIC. In *E. huxleyi*, photosynthesis can therefore be carbon limited at both low and high light.

Figure 4.9 shows the effect of the addition of 500 μM SITS on the rate of $O_2$ production of *E. huxleyi* at different PFD. The PI curves have separated plateaux and distinct initial slopes. Here again, at all PFD, the rate of net photosynthesis is higher at 2 mM DIC than when the carbon supply is limited by SITS, a specific inhibitor of bicarbonate transport (see Chapter 3 for details). Hence, 2 mM DIC limits the photosynthetic rate of *E. huxleyi* at high and low PFD. The control values are those used in figure 4.6.

Figure 4.10 demonstrates that the same results are obtained when cells of *E. huxleyi* are incubated using a sequential experiment at different PFD and DIC concentrations. This sequential experiment comprises four phases :

- A = incubation at a PFD of 500 μmol $m^{-2}$ $s^{-1}$ and 2 mM DIC
- B = reduction of the light to 80 μmol $m^{-2}$ $s^{-1}$
- C = injection of 500 μmol of SITS
- D = increase of the PFD back to 500 μmol $m^{-2}$ $s^{-1}$

The addition of SITS reduced the $O_2$ production rate at both low and high light compared to the control values. T-tests between rates at 2 mM DIC and those with SITS addition give a p value of 0.013 and 0.014 for high and low PFD

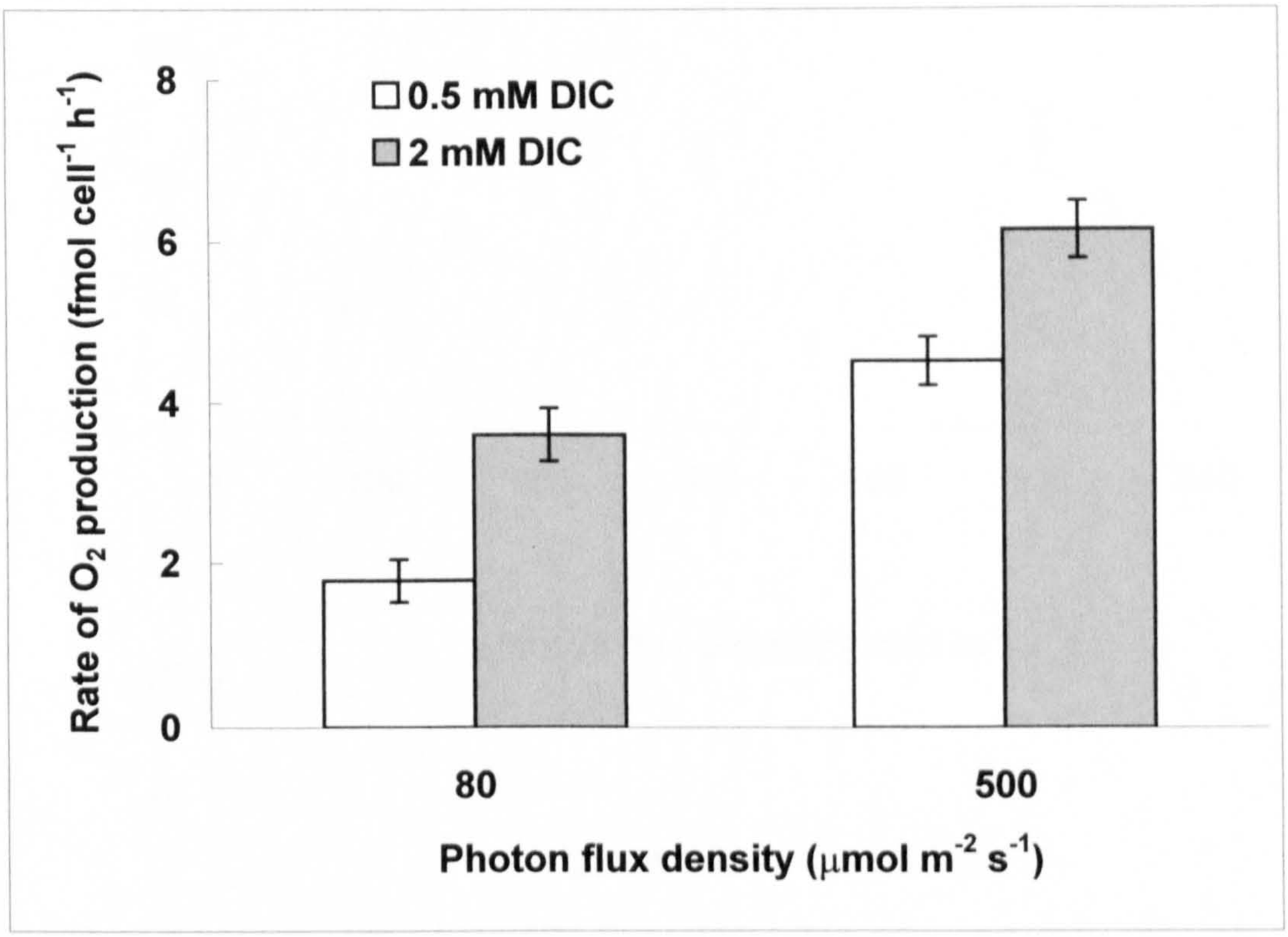



Figure 4.8 : The effect of different DIC concentrations on the rate of $O_2$ production of *E. huxleyi* grown at 2 mM DIC and incubated at a PFD of either 80 or 500 μmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 4-6.

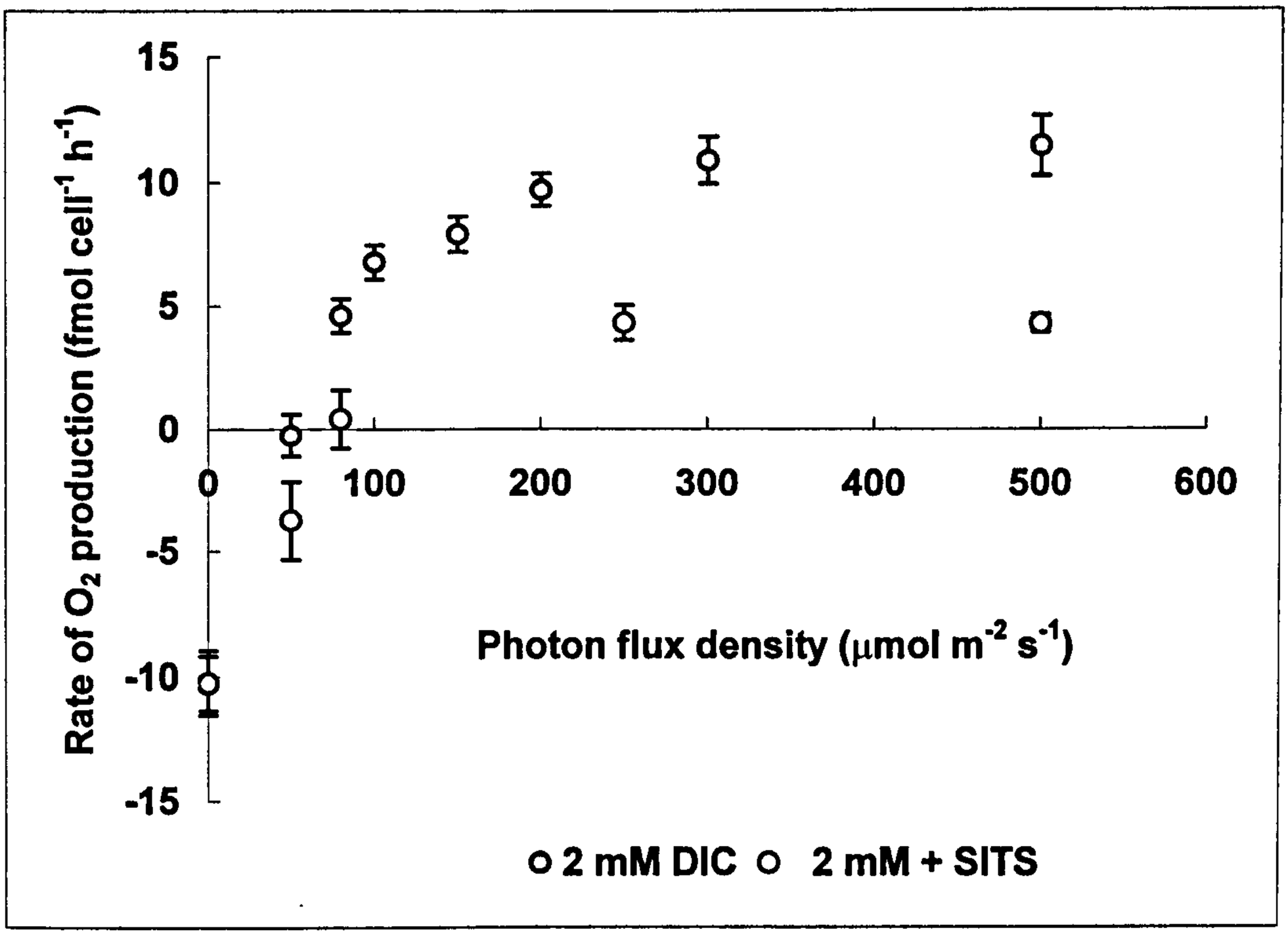



Figure 4.9 : The rate of $O_2$ production of *E. huxleyi* at different PFD with or without 500 μM of SITS. Data represent means ± SE, n = 5-7 for control and n = 4-9 for SITS treatment.

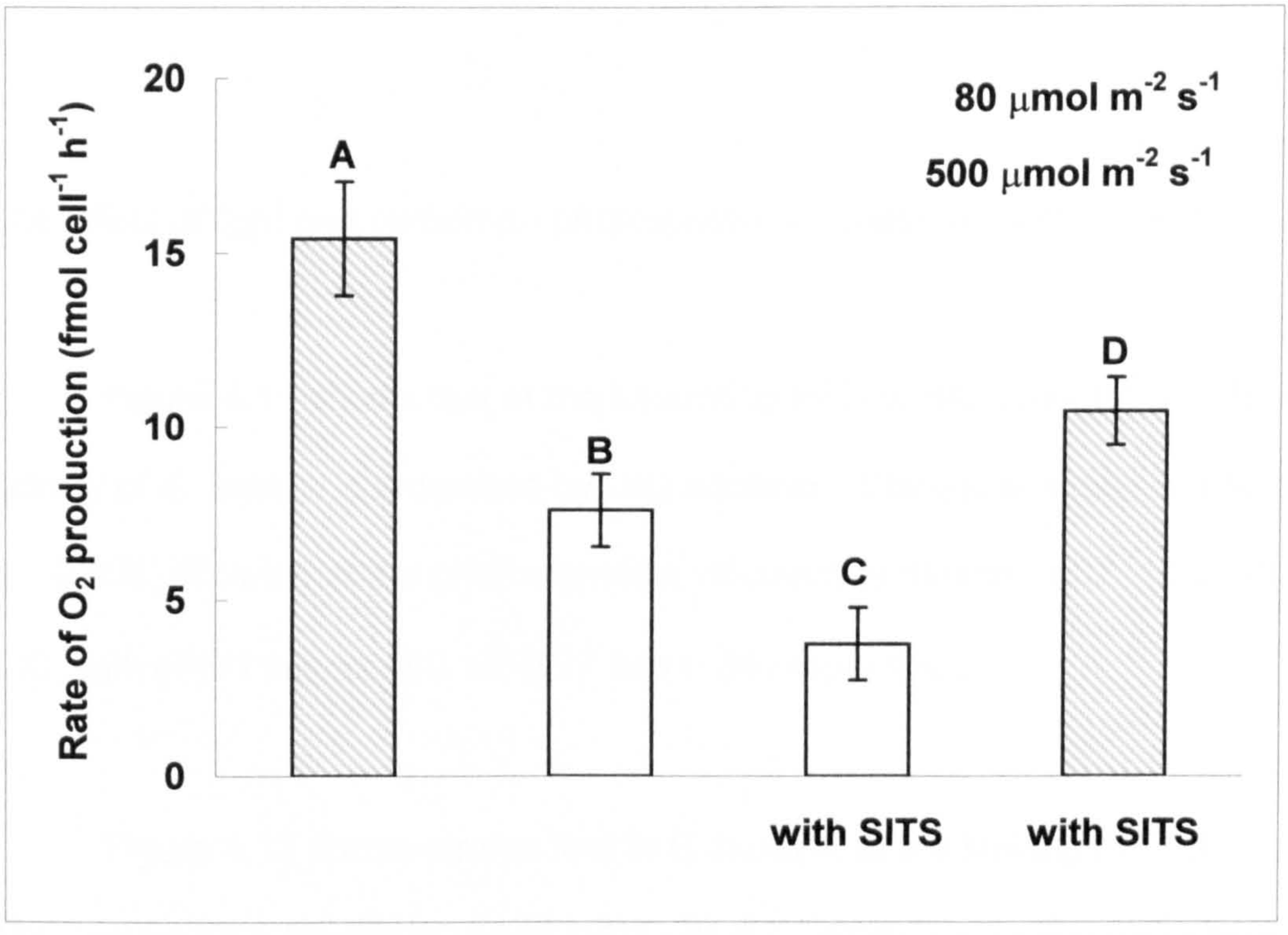



Figure 4.10 : A sequential experiment showing the effect of 500 μmol of SITS on the photosynthetic rate of *E. huxleyi* grown and incubated at 2 mM DIC and exposed to the saturating and limiting PFD of 500 and 80 μmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 11.

respectively. This result confirms that at saturating as well as at limiting PFD, the photosynthetic rate of *E. huxleyi* is carbon dependent.

*The effect of light and carbon on photosynthesis measured with the FMS :*

Figure 4.11 shows that at the saturating PFD of 848 μmol $m^{-2}$ $s^{-1}$, the PSII activity of *E. huxleyi* is enhanced by DIC addition. Statistical analysis (t-test : $p < 0.003$) shows that the photochemical efficiency is different at 2, 10 or 20 mM DIC, with ϕPSII values of 0.16, 0.27 and 0.34 respectively.

Figure 4.12 demonstrates that in *E. huxleyi*, at the limiting PFD of 161 μmol $m^{-2}$ $s^{-1}$, 2 mM DIC limits PSII activity. ΦPSII value increases from 0.55 to 0.65 between 2 and 10 mM DIC (t-test : $p = 0.001$). Although ϕPSII seems to rise from 0.65 to 0.69 between 10 and 20 mM DIC, this is not a statistically significant difference (t-test : $p = 0.053$).

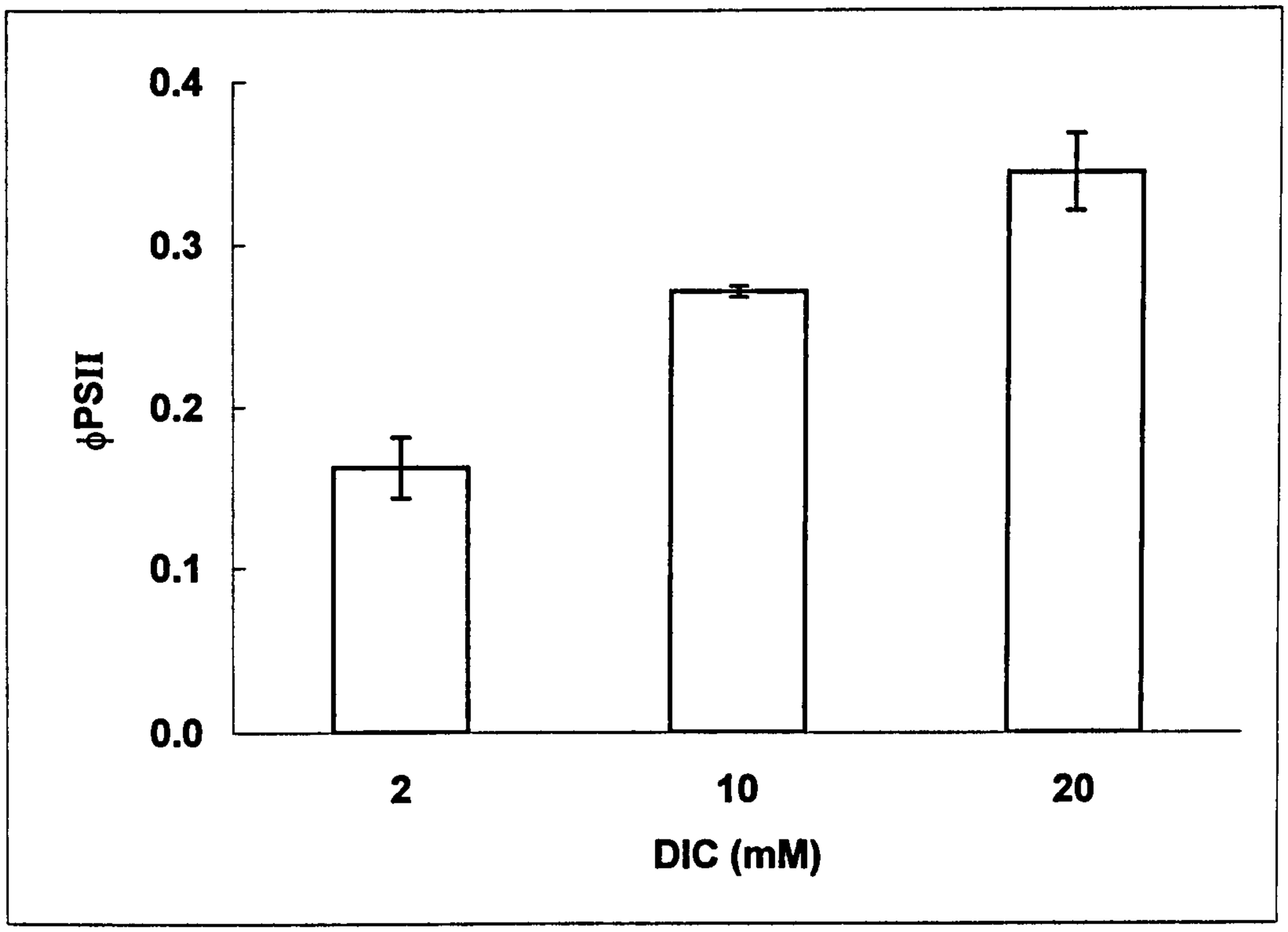



Figure 4.11 : The effect of different DIC concentrations on the PSII activity of *E. huxleyi* at the saturating PFD of 848 μmol $m^{-2}$ $s^{-1}$. PSII activity is expressed as the quantum efficiency of PSII (ϕPSII).

Data represent means ± SE, n = 4.

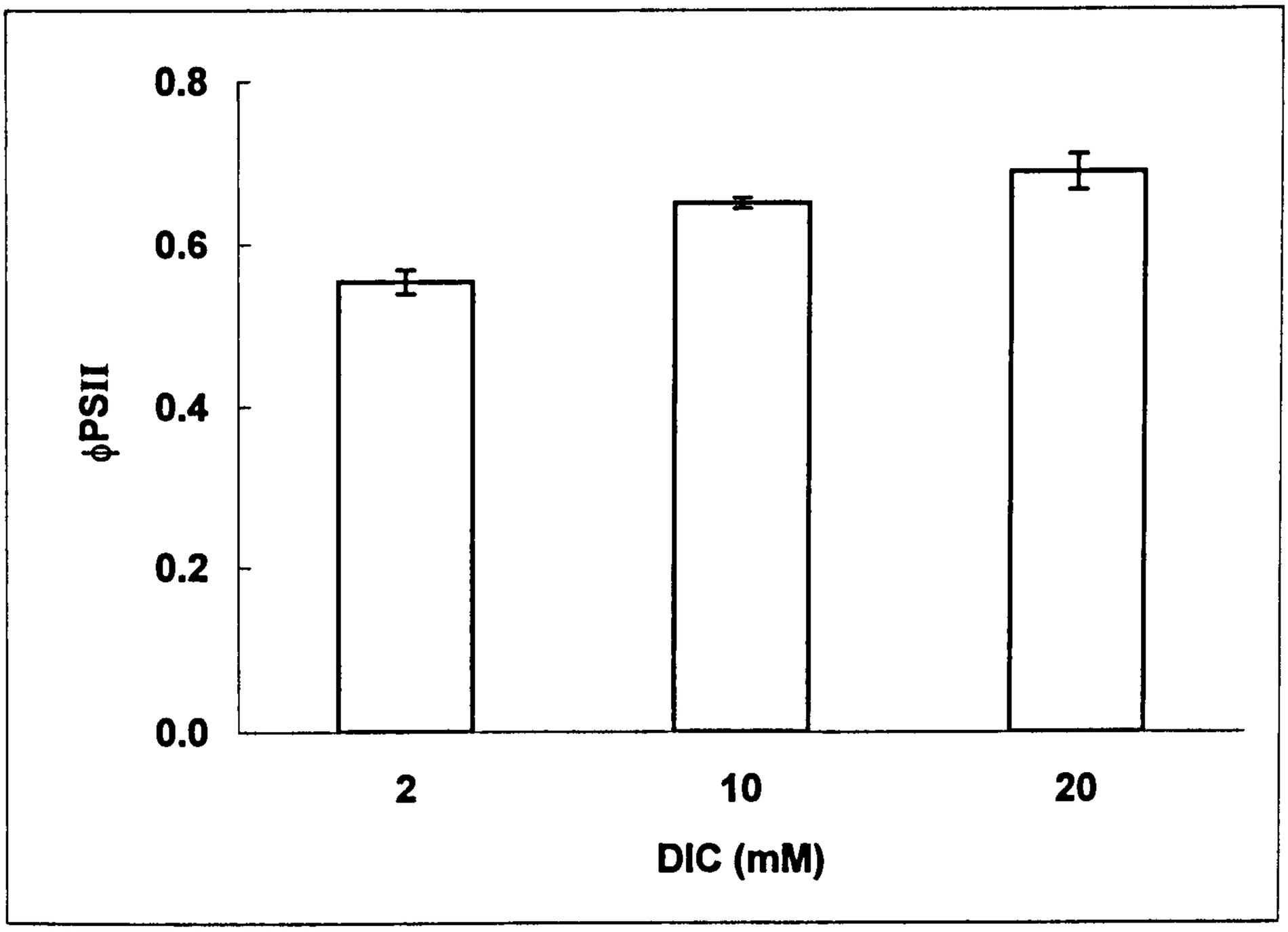



Figure 4.12 : The effect of different DIC concentrations on the PSII activity of *E. huxleyi* at the limiting PFD of 161 μmol $m^{-2}$ $s^{-1}$. PSII activity is expressed as the quantum efficiency of PSII (ϕPSII).

Data represent means ± SE, n = 4.

Figure 4.13 shows the effect of different DIC concentrations on the non-photochemical quenching (qNP) of cells of *E. huxleyi* exposed to the limiting and saturating PFD of 161 and 848 μmol $m^{-2}$ $s^{-1}$ respectively. The t-test analysis confirms that qNP is lower at 10 than 2 mM DIC, at both low and high PFD, with p-values of 0.001 and 0.005 respectively. Although the same pattern seems to occur between 10 and 20 mM, this is not a significant difference at low light, with t-tests giving p-values of 0.11 and 0.05 respectively.

Similarly, figure 4.14 shows that non-photochemical quenching, measured as NPQ, is reduced by DIC addition at both limiting and saturating PFD. Again, t-test analysis establishes that, at 161and 848 μmol $m^{-2}$ $s^{-1}$, NPQ values are significantly different between 2 and10 mM DIC ($p < 0.009$), but not between 10 and 20 mM ($p = 0.16$ and 0.15 respectively).

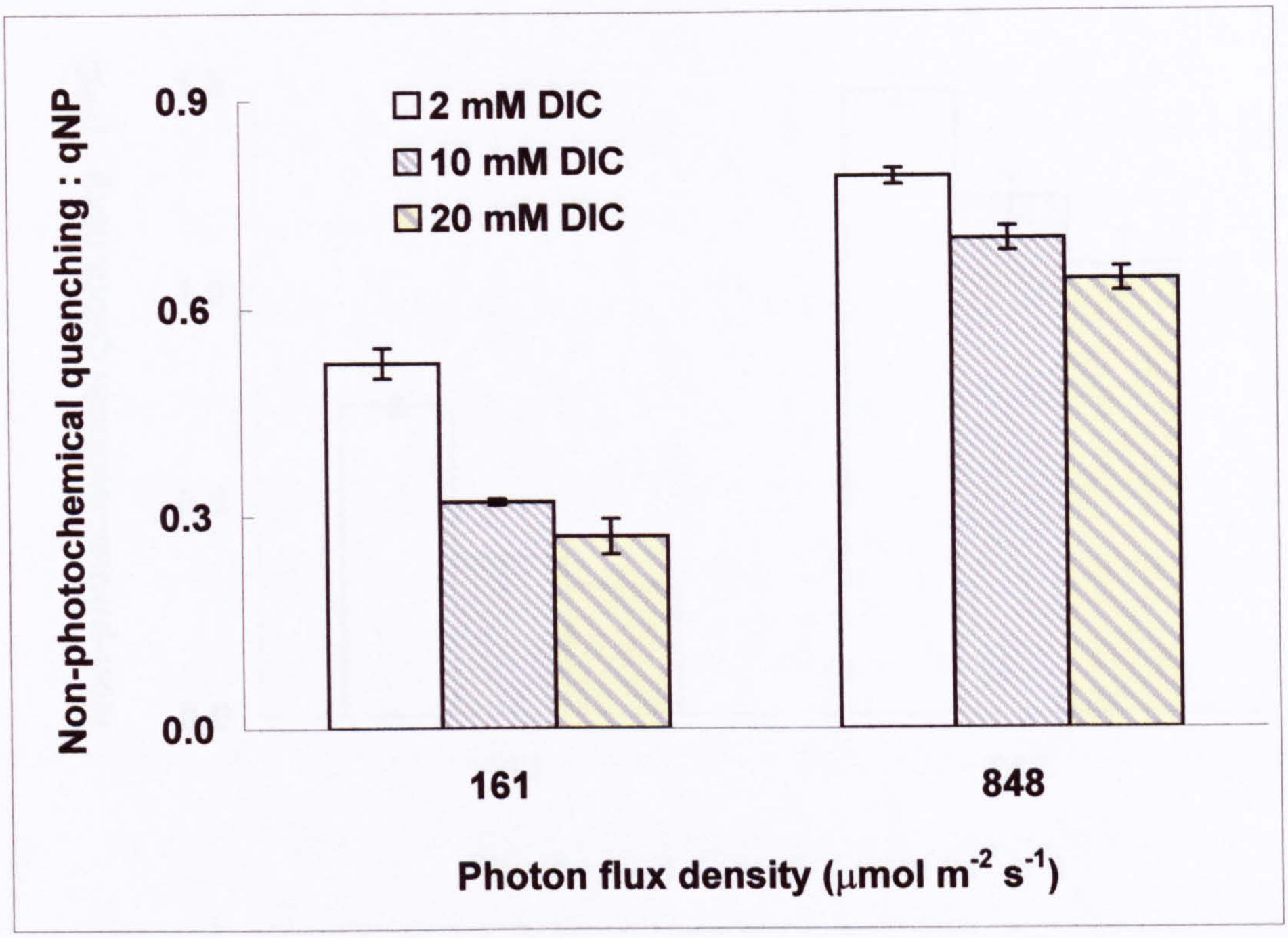



Figure 4.13 : The effect of different DIC concentrations on the non-photochemical quenching (qNP) of cells of *E. huxleyi* exposed to the PFD of 161 and 848 μmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 4.

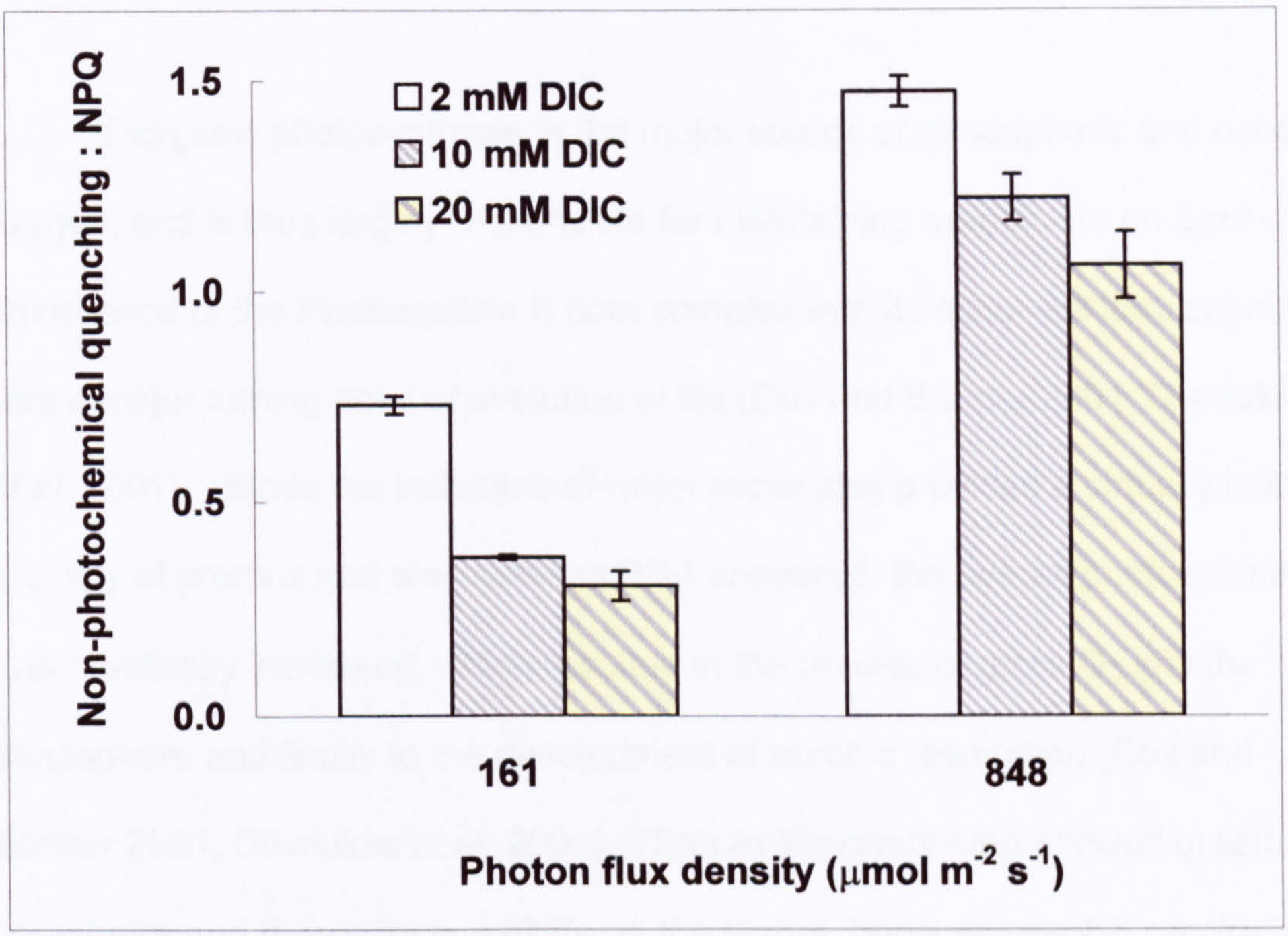



Figure 4.14 : The effect of different DIC concentrations on the non-photochemical quenching (NPQ) of cells of *E. huxleyi* exposed to the PFD of 161 and 848 μmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 4.

## Discussion :

Oxygenic photosynthesis is the major source of atmospheric and oceanic oxygen, and is thus largely responsible for maintaining aerobic life on Earth. The emergence of the Photosystem II core complex with its water-splitting capability was a major turning point of evolution of life (Cox and Bonner 2001, Dismukes *et al.* 2001). Since the reduction of water molecules provides a virtually infinite quantity of protons and electrons, as PSII appeared, the overall photosynthetic rate drastically increased, which resulted in the release of more $O_2$ into the atmosphere and finally to the development of aerobic respiration (Cox and Bonner 2001, Dismukes *et al.* 2001). This evolutionary step allowed greater complexity and diversification of life on the planet, because aerobic respiration yields 18 times more ATP than does anaerobic respiration for each hexose sugar molecule used (Dismukes *et al.* 2001).

Strikingly, as already mentioned in the introduction, the PSII core complex is highly conserved amongst all photosynthetic $O_2$-evolving organisms (Christen *et al.* 1999, Larkum and Howe 1997). This lack of diversity is correlated with the $4e^-/4H^+$ arrangement of the water splitting complex (WSC) necessary for coupled oxidation reaction, which is as Dismukes *et al.* (2001) affirm, 'thermodynamically the most challenging multielectron reaction in biology'. The evolution of pigment molecules into a strong oxidant was required for the emergence of this WSC. Chl. a was thus created by modification of other chlorophyll pigment molecules. The exact origin of chl.a does, however, remain under much debate.

One of the most puzzling questions concerning the development of oxygenic photosynthesis is what was the precursor of the WSC of PSII and how did this structure evolve to produce the universal four-$e^-$ substrate oxidation process (Dismukes *et al.* 2001)?

Upon illumination, the excitation of the chl.a of the PSII reaction centre by a photon leads to charge separation. In order to fill this $e^-$ hole, an intermediate donor molecule, which is a tyrosine amino acid, denoted $Y_z$, quickly reduces the photochemically oxidised $P_{680}$. This $Y_z$ molecule, which becomes strongly electrophilic, regains an $e^-$ through the oxidation of the manganese (Mn) cluster that is associated with the WSC. In fact, each PSII reaction centre contains four Mn atoms, located at the D1 protein (Debus 1992). Mn is a peculiar atom in that it has a wide range of oxidation states (+2 to +7). This characteristic is an essential component of the light reaction, since each transition state S of the WSC of PSII is correlated to a different oxidation form of the Mn cluster (Falkowski and Raven 1997). According to the KoK model, the WSC can take five different oxidation states, denoted $S_0$ to $S_4$ (Kok *et al.* 1970). Each absorption of a photon by $P_{680}$ provokes the concomitant removal from the WSC of one $e^-$ and the increase in positive charge of the Mn cluster. When the $S_4$ state is finally reached, two water molecules are oxidised, which generates four $e^-$, the remaining $H^+$ and one molecule of $O_2$. This causes the return of the WSC to the $S_0$ state and of the Mn cluster to its original oxidised form. The cycle is then ready to repeat itself. As illustrated on figure 4.15, this association between the different oxidised forms of the Mn cluster and the five states of the

WSC allows the transfer of four electrons to the PSII reaction centre for each $O_2$ molecule produced.

The specific structure of the WSC enables the electrons to be 'pulled' from the water by the photochemical oxidation of $P_{680}$. This mechanism by which water splitting provides the $e^-$ required for the reduction of the chl. a of the PSII reaction centre is widely accepted (Lawlor 2001, Falkowski and Raven 1997). Nevertheless, Warburg (1964) proposed the so-called protolyte theory, whereby photosynthetic $O_2$ evolution derived from the reduction of $CO_2$ and not of $H_2O$ molecules. Warburg and Krippahl (1960, 1958) discovered that isolated broken chloroplasts of *Chlorella*, that were not fixing $CO_2$, depended on carbon to perform the Hill reaction. In other words, the electron flow in the Hill reaction was stimulated by the addition of carbon. These authors were thus the first to establish that bicarbonate ions play an important role in the light reaction. This is now referred to as 'the bicarbonate effect'. A stimulation of the Hill reaction does not however indicate whether the bicarbonate binding site is localised on the electron donor or acceptor side of PSII.

Although Stemler and Govindjee (1974) first identified the carbon effect on the electron donor side of PSII, this localisation was considered incorrect for years, as many studies subsequently demonstrated a strong bicarbonate effect on the electron acceptor side of PSII (Eaton-Rye and Govindjee 1988, van Rensen *et al.* 1988, Jursinic and Stemler 1986, Vermaas and Govindjee 1982, Khanna *et al.* 1981, Jursinic *et al.* 1976, Govindjee *et al.* 1976, Wydrzynski and Govindjee 1975). It was first discovered that formate addition reduced the

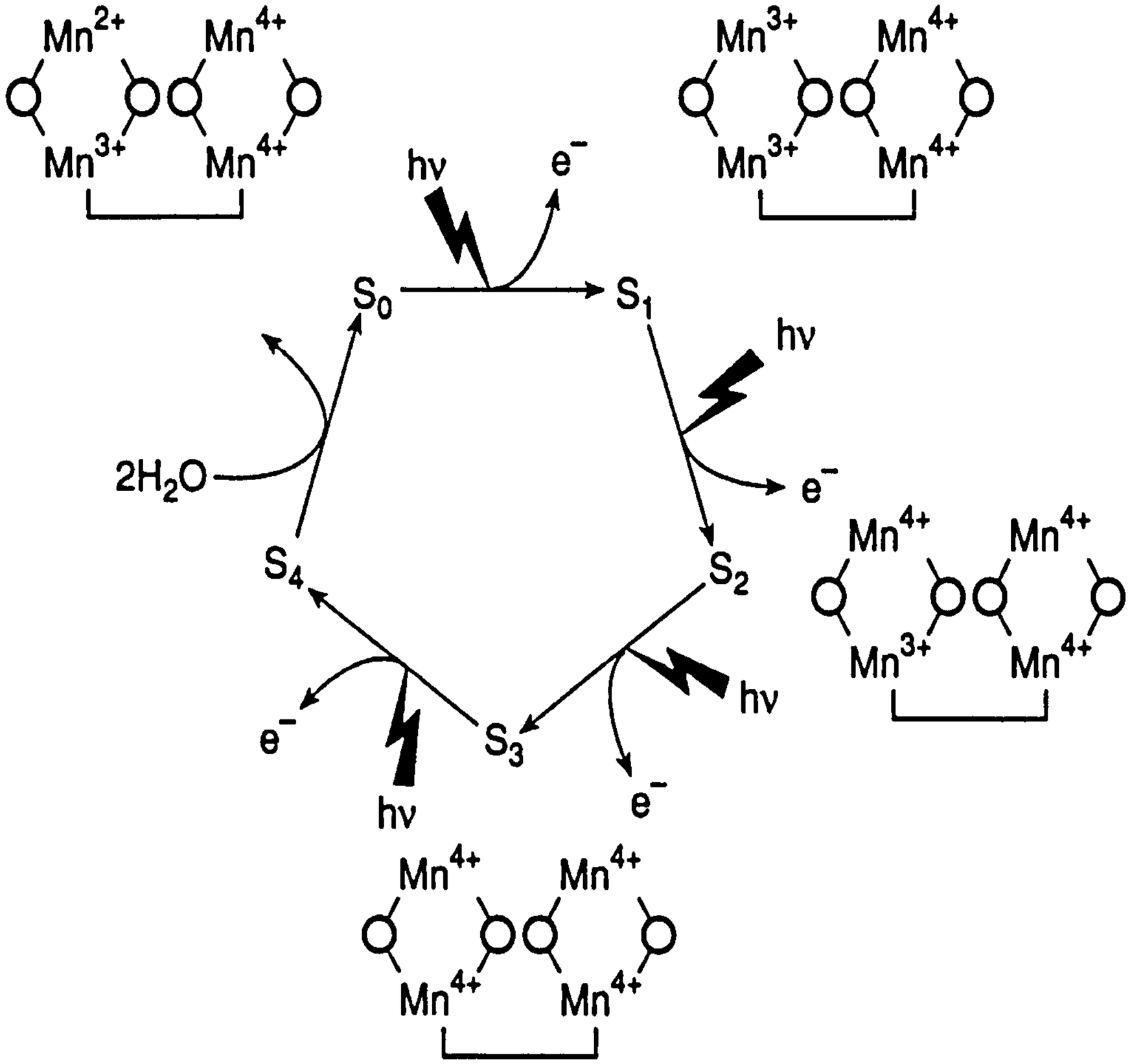



Figure 4.15 : Diagram showing the relationship between the five states of the water splitting complex of the PSII reaction centre and the different oxidised forms of its Mn cluster (from Falkowski and Raven 1997).

electron flow on the acceptor side of PSII and that bicarbonate ions removed this inhibition. Furthermore, Wydrzynski and Govindjee (1975) showed that in the absence of bicarbonate ions, the primary electron acceptor $Q_A$ couldn't be reoxidised. In fact, bicarbonate ions are liganded to the non-heme iron of the $Q_A$-Fe-$Q_B$ complex (Petrouleas *et al.* 1992, Hienerwadel and Berthomieu 1995). The bicarbonate binding site is localised on or close the so-called herbicide-binding protein, to which 3'(3,4-dichlorophenyl)1',1'-dimethyl urea (DCMU) binds competitively with $Q_A$ (Hutchinson *et al.* 1996,Vermass and Govindjee 1982).

It is clear that the bicarbonate effect is located on the acceptor side of PSII. Although Stemler and coworkers continued to provide evidence of a bicarbonate effect on the donor side of PSII (Stemler 1980, Jursinic and Stemler 1988, Jursinic and Stemler 1992), this result was not widely accepted because the potency of the bicarbonate effect was greater on the acceptor side (Govindjee *et al.* 1997). In recent years, numerous studies have confirmed this donor side effect and have demonstrated that bicarbonate ions enhance the light-induced rate of charge separation of the WSC and increase stabilisation at high temperature (Klimov *et al.* 1997, Stemler and Lavergne 1997, Allakhverdiev *et al.* 1997 and Klimov *et al.* 1995a and 1995b). Nevertheless, direct spectroscopic evidence of localisation and characterisation is still missing (Dismukes *at al.* 2001), but because this topic has engendered much controversy since Warburg's discovery, progress has been fast. So, the next decade should shed more light on the exact involvement of bicarbonate during the light reaction of oxygenic photosynthesis.

Already, Zouni *et al.* (2001) have demonstrated that bicarbonate ions constitute an integral part of the WSC by contributing to the efficient functioning of the Mn cluster. This supports the theory of Klimov and co-workers that bicarbonate was the transitional $e^-$ donor in bacteria before PSII as we know it appeared in cyanobacteria (for review see Dismukes *et al.* 2001). In fact, $O_2$ molecules are more easily produced from bicarbonate ions than from pure water, since the former reaction has a free energy 34 % lower than the latter (Dismukes *et al.* 2001). In addition, the bicarbonate concentration of seawater was estimated to have been 30 to 30,000 times greater in the pre-oxygenic photosynthesis era than in contemporary times (Dismukes *et al.* 2001). Mn was also at least two times more concentrated in the Archaeanocean, and its species composition was different. Indeed, high bicarbonate concentration modifies the speciation of Mn ions in solution, so that Achaean ocean was composed of $HCO_3^-$-Mn cluster and not of free $Mn^{2+}$ ions as at present. All those considerations lead Klimov *et al.* (1995b) to suggest that bicarbonate thermodynamically enhances the oxidation of Mn by PSII, by converting non-oxidisable $Mn^{2+}_{aq}$ ions into more easily oxidised forms. This dimangagnese-bicarbonate cluster may have been a determining evolutionary step for the transition from anoxygenic to oxygenic photosynthesis and the bicarbonate effect on the donor side of PSII that is observed nowadays may simply be a relic of past evolution.

More could be said on this rapidly progressing topic, but what is clear is that it is now widely accepted that bicarbonate affects both the donor and the acceptor side of PSII, and by doing so enhances the photosynthetic electron

transport rate. The present study also provides evidence of the bicarbonate effect in *E. huxleyi*. Indeed, the PSII activity of this coccolithophore is enhanced by DIC addition at both saturating and limiting light.

This DIC effect on the photochemistry was expected at saturating PFD. According to the Blackman theory, when photons are in excess, carbon is the only limiting factor in photosynthesis. As mentioned in the introduction to this Chapter, at saturating PFD, the rate of $CO_2$ fixation by Rubisco can limit photosynthetic ETR. Thus, the bicarbonate effect, observed at saturating PFD, is a substrate effect. In other words, a rise in the external DIC concentration leads to an increase in the concentration of $CO_2$ available to Rubisco. Since, in high light, the photochemistry produces excess ATP and $NADPH^+$ molecules, there is no shortage of electrochemical energy to fix this extra carbon. This in turn causes a feedback response that stimulates PSII activity. At saturating PFD, PSII activity is therefore increased by DIC addition, because of a substrate effect.

This finding is supported by the data obtained in experiments where non-photochemical quenching was measured at different DIC concentrations in *E. huxleyi*. These data show that at saturating PFD, bicarbonate addition reduces $q_N$. Since $q_N$ occurs when photochemical quenching is not sufficient to quench excess excitation, this result confirms that DIC addition enables excess amounts of electrochemical energy to be safely dealt with. Thus, at high PFD, because of its substrate effect, bicarbonate promotes ATP and $NADPH^+$

utilisation and consequently diminishes the need for protective mechanisms of photoinhibition, such as $q_I$.

In contrast, the bicarbonate effect observed in *E. huxleyi* at limiting PFD does not follow the law of the limiting factors that were applied to photosynthesis by Blackman (1905), but rather Bose's law, (for review, see Rabinowitch 1951). Bose (1924) postulated that two factors can simultaneously limit photosynthesis. The present study has established that PSII activity is enhanced by DIC addition when light is limited. At low PFD, carbon as a substrate for Rubisco, is in excess. In other words, the photochemistry does not produce enough ATP and $NADPH^+$ molecules, to optimise the use of $CO_2$ during the dark reaction. Thus, increasing the concentration of substrate for Rubisco would not promote photosynthesis, because at low light, photosynthesis is limited by the rate of electron transport. This ultimately depends on the yield of absorbed photons. At limiting light, bicarbonate addition allows the light reaction to use the excitation energy derived from a limiting number of photons more efficiently. Since at ambient temperature, PSI fluorescence is negligible, all the fluorescence measured using the FMS originates from PSII. This shows that the bicarbonate effect observed at low light in *E. huxleyi* is located at the PSII reaction centres. This technique does not however indicate whether this is a donor or an acceptor side effect, but in view of all the numerous studies previously mentioned, it is likely that bicarbonate ions affect both sides of PSII in *E. huxleyi*. It is conceivable that, when photosynthesis is light saturated, the bicarbonate effect on PSII, as well as the substrate effect on Rubisco takes place simultaneously, but when the FMS is used on the whole organism, the bicarbonate effect on PSII

can only be observed at low light. The bicarbonate effect on PSII can only be identified at high light if isolated thylakoids lacking Rubisco are used.

Furthermore, at limiting PFD, bicarbonate also reduces non-photochemical quenching. It was mentioned in the introduction to this Chapter that one form of $q_N$, denoted $q_E$, occurs independently of the presence or absence of excess light to provide photoprotection (Osmond *et al.* 1999). So, the bicarbonate effect on PSII decreases the need for photoprotection.

$O_2$ production measurements have also confirmed that the photosynthetic rate of *E. huxleyi* is limited at low PFD by both light and carbon. When the rate of bicarbonate uptake was reduced by the addition of SITS, a specific inhibitor of the anion exchanger AE1(see Chapter 3 for details), the PI curves showed separate saturation plateaux and distinct initial slopes. The photosynthetic rate of *E. huxleyi* is therefore lower when the entry of carbon is restricted at both low and high PFD. This, which corresponds to a 'Bose type' curve, confirms that bicarbonate promotes photosynthesis at low as well as high PFD. Furthermore, dark and light reactions can be visualised with a PI curve. In fact, $P_{max}$ is determined by the rate of $CO_2$ fixation by the Calvin cycle, while the initial slope of a PI curve depends on the photochemical reactions. Hence, the PI curve data presented here show that both the rate of carbon reduction during the dark reaction and the rate of electron transport of the light reaction can be DIC limited in *E. huxleyi*.

The same result was obtained in the sequential experiment where cells were first incubated at 2 mM DIC at high and low light and then with 500 μM SITS at low and high light (figure 4.5). Additional measurements of $O_2$ production at 0.5 and 2 mM DIC confirm that photosynthesis is enhanced by DIC at both saturating and limiting PFD. So, all the above data, obtained using a well-established technique, confirm that photosynthesis can be limited by the DIC concentration of the medium when light is not saturating. This result is in agreement with that of Nielsen (1995) who also showed that $O_2$ production is increased by elevated DIC concentrations at low and high pfd. It is however the first time that induced fluorescence is used in such study.

To sum up, this work has demonstrated that bicarbonate ions enhance the photosynthetic rate of *E. huxleyi*. This effect can be divided into two mechanisms : a bicarbonate effect on PSII and a substrate effect on Rubisco. Limiting and saturating PFD enable the detection of the former and the latter respectively. Moreover, these two carbon effects reduce the need for non-photochemical quenching processes such as photoprotective and photoinhibitory mechanisms. This involvement of DIC in the light reaction, explains why the photosynthetic rate of *E. huxleyi* can be carbon limited at low light. Hence, the well-established Blackman theory, which states that photosynthesis can only be limited by one factor at the time, is not applicable in the interaction between photosynthesis, light and DIC in *E. huxleyi*. The bicarbonate effect, which was first discovered by Warburg in the early 1960s, is usually investigated merely in a biochemical context. The present study represents the first attempt to place this bicarbonate effect in a physiological perspective.

Furthermore, since *E. huxleyi* is of great importance for the geochemical cycle of the planet (see Chapter 1 for details), the physiological consequences of the bicarbonate effect in this alga can also be viewed in an ecological context. Coccolithophores are widespread in all oceans (with the exception of polar waters). Their abundance in temperate regions is correlated with the beginning of the stratification of the water column in the spring (Brand 1994, Hulburt *et al.* 1960), which causes the formation of large blooms, observable by satellite imagery. Because of the back-scattering properties of their coccoliths (see Chapter 5), they are microorganisms that are visible from space. An increase in the outbreaks of large-scale blooms of *E. huxleyi* have been observed in recent years and is associated with the change in coastal seawater nutrient composition resulting from river input (Lancelot *et al.* 1987) and the emergence of very warm springs, which is a consequence of global warming. Such blooms have interested climatologists, because *E. huxleyi* affects the ocean-atmosphere climate through its participation in the Earth carbon cycle, by altering the heat exchange between the seawater and the atmosphere, and by contributing to the emissions of dimethyl sulphide (DMS), a major source for cloud condensation nuclei (see Chapter 1 for details).

The Industrial Revolution that started 250 years ago is characterised by an increase in deforestation and fossil fuel burning. These anthropogenic activities led to a rise of the yearly atmospheric carbon input, from 0.5 Pg in the pre-industrial era to 7 Pg nowadays (Raven and Falkowski 1999) thus affecting the global carbon cycle. Because the $CO_2$ concentration of surface seawater co-varies with atmospheric $CO_2$ levels, the last 2.5 centuries

have also been marked by an increase of the surface seawater $CO_2$ partial pressure ($_pCO_2$) (Broeker and Peng 1982). Rising $_pCO_2$ causes an increase in the overall DIC concentration of seawater. It is noteworthy that the predicted rise in $_pCO_2$ will not change the fact that seawater is supersaturated with respect to aragonite and calcite. Alkalinity will therefore remain constant (Stumm and Morgan 1981). Rising $_pCO_2$ will, however, affect the pH of seawater, which is predicted to decrease by 0.35 units, hence leading to a 50 % diminution in the concentration of $CO_3^{2-}$ ions (Riebesell *et al.* 2000). Since alkalinity will remain constant, the decrease in concentration of $CO_3^{2-}$ ions will be compensated by an increase in bicarbonate ion concentration. So, as long as seawater remains supersaturated with respect to aragonite and calcite, rising atmospheric $CO_2$ concentration will eventually result in an increase in concentration of bicarbonate ions.

Since the doubling of the $_pCO_2$ value of 1880 is predicted by about 2030-2050 (Perry1982), the DIC concentration could rise to a level that would have physiological effect on *E. huxleyi*. Although cells of *E. huxleyi* move up and down within the water column, at any given time, only those located in surface waters receive saturating light for photosynthesis. This study has shown that DIC enrichment would not only enhance the photosynthetic rate of these cells, but also of those situated deeper within the photic zone. So, although cells are in constant movement within the mixed layer, whatever their position within the photic zone, their photosynthetic rate would be stimulated by the predicted increase in seawater DIC concentration. The productivity of the whole blooms of *E. huxleyi* may thus be enhanced by DIC enrichment. In the future, the effect of

*E. huxleyi*'s blooms on the global carbon may be more pronounced than initially expected, because of the stimulation of PSII by bicarbonate at very low light.

To sum up, with the predicted rise in the DIC concentration of seawater, the bicarbonate effect on PSII may have profound implications for the photochemical events of the light reaction of photosynthesis in *E. huxleyi*, and may result in increased productivity.

# Chapter 5 :

# The function of coccoliths

## Introduction :

Metal ions perform numerous vital functions in living organisms. One of the major tasks of cells is to sustain an optimum intracellular concentration of useful metal ions and remove toxic ones. Metal traps are intra and extracellular isolation compartments where unwanted metals are accumulated and can undergo transformation processes such as alkylation, dealkylation, oxidation or reduction (Westbroek *et al.* 1985).

The process by which dissolved ions or molecules are converted into solid minerals by living organisms is called biomineralisation. Biominerals can have an amorphous or a crystalline form. An amorphous mineral lacks order over distances longer than 10 Å (Mann *et al.* 1983), whereas a crystal shows symmetry. Mineral formation is possible because the standard free energy ($G^0$) of a solid is lower than that of its constituents in solution. Since the lowering of $G^0$ upon solid formation is positively correlated with mineral stability, and crystal formation entails a greater reduction of $G^0$, crystals are thermodynamically favoured and thus more stable than amorphous minerals (de Vrind-de Jong and de Vrind 1997). One the other hand, amorphous forms are kinetically favoured because less energy is required for their formation. Living organisms use several mechanisms to lower the energy barrier of crystal formation and thus kinetically to enhance crystallisation. Whether the result is amorphous or crystalline, precipitation only occurs under conditions of supersaturation, i.e. when concentrations of constitutive ions are greater than their solubility product

(de Vring-de Jong and de Vring 1997). The transformation from a supersaturated solution into a crystal starts with the aggregation of molecules into a stable ionic cluster, also called nuclei (Heywood and Mann 1994). If the nucleus is below a critical size it will redissolve, but above, it will grow. This critical size depends on the degree of supersaturation. Two sorts of nucleation occur. When a solution is pure, i.e. when only the crystal constituents are present, homogeneous nucleation takes place. Heterogeneous nucleation happens when a solution contains additional "foreign" particles. These non-constitutive particles can stabilize and bind the crystal nuclei by reducing the level of supersaturation necessary for the formation of crystals (de Vrind-de Jong and de Vrind 1997).

Metal ions behave differently depending on whether they are in an inorganic or an organic environment (Westbroek *et al.* 1985). Biomineralisation can be biologically induced by the creation of supersaturated conditions by the living organism. It can also be biologically controlled. Living organisms can interfere in the crystallisation process by forming specialised vesicles or organic matrices that can be used as templates (de Vrind-de Jong and de Vrind 1997). *E. huxleyi* is a good example of an organism that carries out biomineralisation. These calcium carbonate plates are produced inside the cell, and extruded to add to the coccosphere. Each coccolith is formed of 30 to 40 single-crystal calcite elements (Mann and Sparks 1988). Each unit element is made of 4 sub-unit elements : the lower or proximal shield element, which is the flattened lower element of about 0.96 μm in length; the upper or distal shield element, which is the hammer-shaped element of 0.6 μm in length; the vertical element also called

the inner tube; and finally the central element. The central element is connected to proximal and distal shields by the inner tube as shown on figure 5.1.

Calcification is carried out in the Coccolith Production Compartment (CPC) (Westbroek *et al.* 1989), which is formed by fusion of Golgi-derived vesicles (Van Emburg *et al.* 1986). The CPC is closely apposed to the nucleus and the reticular body. The biochemical mechanisms supporting calcification in *E. huxleyi* remain largely unknown. Corstjens *et al.* (1998) have identified in *E. huxleyi* a protein named GPA because its high percentage of glutamic acid, proline and alanine. Because of the high calcium ion binding capacity of this protein, these authors have suggested that it plays a role in the nucleation and regulation of calcite crystal formation or in the transport of calcium into the calcifying vesicle. The exact role of the GPA protein is still not clear and further investigations are necessary. On the other hand, the coccolith polysaccharide has been extensively studied and is known to be involved in the induction, inhibition and stabilisation of calcite crystals in *E. huxleyi*. The coccolith polysaccharide of *E. huxleyi* is a very complex polymer made of 13 different monosaccharides with non-polar and acidic properties (Fichtinger-Schepman *et al.* 1981). The coccolith polysaccharide is present in the Golgi apparatus, the CPC, the organic base-plate and as a thin layer lining coccolith elements (Corstjens *et al.* 1998). Borman *et al.* (1982) have shown that in vitro crystallisation of calcium carbonate is impossible in supersaturated conditions when the polysaccharide is absent. All these considerations suggest a strong link between calcification and the coccolith polysaccharide in *E. huxleyi*.

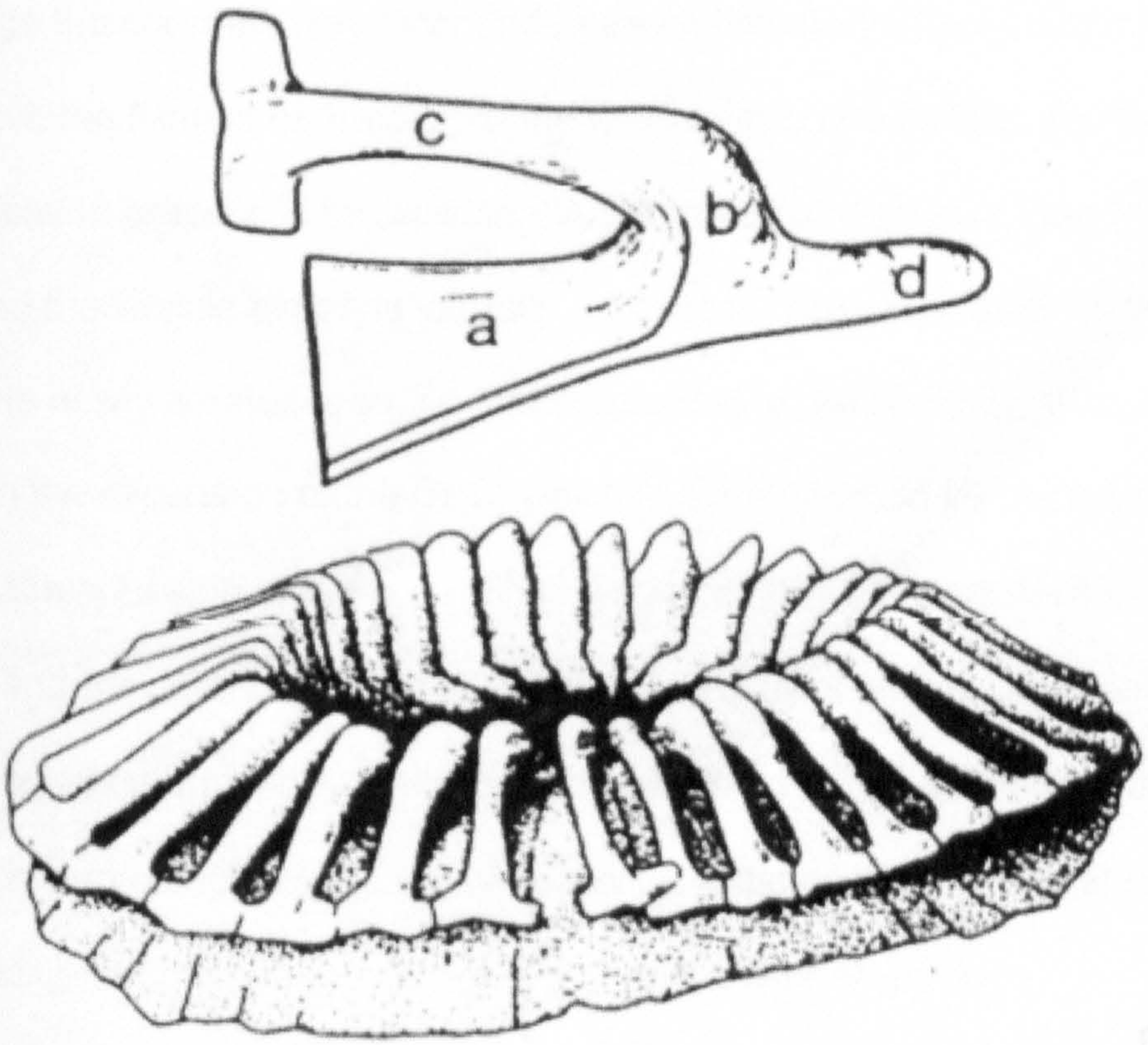



Figure 5.1 : Drawing of a calcite crystal of a coccolith of *E. huxleyi* (from Westbroek *et al.* 1985). The central element (d) is connected to proximal (a) and distal (c) shield by the inner tube (b).

Several studies have described the steps of coccolith formation in *E. huxleyi* (de Vring-de Jong *et al.* 1994, Westbroek 1991, Westbroek *et al.* 1985). A brief summary of the main stages is given below. Lumen-localised coccolith polysaccharide attaches threads to the CPC membrane. Because at this stage the coccolith polysaccharide has an inhibitory effect on crystal formation, the fixation of threads on the CPC membrane inhibits the crystallisation process in the vesicle. An organic base-plate is then formed in the CPC and the vesicle expands laterally. Coccolith polysaccharide threads cannot reach the newly created area, so that crystallisation takes place. Crystals grow owing to the expansion of the CPC, which is accomplished by the cytoskeleton. Finally, the adhesion of the coccolith polysaccharide to the crystal stops further growth and provides protection to the newly formed element. The characteristic and elaborate morphology of the crystal elements as well as the intricate formation and complex structure of coccoliths indicate strong cellular control of the crystallisation process in *E. huxleyi* (Corstjens *et al.* 1998).

The strong cellular control over coccoliths formation observed in this alga suggests that calcification is an essential process for the alga. This study examined the effect of magnesium, calcium and light on coccolith morphology, photosynthesis and calcification in order to try to determine the function of coccoliths in *E. huxleyi*.

Magnesium was chosen because of its unexpected presence in the crystal elements of coccoliths. In fact, 3-4 % of $Mg^{2+}$ is crystallised in the calcite crystal elements even though $Mg^{2+}$inhibits calcite and favours aragonite formation

(Fallini 1994, Fork 1974, Paasche 1966). Magnesian-calcite is unstable, so the presence of $Mg^{2+}$ in calcite crystals of coccoliths suggests that there is a strong biological influence to stabilise the system. Why is *E. huxleyi* incorporating $Mg^{2+}$ with calcite? What is the advantage of magnesium crystallisation for the alga? Fernández-Diaz *et al.* (1996) and Mitsuguchi *et al.* (1996) have demonstrated that $Mg^{2+}$ modifies the morphology of calcite precipitates. $Mg^{2+}$ may then participate in creating the special shape of coccolith crystal elements. Commonly, calcite crystals have a rhombohedral shape, which means that they are hexagonal calcite prisms (de Vring-de Jong and de Vring 1997). The coccoliths of *E. huxleyi* are called heterococcoliths (Paasche 1964) because their calcite elements are much more elaborate with rounded crystal faces. Moreover, in the early stage of development, the newly nucleated proto-coccolith ring is made of calcite rhombohedrons (de Vring-de Jong *et al.* 1994) and only later will it become a heterococcolith. $Mg^{2+}$ may interfere in this transformation of calcite from rhombohedral to heterococcoliths. The detailed structure of coccoliths of *E. huxleyi* cells grown in artificial seawater medium containing different $Mg^{2+}$ concentrations was therefore examined.

Originally, calcium was selected for the current study because of its major role in calcification. Weinberg-Flax (2000) demonstrated that the growth rate of *E. huxleyi* was highest in seawater medium containing 5 mM calcium and was lower at 10 mM, and because the ambient seawater concentration lowers the growth rate of this coccolithophore, in ambient seawater calcium could be regarded as toxic to this alga. This suggests that calcium toxicity control may have been the original reason for calcification. Kazmierczak *et al.* (1985)

hypothesised that the massive increase in the calcium concentration of seawater during the Precambrian could explain the sudden emergence of biocalcification. The present study investigated the short-term effect of different calcium concentrations on photosynthesis and calcification rates and its long-term effect on the structure of coccoliths in *E. huxleyi* in order to test this idea.

Calcification in *E. huxleyi* is also strongly influenced by light. No or little calcification takes place in the dark (Paasche 1966, Paasche 1964). Because cells on a day-light regime divide in the dark, de Vring-de Jong and de Vring (1997) argued that the lack of calcification in the dark is related to cell division. During mitosis the nucleus cannot appose itself to the CPC, which is a crucial step in the early stage of coccolith formation. However, this alone cannot explain why *E. huxleyi* does not calcify in the dark because as de Vring-de Jong and de Vring (1997) noticed, calcification remains inhibited once mitosis is finished and the nucleus again becomes fully operational. Hence, calcification may not be dark inhibited, but rather light enhanced. To determine if the presence of coccoliths was related to light, photosynthetic rates were measured at different PFD in coccolith-bearing and coccolith-less cells of *E. huxleyi*.

## Materials and methods :

### *Growth media :*

A calcifying strain of *E. huxleyi* (Lohmann) Hay and Mohler (PCC.B 11) provided by the Marine Biological Association Culture Collection (Plymouth) was cultured as described in Chapter 2. Cells were grown in artificial seawater media containing the following different calcium or magnesium concentrations :

Calcium (mM) : 0 / 1 / 2 / 4 / 10 / 20

Magnesium (mM) : 0 / 5 / 15 / 29 / 58 / 87 / 116

For concentrations higher than those of seawater (i.e. calcium : 20 mM and magnesium : 87 and 116 mM) the addition of calcium or magnesium was performed by sterile filtration after autoclaving.

Cells were sub-cultured 3 times in the different calcium or magnesium concentrations before treated for electron microscopy.

*Sample preparation for single coccolith micrographs :*

This protocol is described in Chapter 2. The photographs were taken by Miss E. Loste and Dr. F. Meldrum (School of Chemistry, Queen Mary University of London, UK)

Plate 5.1 shows the manner in which the measurements of length and width of the distal shield element of coccoliths of *E. huxleyi* were taken.

*Sample preparation for micrographs of intact coccoliths:*

Chapter 2 details the protocol used for sample preparation of intact coccoliths.

*Measurement of calcification and photosynthetic rates at different DIC concentrations :*

Cells were grown in Harrison's medium (appendix 1) as described in Chapter 2. After centrifugation for 10 minutes at 150 X g, cells were resuspended in artificial seawater medium containing different calcium concentrations (0-50 mM). The artificial seawater medium had been previously buffered with 25 mM Bicine and its pH adjusted to 8.2. Calcification and photosynthesis rates were measured as described in Chapter 2.

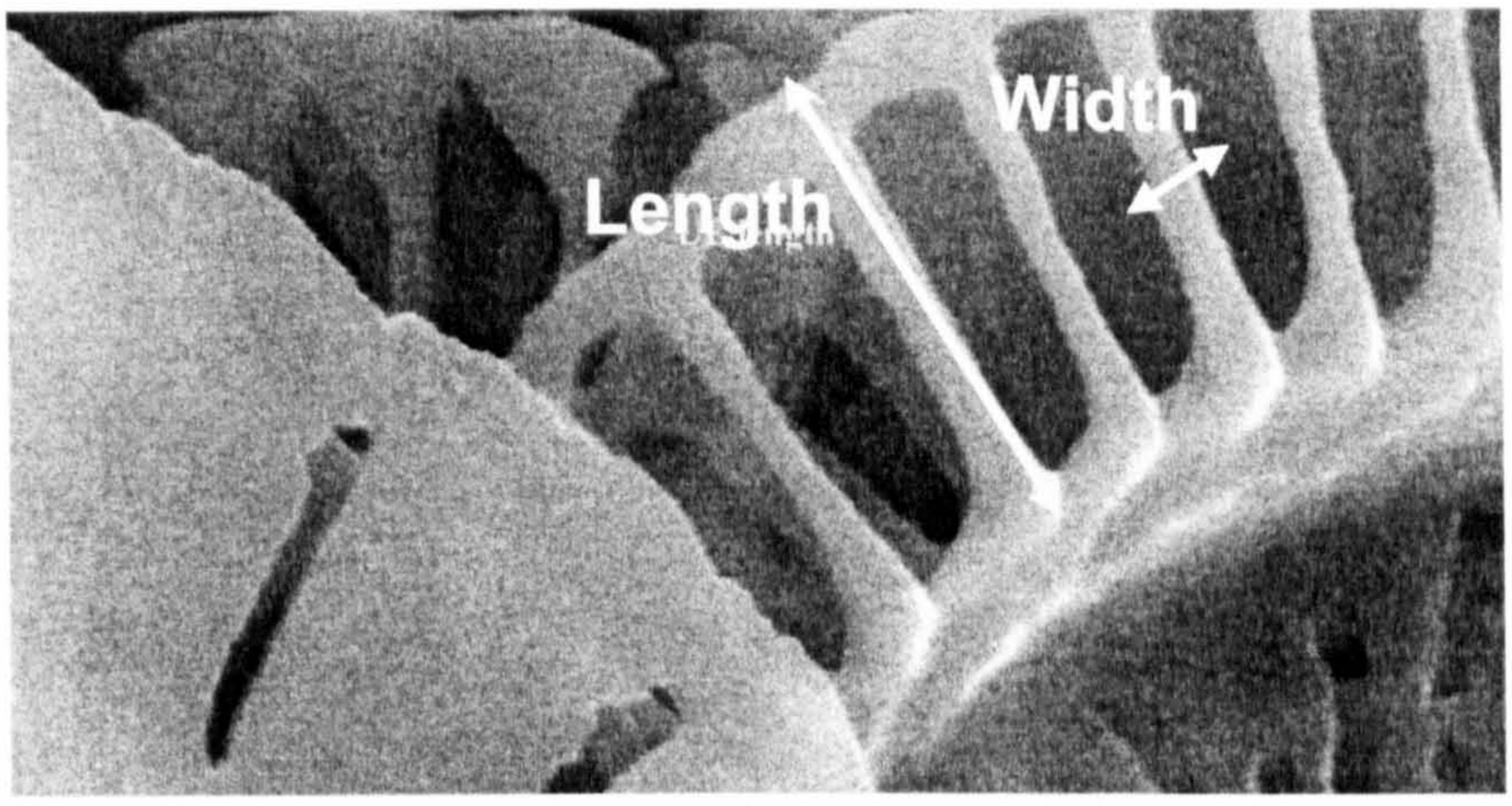



Plate 5.1 : An example of the manner in which the measurements of length and width of the distal shield element of coccoliths of *E. huxleyi* were taken.

Results :

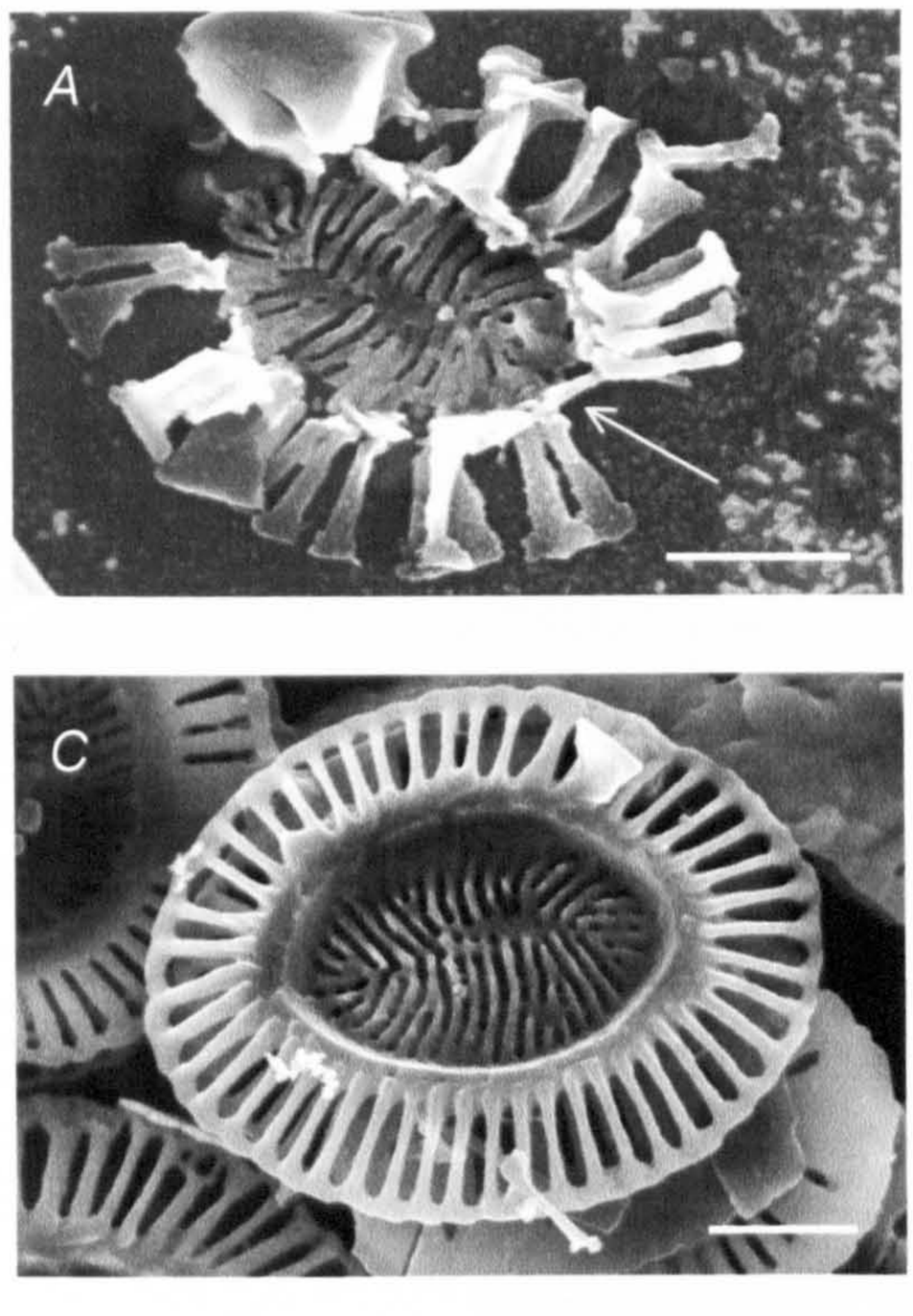
*Coccolith morphology with different magnesium concentrations :*

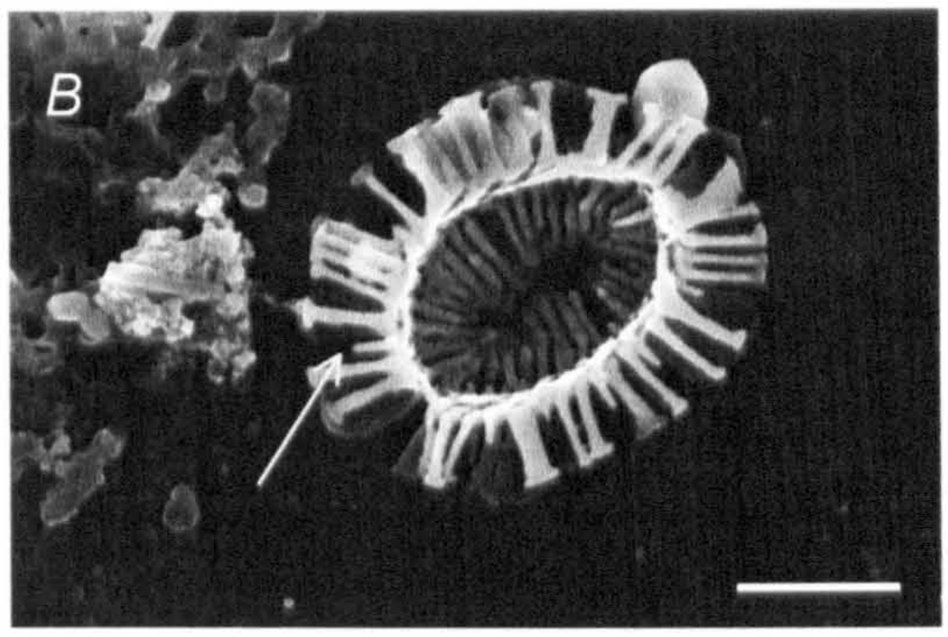
Plates 5.2A to 5.2G show coccoliths of cells of *E. huxleyi* grown in artificial seawater containing 0 / 5 / 15 / 29 / 58 / 87 and 116 mM magnesium respectively.

Very few coccoliths were present in the medium containing 116 mM $Mg^{2+}$. Coccolith rings were incomplete with wholly or partially broken distal and proximal shields. The heads of the hammer elements were also often absent. Similar features were observed at 87 mM $Mg^{2+}$, even though more calcification takes place as fewer distal shield elements were missing. However, a large number of those were still incomplete.

Cells incubated in the intermediate concentrations of 58 and 29 mM $Mg^{2+}$ all displayed normal coccoliths with perfect structures.

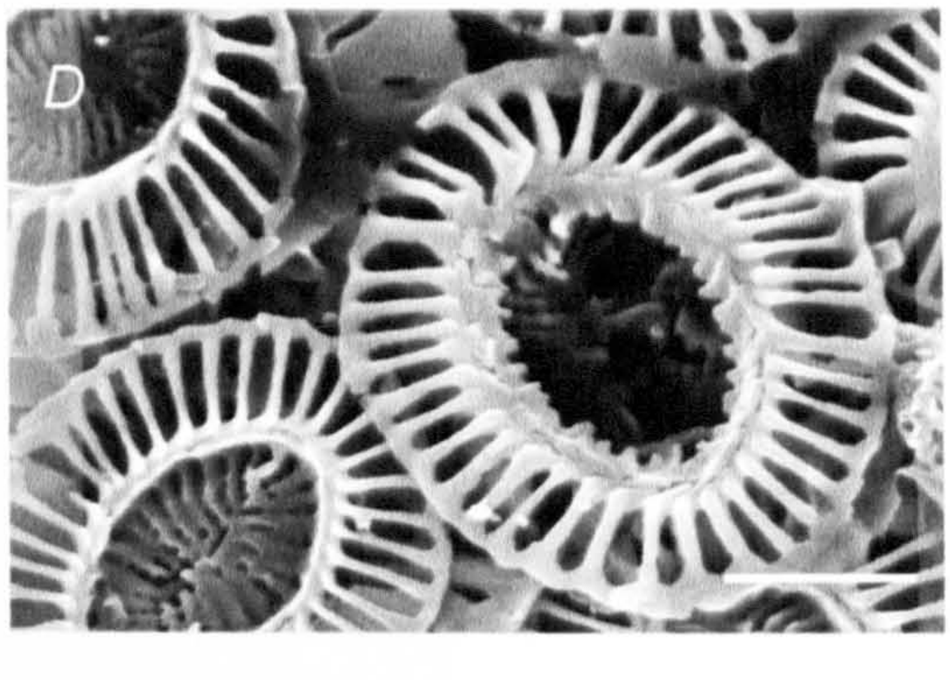
Cells incubated in low $Mg^{2+}$ concentrations also show signs of undercalcification similar to those noticed at high Mg concentrations. The distal shield elements were absent or incomplete and the hammer heads were missing. Nevertheless, calcification is less affected at low than at high $Mg^{2+}$ concentrations.



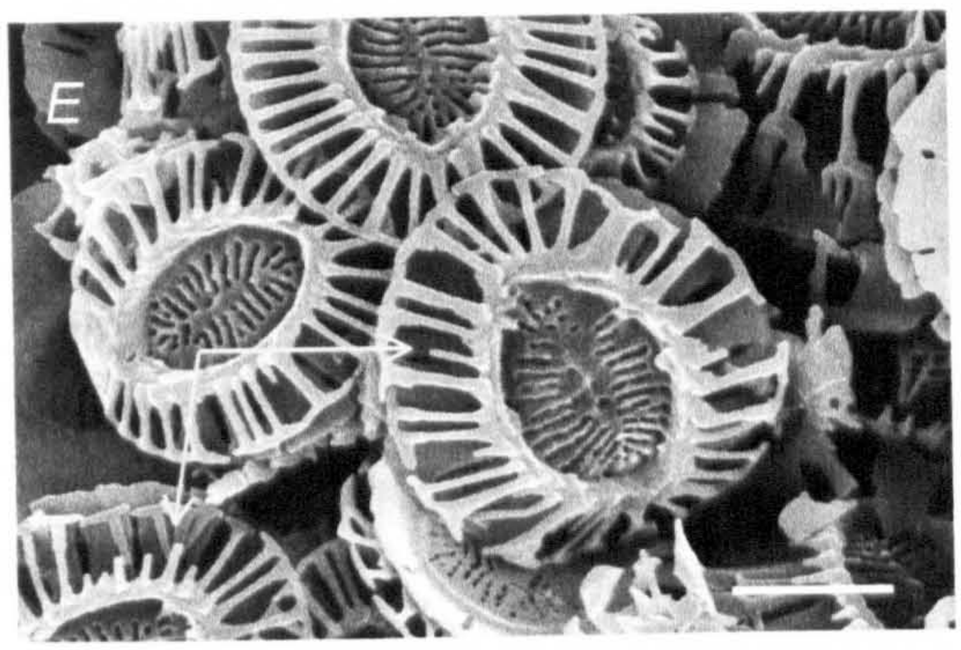



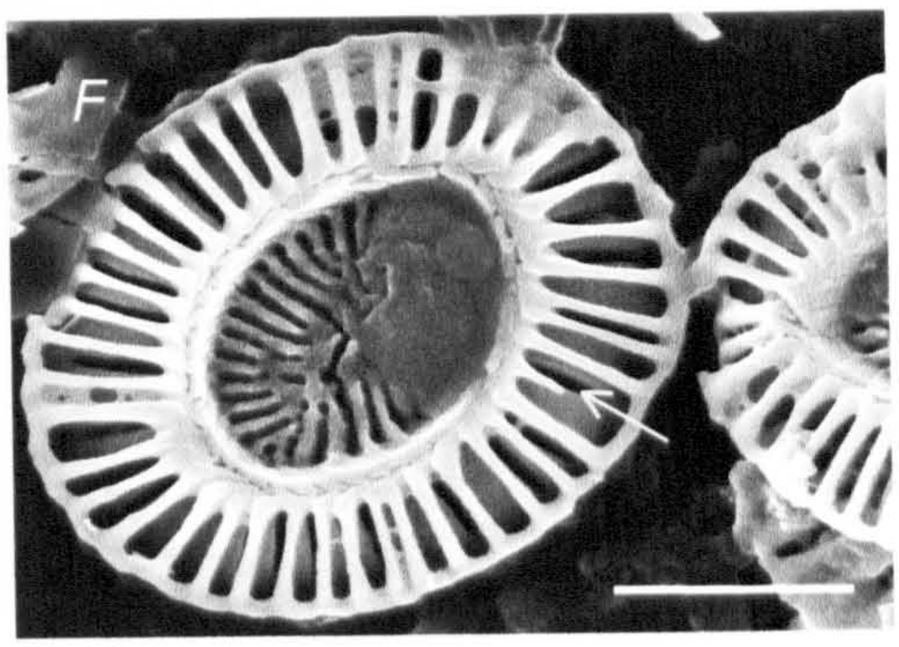


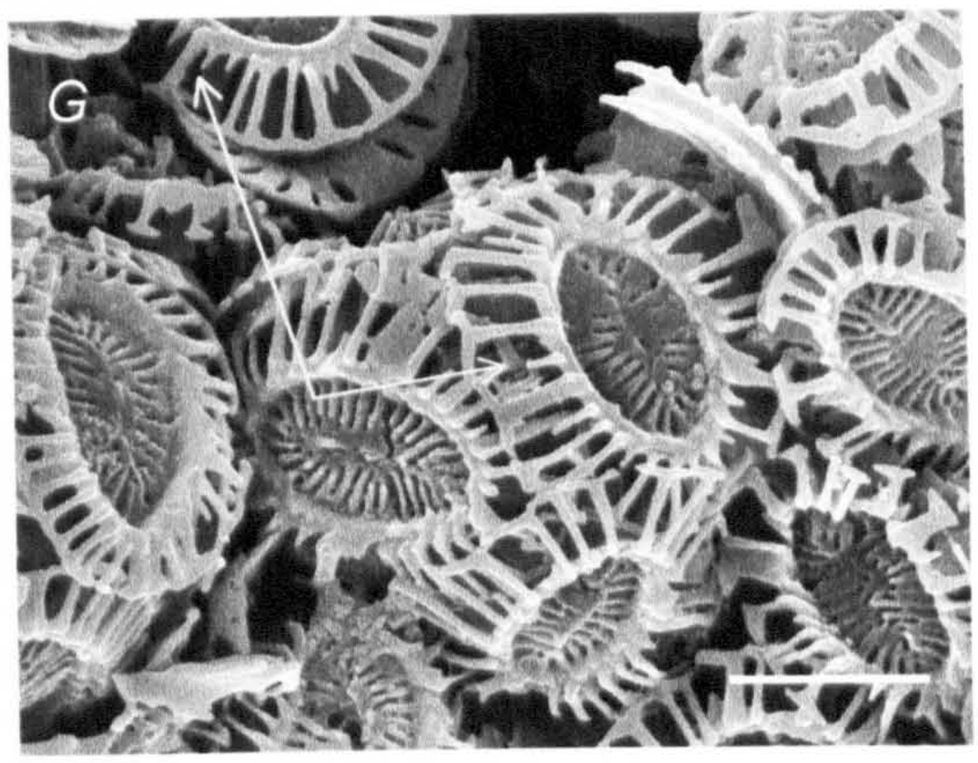





Plates 5.2A to 5.2D : The effect of different intermediate and high magnesium concentrations on the morphology of coccoliths of *E. huxleyi*. Plates A, B, C and D show coccoliths of cells grown in medium containing 116, 87, 58 and 29 mM $Mg^{2+}$ respectively. Arrows indicates examples of incomplete or missing elements. Bars = 1 μm.




Plates 5.2E to 5.2G : The effect of different low magnesium concentrations on the morphology of coccoliths of *E. huxleyi*. Plates E, F and G show coccoliths of cells grown in medium containing 15, 5 and 0 mM $Mg^{2+}$ respectively. Arrows indicate examples of incomplete or missing elements. Bars = 1 μm.

Figure 5.2 shows the effect of different $Mg^{2+}$ concentrations on the length of the distal shield elements of coccoliths for 3 batches of culture of *E. huxleyi*. In all 3 batches, the maximum length was measured in medium containing 58 mM $Mg^{2+}$, which corresponds to the ambient $Mg^{2+}$ concentration of seawater. A large variability was observed between each batch, but similar pattern (i.e. highest length at 58 mM $Mg^{2+}$) was detected in all batches.

No variation in the width of distal shield elements were measured with different $Mg^{2+}$ concentrations (data not shown).

*Coccolith morphology with different calcium concentrations :*

Plates 5.3A to 5.3F show coccoliths of cells of *E. huxleyi* grown in seawater medium containing 0 / 1 / 2 / 4 / 10 and 20 mM calcium respectively.

In calcium-free medium, no coccoliths could be seen around cells of *E. huxleyi*.

Cells grown in medium containing 1 and 2 mM calcium showed serious signs of undercalcification, with incomplete distal shield elements and split proximal shield elements. At 4 mM calcium, slightly under-calcified coccoliths were observed, but perfectly structured coccoliths without splits were seen at 10 and 20 mM calcium.

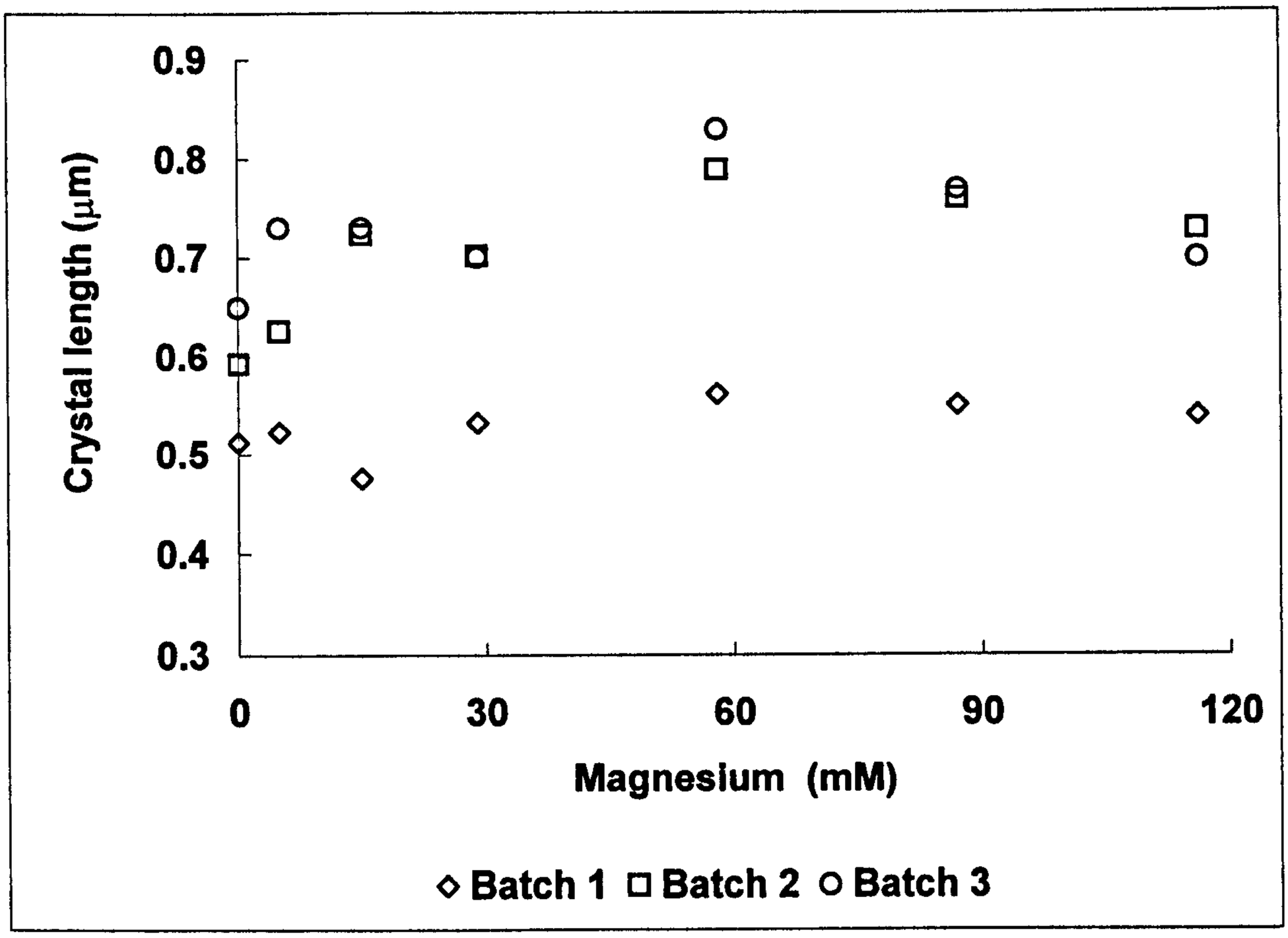
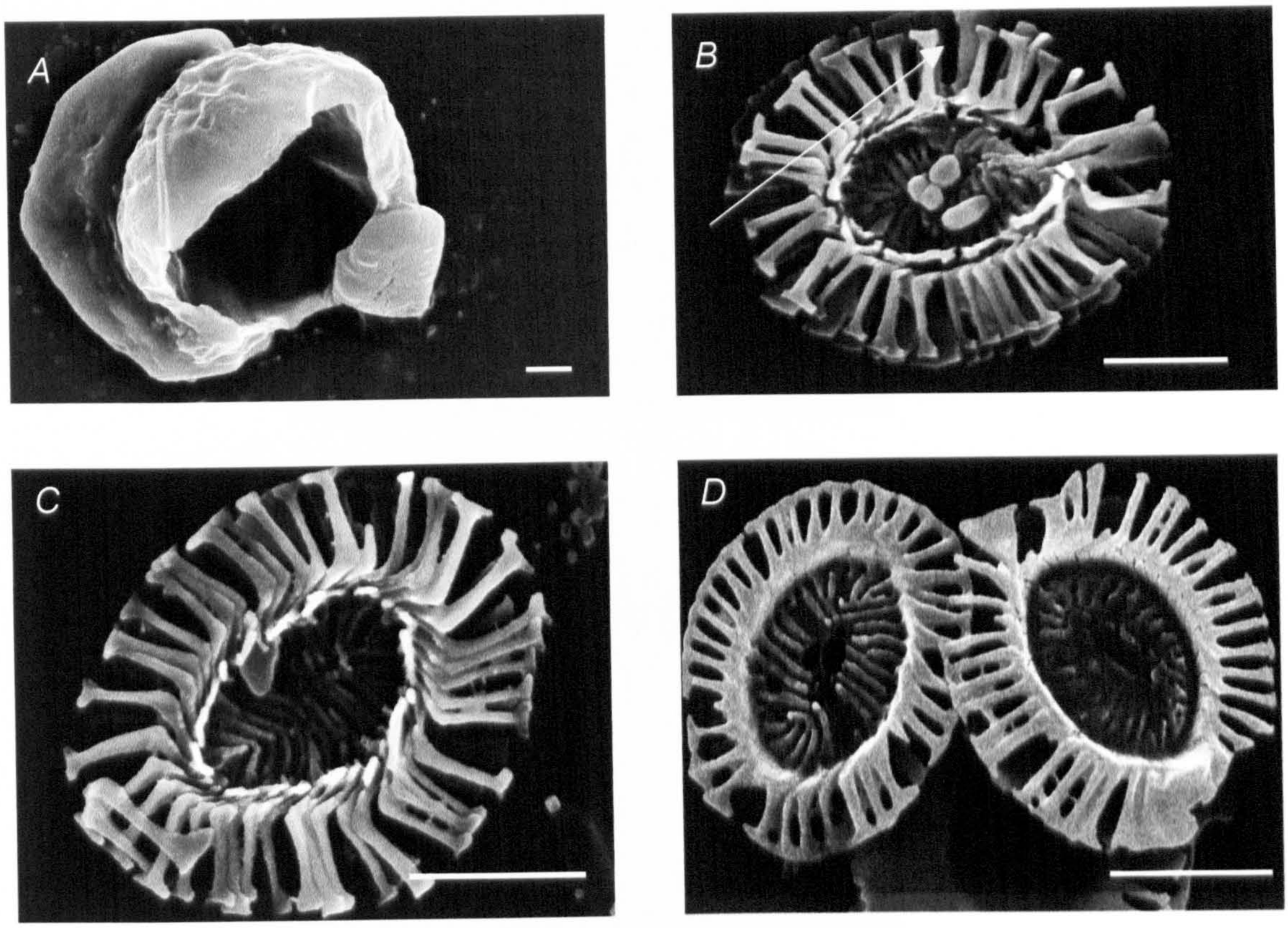
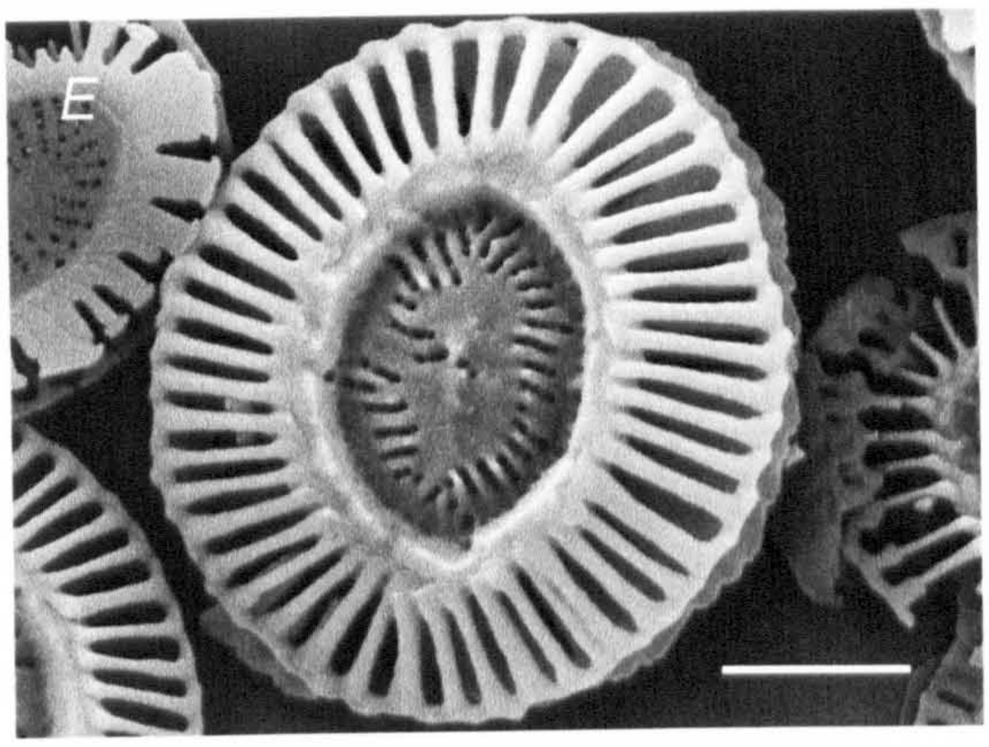


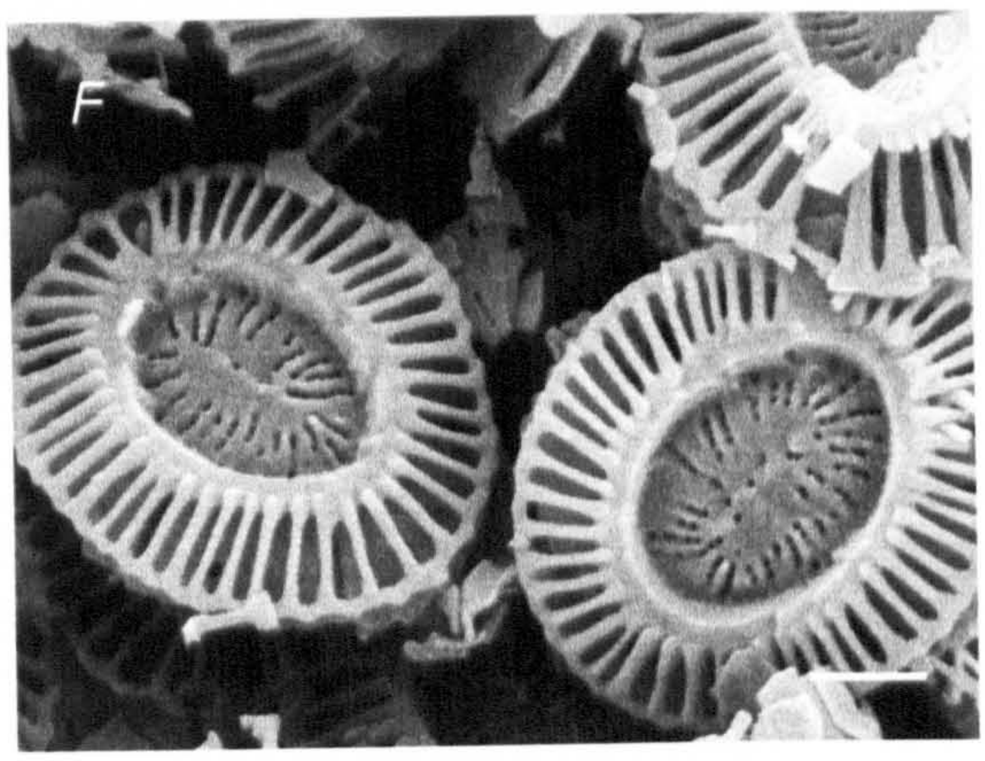

Figure 5.2 : The effect of different magnesium concentrations on the length of the distal shield elements of coccoliths of *E. huxleyi*.

Data represent means, n = 300.




Plates 5.3A to 5.3D : The effect of different low calcium concentrations on the morphology of coccoliths of *E. huxleyi*. No coccoliths are present at 0 mM $Ca^{2+}$ (plate A). Plates B, C and D show coccoliths of cells grown in medium containing 1, 2 and 4 mM $Ca^{2+}$ respectively. Bars = 1 μm.




Plates 5.3E and 5.3F : The effect of different calcium concentrations on the morphology of coccoliths of *E. huxleyi*. Plates E and F show coccoliths of cells grown in medium containing 10 and 20 mM $Ca^{2+}$ respectively. Bars = 1 μm.

Figure 5.3 shows the effect of different calcium concentrations on the width of the distal shield elements of coccoliths for 3 batches of culture of *E. huxleyi*. The largest width of the distal shield elements was measured at 10 mM calcium for the first batch of culture whereas it was at 20 mM for the second and third ones. So, all 3 batches showed maximum width at high calcium concentrations. No variation in the length of the distal shield element was observed with different calcium concentrations (data not shown).

Plates 5.4A to 5.4E show the overall aspect of the coccosphere *E. huxleyi* grown at different calcium concentrations. Like the analysis of the morphology of single coccoliths, these pictures demonstrate that no coccoliths are present at low calcium concentrations whereas the coccosphere is composed of many layers of plates at high calcium concentrations. Plates 5.4A and 5.4E are the same as plates 3.1 and 3.2 in Chapter 3.

The effect of different calcium concentrations on the rate of $^{14}C$ incorporation during photosynthesis and calcification is presented in figure 5.4a. Both processes were strongly inhibited when the cells were incubated for 3 hours in a medium containing 50 mM calcium. Figure 5.4b shows the same data but with a reduced range of calcium concentrations (0 - 20 mM). Clearly photosynthetic rate remains constant between 0 and 20 mM calcium, while calcification is enhanced by increasing calcium concentrations. Photosynthetic rates at 0 and 10 mM calcium are the same data as those presented in figure 3.6 in Chapter 3.

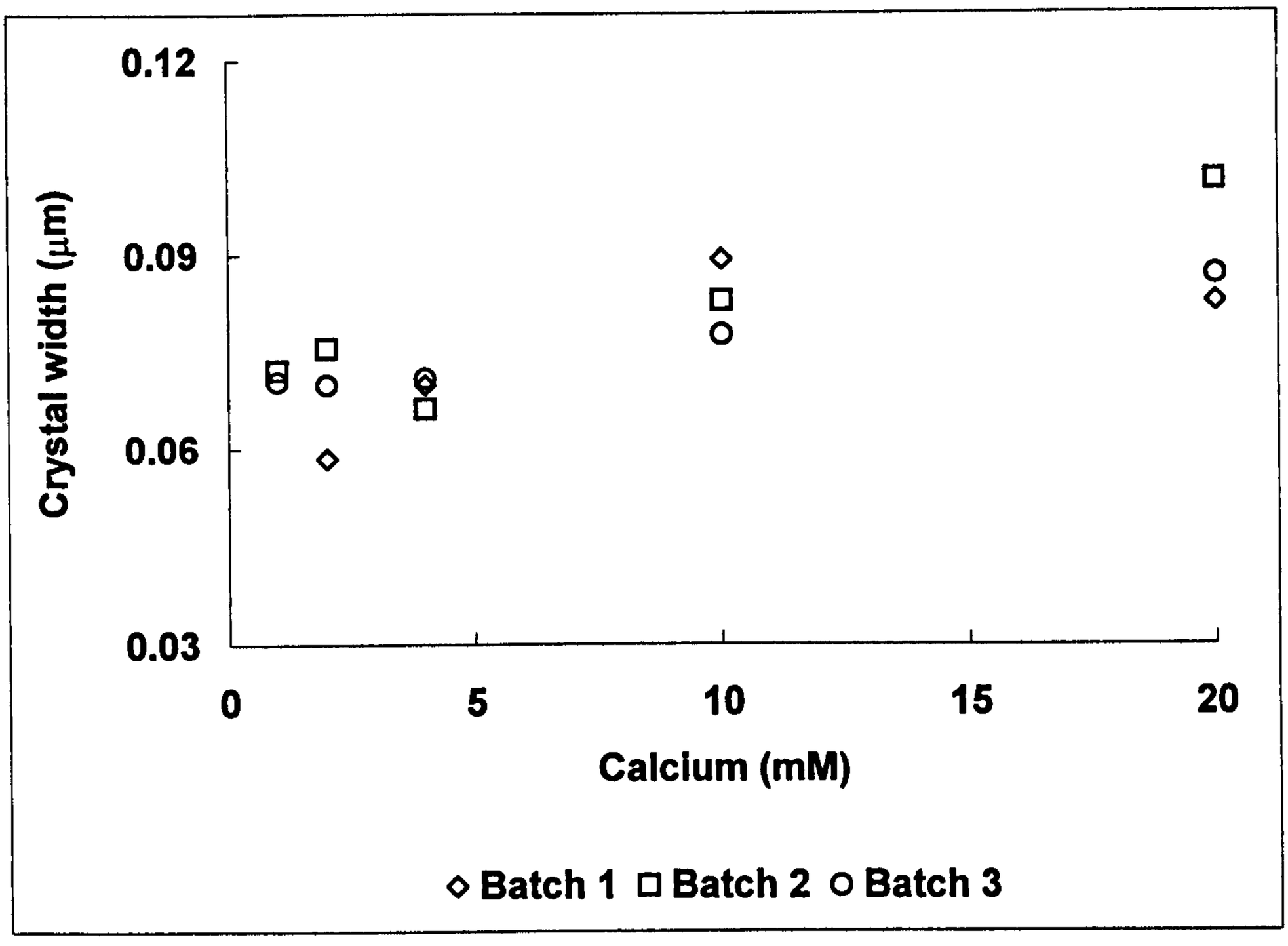
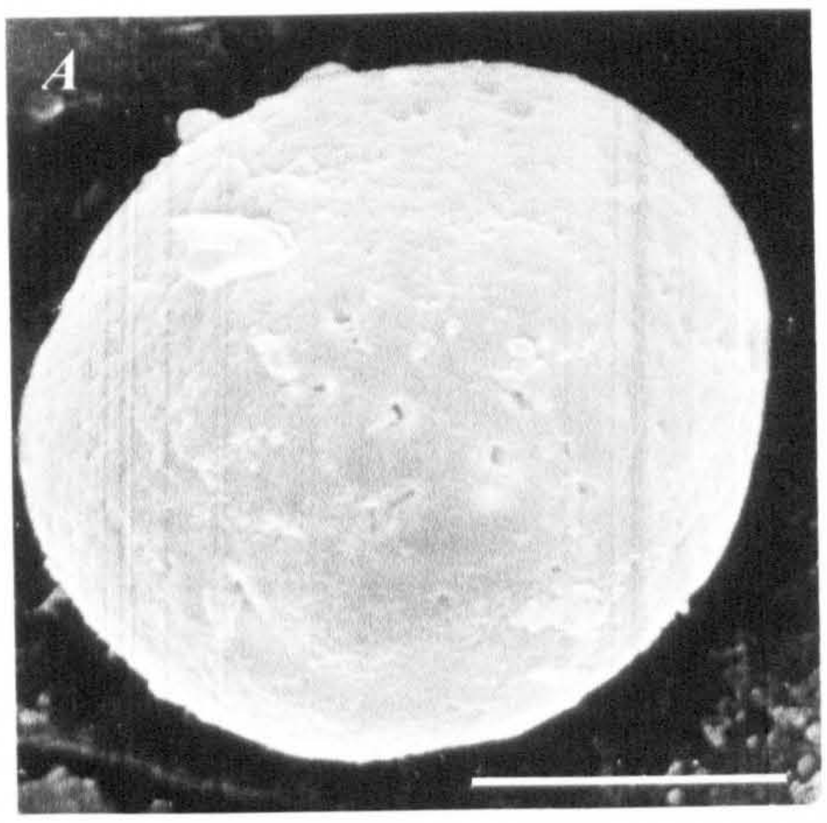
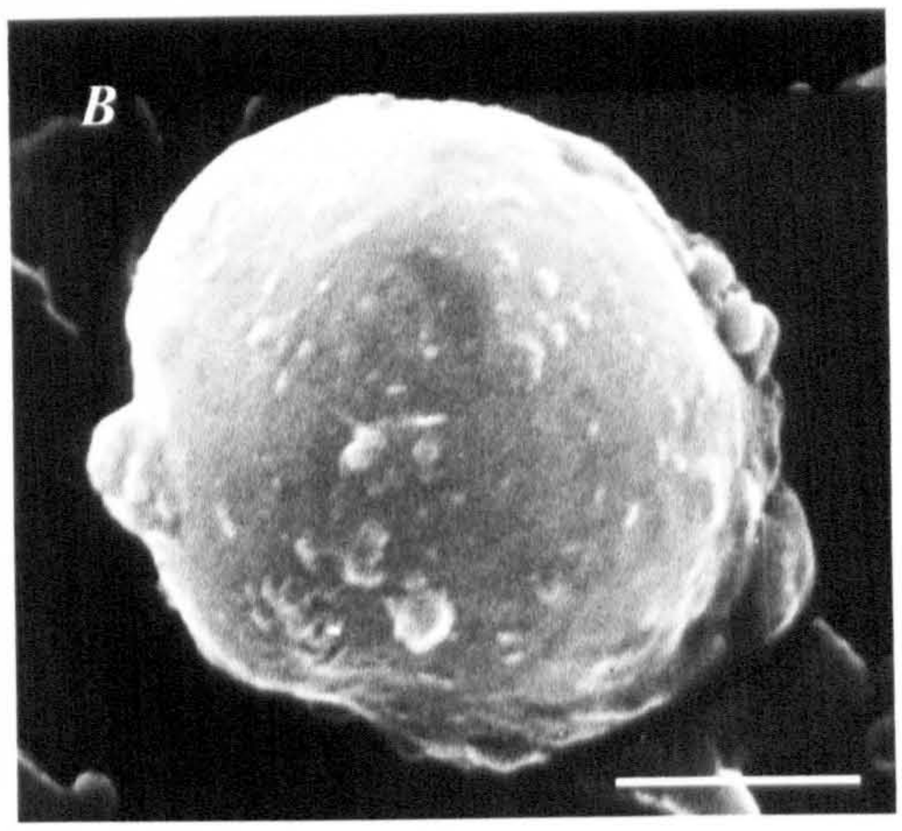
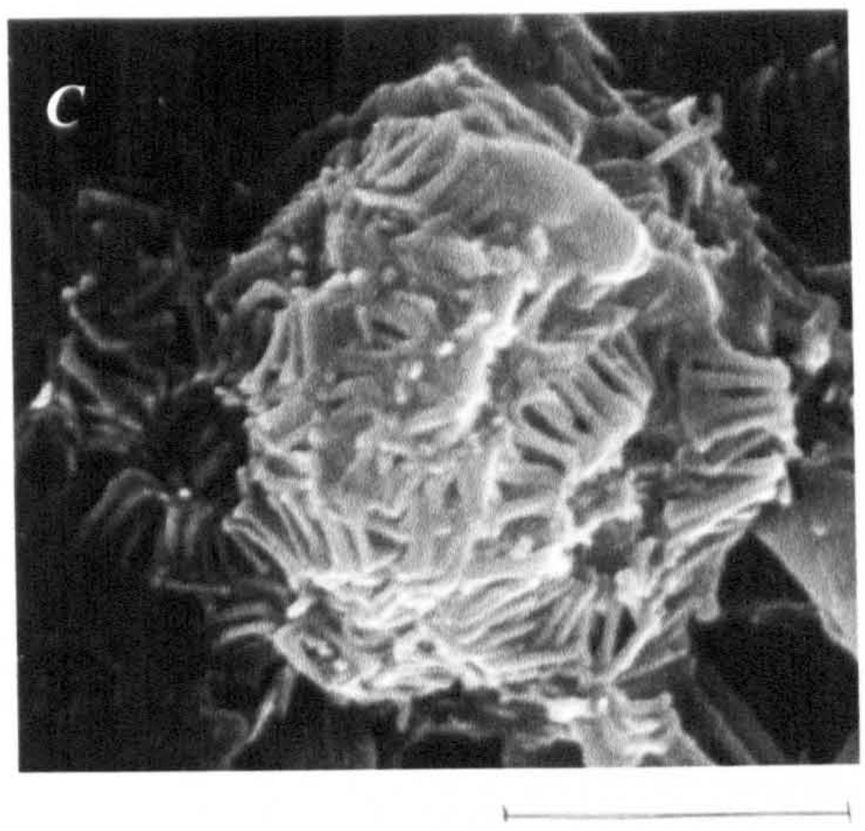


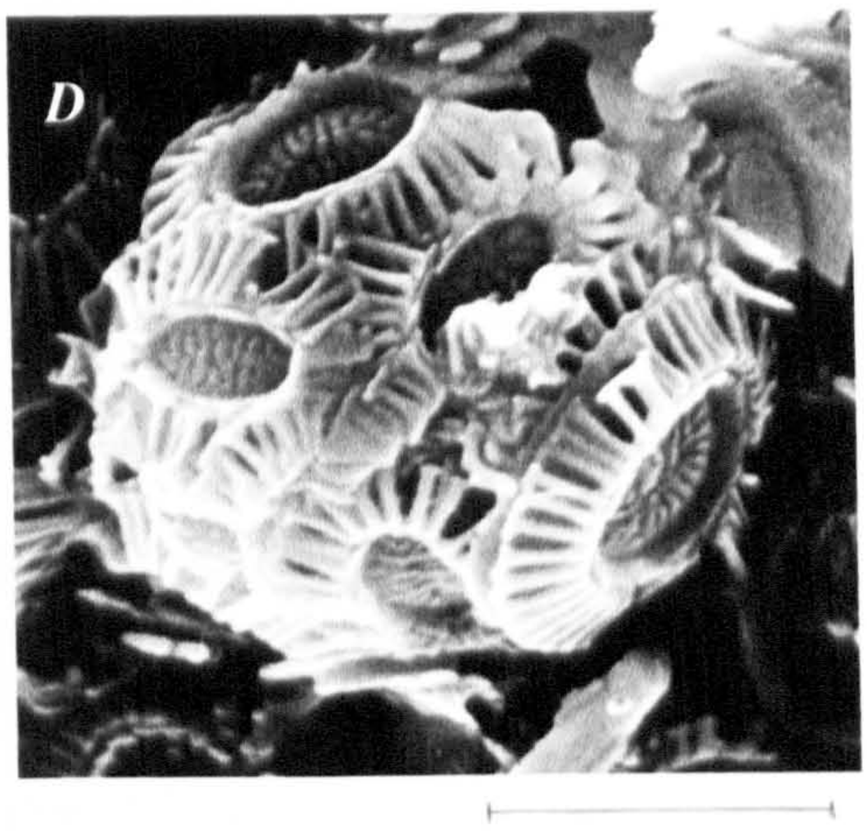

Figure 5.3 : The effect of different calcium concentrations on the width of the distal shield elements of coccoliths of *E. huxleyi*.

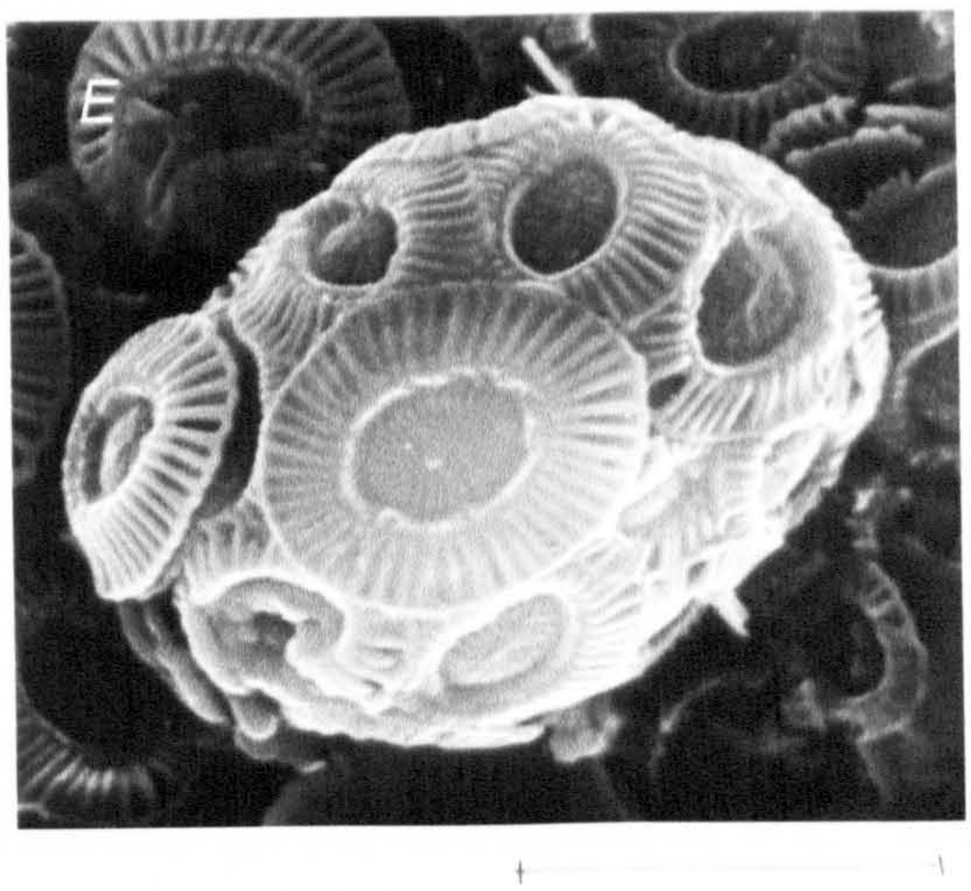
Data represent means, n = 300.




Plates 5.4A to 5.4E : The appearance of the *E. huxleyi* coccosphere with different calcium concentrations. Plates A, B, C, D and E show cells grown in medium containing 0, 1, 2, 4 and 9 mM calcium respectively. Bars = 5 μm.

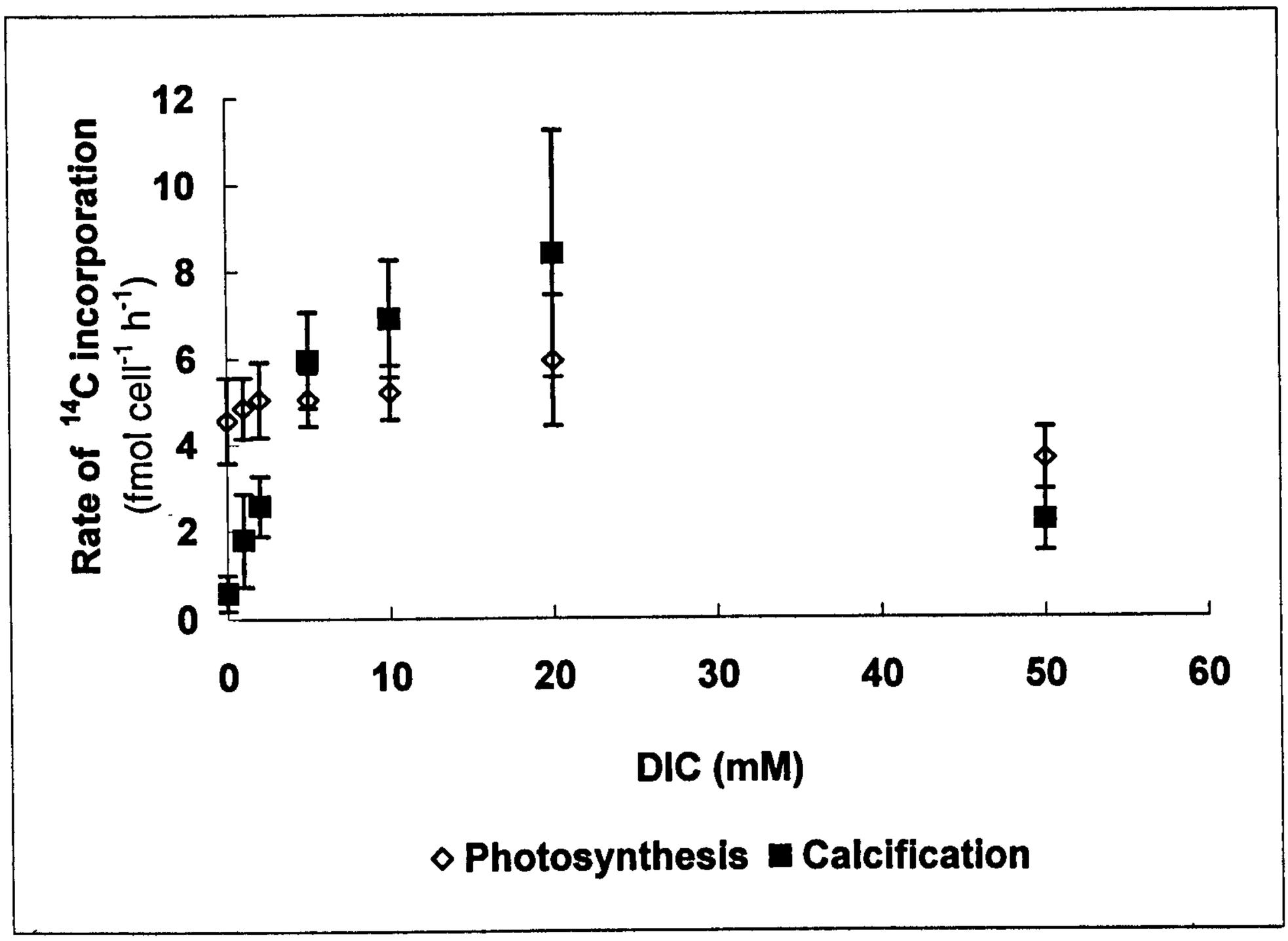



Figure 5.4a : The rate of $^{14}C$ incorporation in *E. huxleyi* during photosynthesis and calcification over a wide range of calcium concentrations (0 – 50 mM). Data represent means ± SE, n = 4.

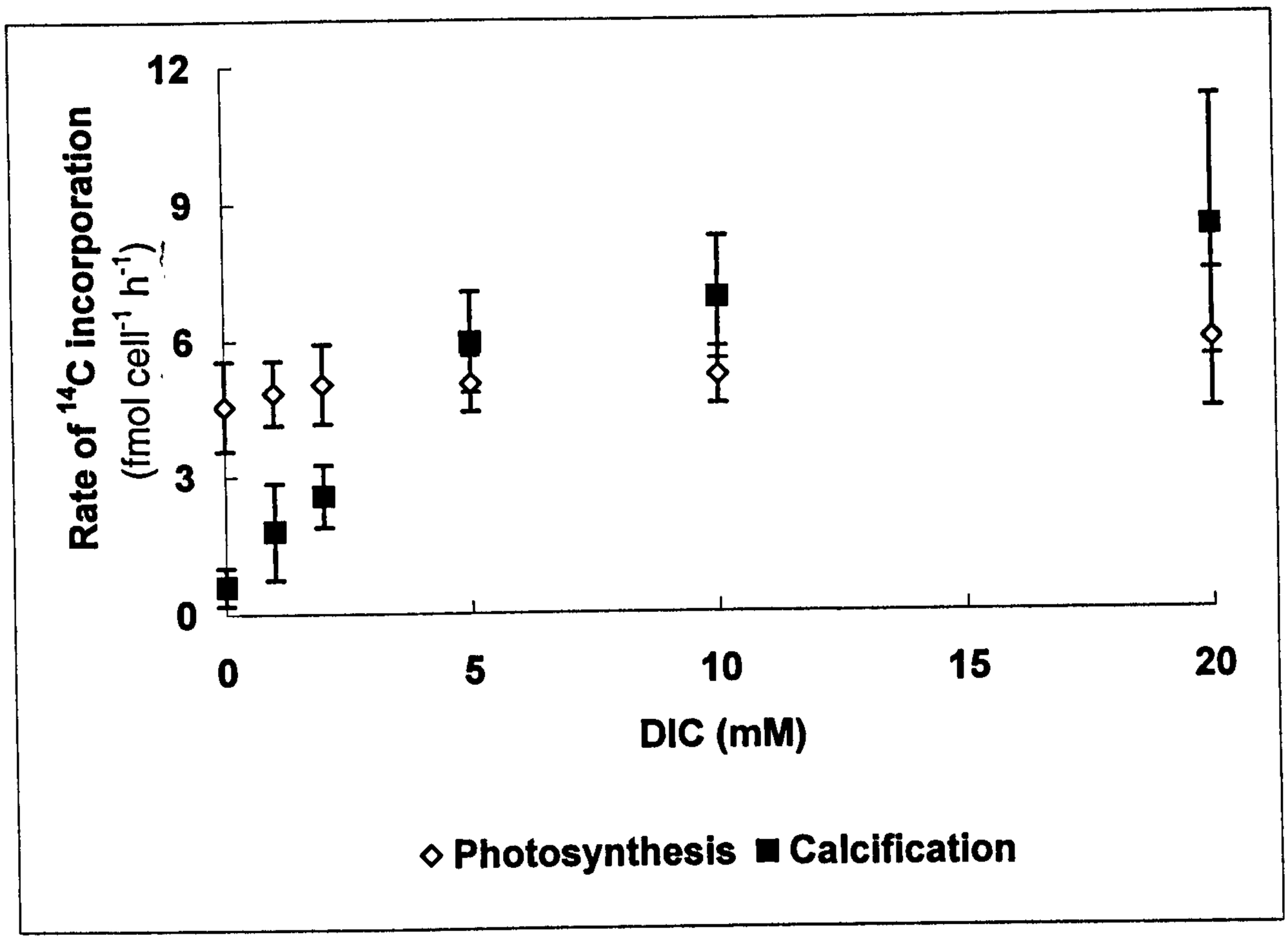



Figure 5.4b : The rate of $^{14}C$ incorporation in *E. huxleyi* during photosynthesis and calcification over a reduced range of calcium concentrations (0 – 20 mM). Data represent means ± SE, n = 4.

*The effect of light on photosynthesis in coccolith-bearing and coccolith-less cells :*

Figure 5.5 shows the $O_2$ production rate of coccolith-bearing and coccolith-less cells of *E. huxleyi* incubated at 3 different PFD : 50, 80 and 500 μmol $m^{-2}$ $s^{-1}$. At low PFD, the photosynthetic rate is higher in the coccolith-less than coccolith-bearing cells, but at the PFD of 500 μmol $m^{-2}$ $s^{-1}$, the two types of cell photosynthesise at the same rate.

Figure 5.6 shows the quantum efficiency of PSII (ϕPSII) of coccolith-bearing and coccolith-less cells measured at different PFD. Actinic values of 5 / 10 / 15 / 20 / 25 / 30 / 35 and 40 were used, which correspond to PFD of 33 / 67 / 161 / 316 / 545 / 848 / 1233 and 1708 μmol $m^{-2}$ $s^{-1}$ respectively. For more details on the calibration see Chapter 2. ΦPSII values are statistically higher for coccolith-bearing than coccolith-less cells for PFD over 316 μmol $m^{-2}$ $s^{-1}$. These measurements, as well as those presented in figures 5.7a and 5.7b, were made by B. Thake (School of Biological Sciences, Queen Mary University of London, UK).

Figures 5.7a and 5.7b, which show the effect of different PFD on non-photochemical quenching (qNP and NPQ), also confirm that photosynthetic activity is different in coccolith-bearing and coccolith-less cells. Both graphs show that at low light, non-photochemical quenching is higher in coccolith-bearing than coccolith-less cells.

Coccolith-less cells were always incubated during experiments in calcium-free medium to prevent any calcification to occur, while calcium was present in the medium used for coccolith-bearing cells.

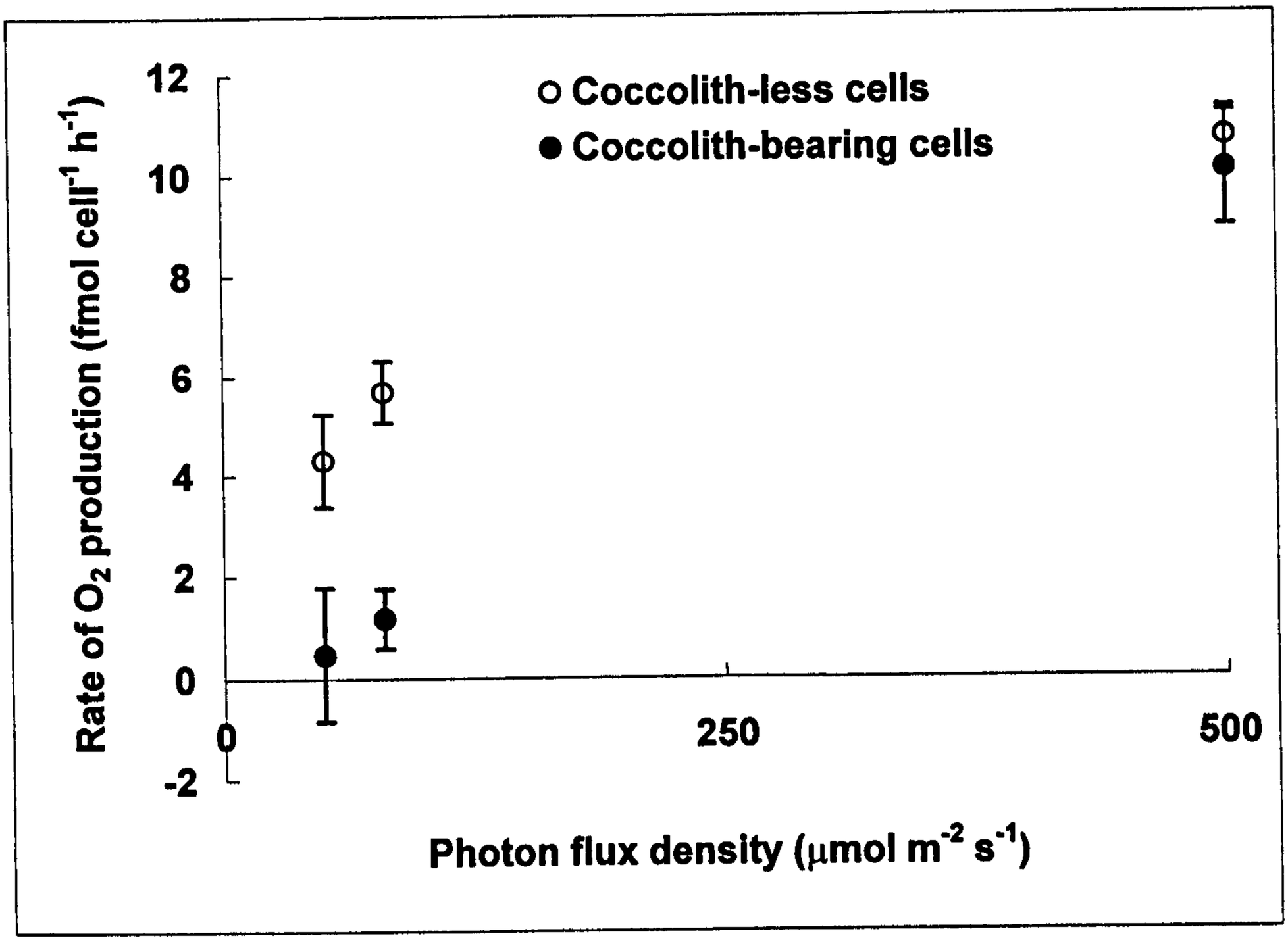



Figure 5.5 : The $O_2$ production rate of coccolith-bearing and coccolith-less cells of *E. huxleyi* incubated at 50, 80 and 500 μmol m$^{-2}$ s$^{-1}$.

Data represent means ± SE, n = 6-11.

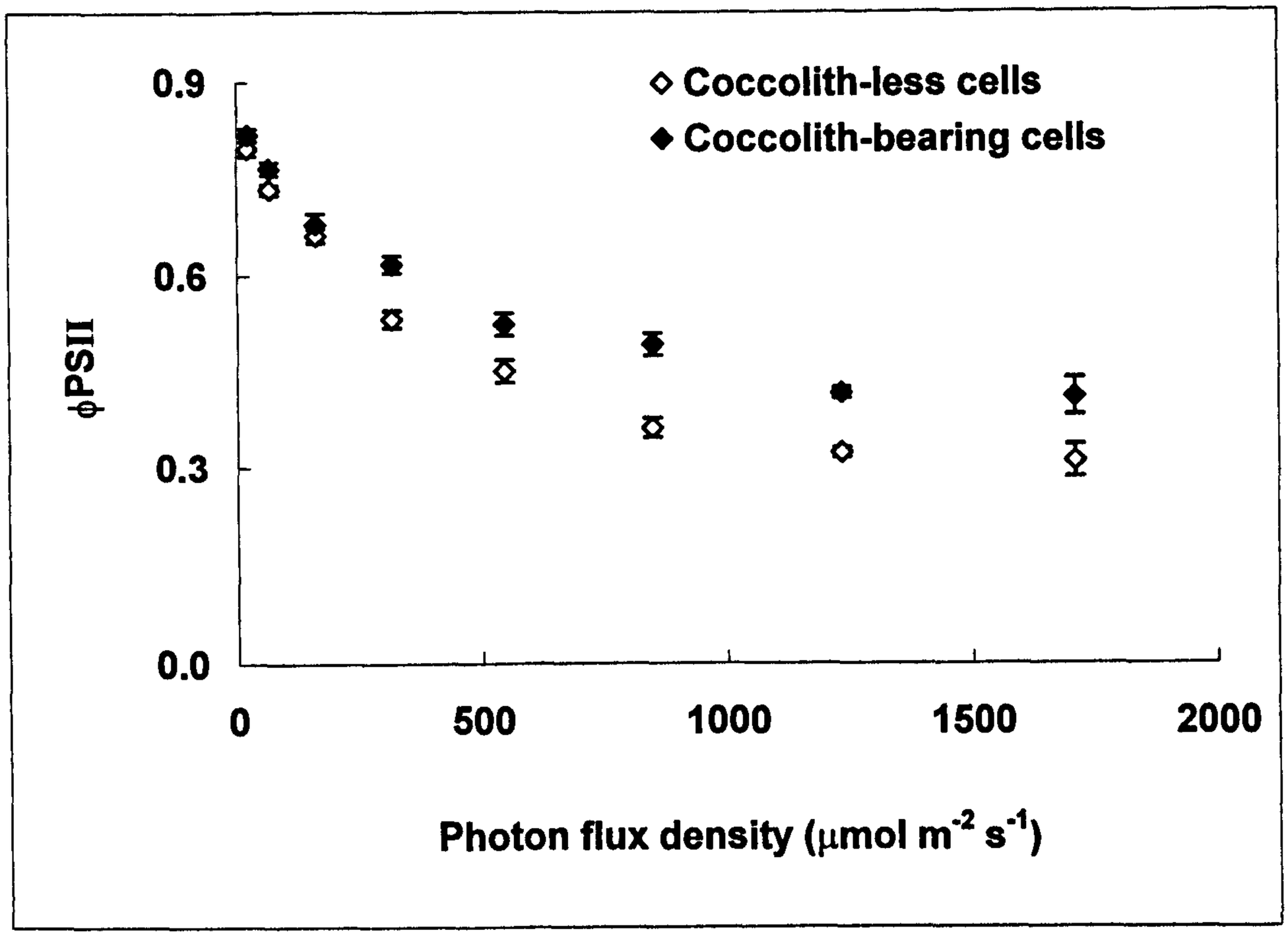



Figure 5.6 : The effect of PFD on the quantum efficiency of PSII of coccolith-bearing and coccolith-less cells of *E. huxleyi*.

Data represent means ± SE, n = 5.

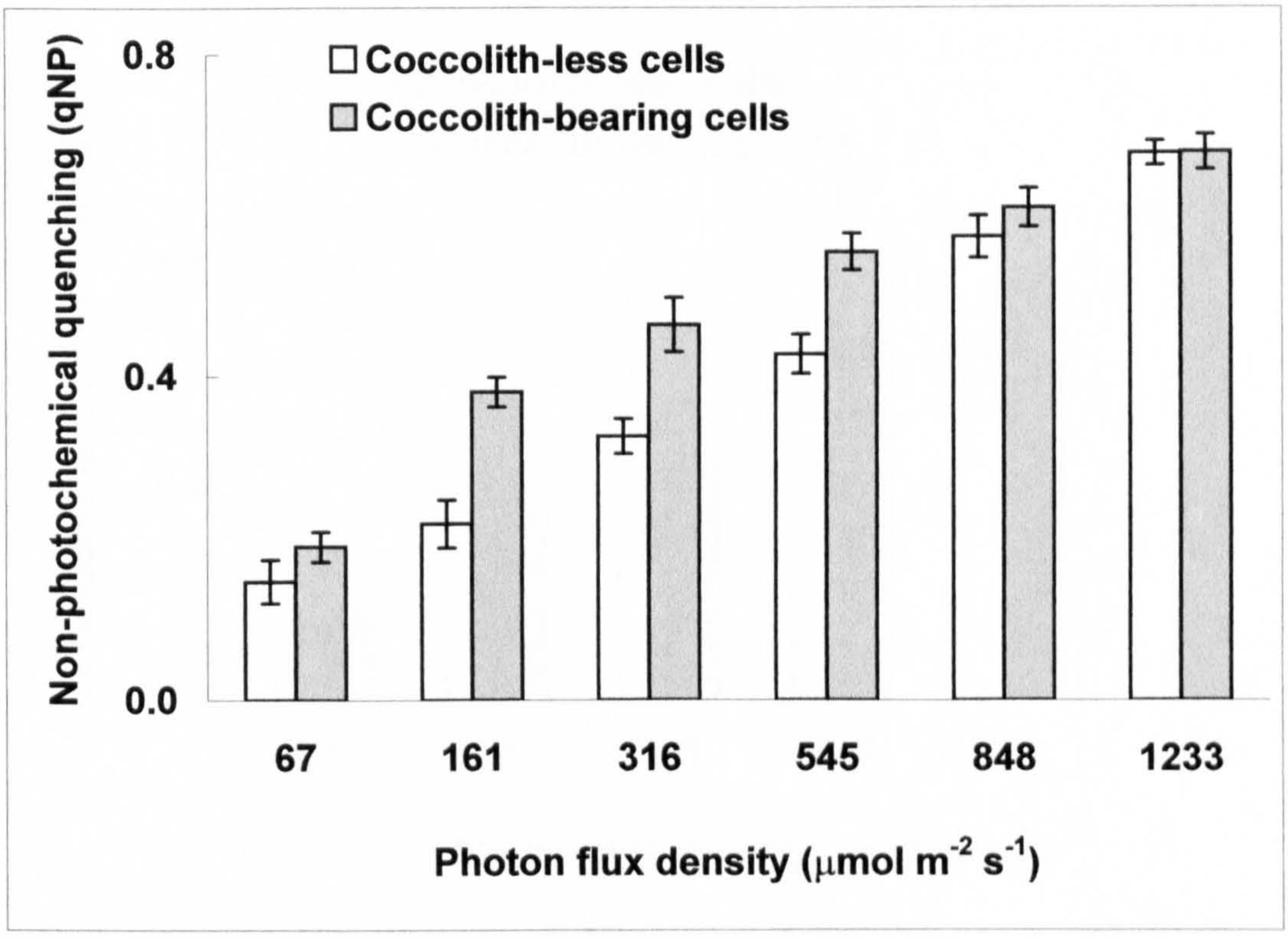



Figure 5.7a : The effect of different PFD on non-photochemical quenching (qNP) in coccolith-bearing and coccolith-less cells of *E. huxleyi*.

Data represent means ± SE, n = 5.

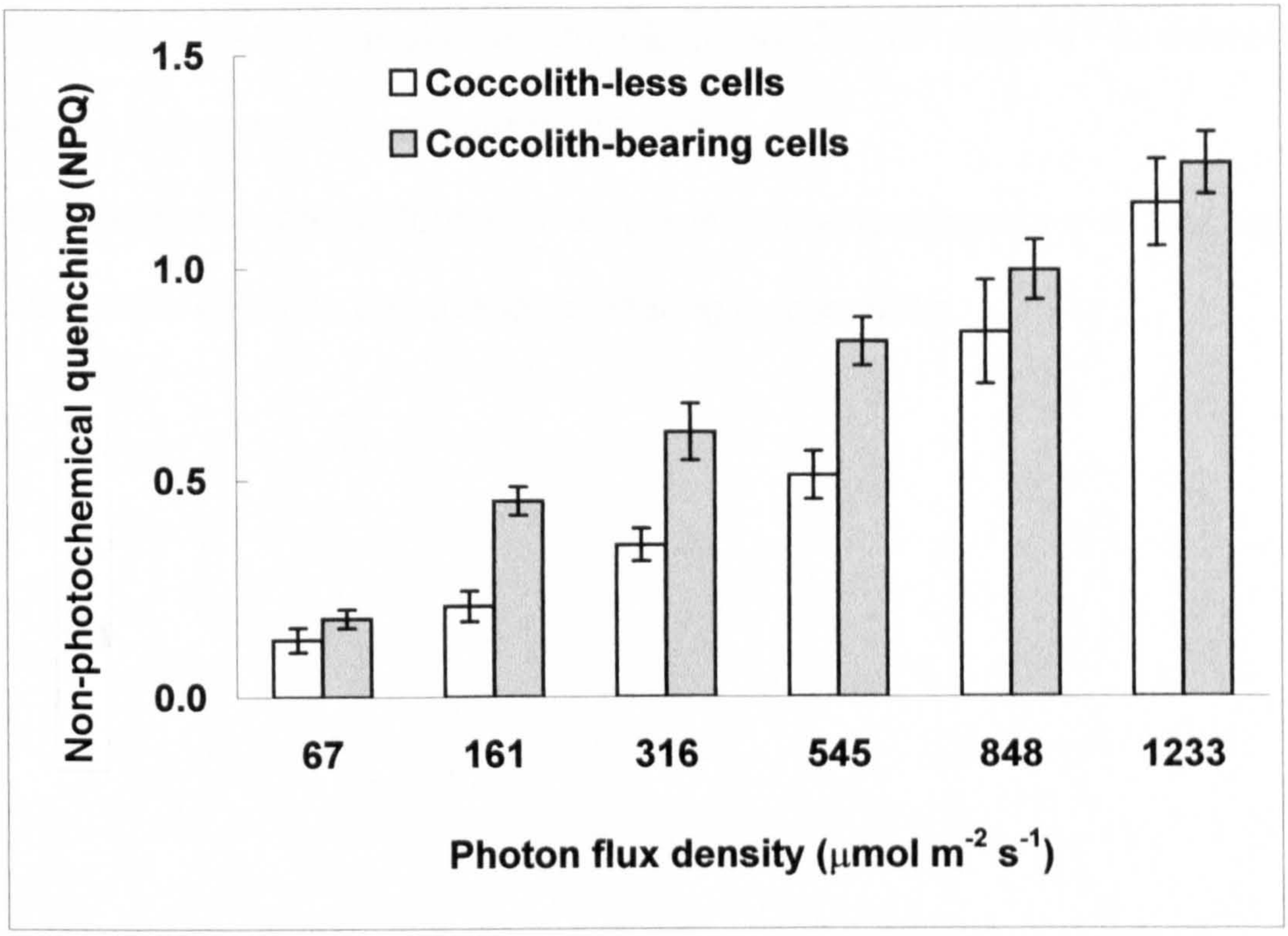



Figure 5.7b : The effect of different PFD on non-photochemical quenching (NPQ) in coccolith-bearing and coccolith-less cells of *E. huxleyi*.

Data represent means ± SE, n = 5.

Figure 5.8 shows that absorbance values measured over a wide range of wavelengths (400-700) are higher for coccolith-bearing than coccolith-less cells. nm Cells concentration was similar with $3.9 \times 10^6$ and $3.7 \times 10^6$ cells $ml^{-1}$ for coccolith-bearing and coccolith-less cells respectively.

Measurements were performed in a standard spectrophotometer so that high absorbance might be due to light-scattering by coccoliths.

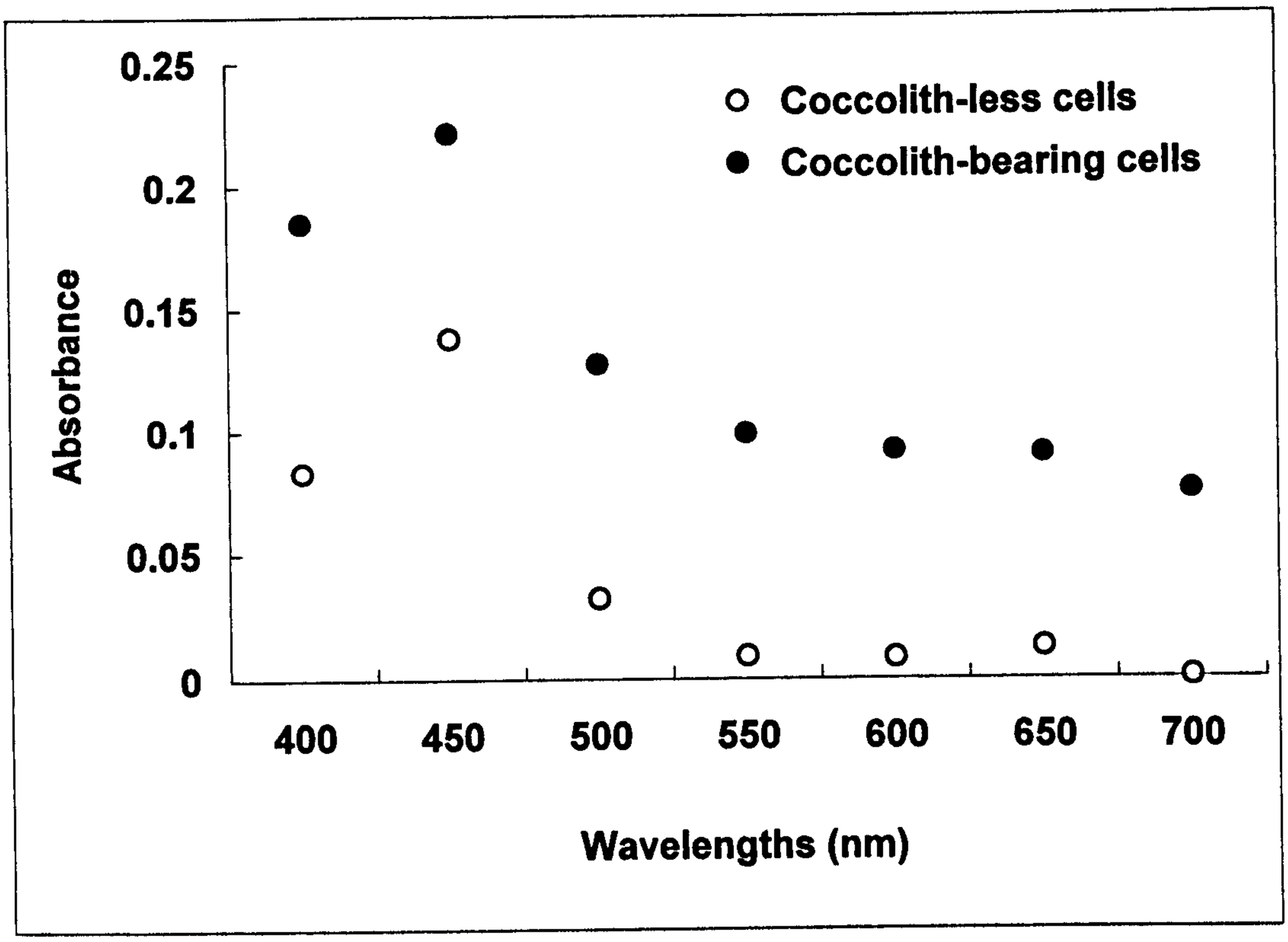



Figure 5.8 : The absorbance spectrum of coccolith-bearing and coccolith-less cells of *E. huxleyi* measured over a wide range of wavelengths (400-700nm).

Discussion :

More than 40 different minerals have been identified in living organisms (Mann 1988). The coccolithophorid *E. huxleyi* crystallise calcium carbonate and form minute calcite plates called coccoliths. This planktonic alga is considered to be the most productive lime-secreting species on Earth (Westbroek *et al.* 1985). The exact biological function of coccoliths remains under much debate. Some authors argue that calcification takes place in order to promote photosynthesis through the production of $CO_2$ and protons (Buitenhuis *et al.* 1999, Nimer *et al.* 1996, McConnaughey 1994, *Sikes et al.* 1980). The present study (Chapter 3) has demonstrated that this is not true for *E. huxleyi*. Calcification does not enhance photosynthesis by providing $CO_2$ or protons.

The protective aspect of the coccoliths has also been suggested as their biological function. Manton (1986) claimed that calcite plates act as a physical boundary between the cell and its environment, thus enabling *E. huxleyi* to get a greater resistance against chemical, osmotic and mechanical shocks. She suggests that the protective propriety of the coccosphere is related to the water trapped between cell membrane and calcite plates. Coccoliths have also been thought to participate in the buoyancy control of *E. huxleyi*. At the surface, the limited concentration of nutrients does indeed promote coccolith formation, so that cells of *E. huxleyi* become covered with many layers of calcite plates. Since coccolith-bearing cells sink faster than coccolith-less ones (Fritz and Balch 1996, Linschooten *et al.* 1991), the alga sinks and reaches the light limited / nutrient

rich zone of the water column. Since calcification stops in high nutrient concentrations (Fritz 1999), only a few coccolith remain in the coccosphere. Fernández *et al.* (1996) have shown that *E. huxleyi* has a high cellular concentration of lipid that can reach 60 % of the total cellular organic carbon. So, when the cell has very few coccoliths it tends to 'float' back to surface waters. In this model, coccoliths play an essential role in the regulation of the cell's vertical movements in the water column. The limitation of this model lies in the fact that seawater is in constant motion, and therefore this motion regulatory system may only have a very limited influence in controlling the location of the coccolithophore in the water column. The aim of this study was to supply experimental evidence to determine the functions of coccoliths in *E. huxleyi.*

It was first necessary to determine if calcification is such an essential process for *E. huxleyi* that it requires a strong biological control, or if calcification is simply taking place because carbon and calcium are present at supersaturated concentrations inside the cell. The formation of calcite crystals could be purely a chemical reaction with little biological influence. Although, as described in the introduction of this chapter, the numerous steps required for coccolith formation makes this highly unlikely for *E.huxleyi*, the saturation state theory, whereby calcium carbonate precipitation depends solely on the concentration of carbonate and calcium ions in seawater, is widely accepted for hermatypic corals (Marubini *et al.* 2001, Langdon *et al.* 2000, Gattuso *et al.* 1998).

The level of biological control in *E.huxleyi* was thus assessed by examining the effect of different magnesium concentrations on calcification.

High $Mg^{2+}$ concentrations provoke strong under-calcification in coccoliths. This result was expected since $Mg^{2+}$ is known to inhibit calcite and to favour aragonite formation (Fallini, 1994, Fork 1974, Paasche 1966). More surprisingly, low $Mg^{2+}$ concentrations also cause signs of under-calcification of calcite crystals in *E. huxleyi*. Perfectly formed coccoliths with the longest length of distal shield elements were only made when this alga was grown in the ambient seawater concentration of $Mg^{2+}$. Hence, calcification in *E. huxleyi* seems to have a strong biological influence that optimises the use of the $Mg^{2+}$ present in seawater. The coccolithophore may have adapted its calcification process so that the ambient concentration of $Mg^{2+}$ of seawater does not have any deleterious effects on calcification.

The strong biological control exerted by *E. huxleyi* on calcification suggests that this is an essential process for the alga. Because calcium is a major component of calcification, a similar investigation using this ion was designed in order to examine the possible role of coccoliths.

At a concentration of 10 mM, calcium is the fifth most abundant constituent of seawater (Lobban *et al.* 1985). The cytosolic calcium concentration of eukaryotic cells ranges from $10^{-8}$ to $10^{-7}$ M (Ashley and Campbell 1979). Brownlee *et al.* (1994) measured a cytosolic calcium concentration of $10^{-7}$ M in *E. huxleyi*. Calcification may be responsible for maintaining the$10^{5}$ fold difference in calcium concentration between the cytosol and seawater. High calcium concentrations in the medium greatly affect the physiology of *E. huxleyi*, although the cells are still alive. Calcification and photosynthetic rates were

indeed strongly inhibited when cells were incubated for 3 hours in medium containing 50 mM calcium. The long-term effect is even more dramatic since the addition of 50 mM calcium to the growth medium kills *E. huxleyi*. In addition, Weinberg-Flax (2000) has shown that the highest growth rate of *E. huxleyi* is obtained with 5 mM calcium and not with the ambient seawater concentration of 10 mM. All these considerations suggest that high calcium concentrations may be toxic to *E. huxleyi*. Moreover, when cells of *E. huxleyi* were incubated in different sub-lethal calcium concentrations (0-20), the calcification rate increased with high calcium concentrations, whereas the photosynthetic rate remained constant. Photosynthesis may be unaffected by high calcium concentrations because of protection given by calcification against the deleterious effects of excess calcium. Although, as already stated in Chapter 3, since the kinetics of calcification and photosynthesis are not similar when cells are incubated at different calcium concentrations this study resolves the disagreement in the literature in favour of Paasche (1964), further work does however remain necessary to ascertain if as the current data seems to suggest calcification is a mechanism by which toxic cytosolic calcium is removed.

One could argue that the association of coccoliths with the simple function of removing $Ca^{2+}$ is not realistic if one considers the elaborate structure of the calcite plates. Indeed, why would the alga produce such perfect arrangements of crystal if their only role were to remove calcium from the cytoplasm? It is true that in normal seawater the coccoliths formed by the alga have perfect symmetry. However, when calcium is sparse, coccoliths display signs of under-calcification. If the most important feature of coccoliths was their crystal structure, when

calcium is in more limited supply, the alga would produce fewer calcite plates, but with perfect morphology. This is not the case, since at low calcium concentrations *E. huxleyi* makes many malformed coccoliths. Hence, it appears to be important to produce crystals of calcite, whether they are fully formed or not. This again suggests that the primarily biological function of coccoliths in *E. huxleyi* may be to remove excess toxic cytosolic calcium. However, this does not rule out any subsequent additional role of coccoliths.

One of the most striking features of *E. huxleyi* is its capacity to scatter light. Blooms of *E. huxleyi* have been identified by satellite imagery because of the light scattered by the coccoliths (Linschooten *et al.* 1991). Light scattering is defined as a change in the direction of the light without a change in wavelength. This happens when the wavelength of the incident light is in the same order as the particle size. Light scattering can have a forward direction, but backscattering can also take place. When *E. hux*leyi is present in the top 40 m of seawater, calcite can cause up to 70 % of backscattering. Since coccoliths have a diameter of 1-2 μm, they are the optimum size for maximum scattering effect (Balch *et al.* 1996). Detached coccoliths are thus much more efficient at scattering light than those that are still part of the coccosphere. So, light that reaches *E. huxleyi* may not be scattered much by the cell itself and thus photons are able to penetrate the alga. In comparison, detached coccoliths in suspension in the water column scatter much light in a forward and backward direction. This in turn increases the number of photons reaching the alga.

In this study, cells of *E. huxleyi* were always resuspended in fresh medium before an experiment. Photosynthetic rates were therefore measured in the absence of detached coccoliths. The similar rate of $O_2$ production obtained in coccolith-bearing and coccolith-less cells at a PFD of 500 μmol $m^{-2}$ $s^{-1}$ shows that saturating number of photon penetrates a cell of *E. huxleyi* whether or not it is covered with coccoliths. Coccoliths are thus no hindrance to photosynthesis, since they do not reduce light penetration when still in the coccosphere, but by scattering light once detached they may maximise the use of incident light in the water.

Since detached coccoliths might be more useful for optimising light by scattering, why is the alga not shedding all its coccoliths? First of all, when the PFD is saturated for photosynthesis and at high light, coccoliths protect the photochemical apparatus of the cells. The quantum efficiency of PSII, measured as ϕPSII, is higher in coccolith-bearing than coccolith-less cells. This disagrees with data from previous studies. Paasche (1964) found no difference in the rate of photosynthesis between coccolith-bearing cells of *E.huxleyi* and cells which had been previously decalcified by acid treatment. However, this author suggests that his result should be considered with caution since McAllister (1961) showed that measurements of $O_2$ evolution and $^{14}C$ can provide very different data when shade adapted cells are exposed to high PFD. Nanninga and Tyrrell (1996) measured photosynthesis by monitoring rates of $O_2$ evolution in calcifying, non calcifying and decalcified cells of *E.huxleyi* and also showed that coccoliths do not provide any protection against high light. It is interesting to note that although ~~this~~ These two studies suggest the same conclusion, their data are, however,

dissimilar. In contrast to the lack of difference in photosynthesis observed by Paasche, in the experiment of Nanninga and Tyrrell the rate of $O_2$ production was much higher in coccolith-less cells than coccolith-bearing ones. Induced fluorescence was used in the present study because this relatively recent technique gives clear indications of the activity of PSII. Direct monitoring of the photochemistry has provided the first evidence to support of the idea that coccoliths protect the cell against high light.

In addition, at high light, non-photochemical quenching is however lower in coccolith-less than coccolith-bearing cells and become equal at high PFD. So, attached coccoliths seems to protect against photochemical damage when light is in excess by stimulating PSII activity, but does not reduce the need for non-photochemical quenching mechanisms. This is a different pattern from that observed in Chapter 3, where increased PSII activity was correlated with a reduction of non-photochemical quenching. This might be because bicarbonate directly affects the photochemistry, while coccoliths simply provide external protection against damaging light.

Furthermore, when light was limiting, rates of $O_2$ production were higher for coccolith-less than for coccolith-bearing cells. In addition, when grown in limiting light for photosynthesis, coccolith-bearing cells contained more chlorophyll than coccolith-less ones. This shows that at low PFD, coccoliths are shading the light, since a classic 'shade response' is for plants to compensate by producing more chlorophyll. Attached coccoliths seem therefore to allows cells to photosynthesise at high light by protecting the photochemistry, but they

negatively affect photosynthesis when the PFD is low. Three points are, however, important to consider. Firstly, in turbulent mixing, cells located deeper in the photic zone may rapidly be exposed to the high PFD of surface waters, hence receiving on average saturating quantum of light. Secondly, since suspended coccoliths are efficient are scattering and backscattering light, the PFD below surface waters may be higher in an *E. huxleyi*-rich water column. And finally, the area of the water column where light limitation occurs may be more nutrient rich. Since high nutrient concentrations repress calcification, in light limited conditions, cells of *E. huxleyi* may be coccolith-less or covered by very few coccoliths. All those considerations suggest that photosynthetic inhibition by coccoliths at low PFD may not actually occur in the field.

Further work will be carried out using the same light source for both induced fluorescence and $O_2$ evolution measurements to determine if some of the discrepancy in the data gained with the two techniques originates from the use of different light sources.

In summary, the present work has supplied evidence that calcification is an essential process for *E. huxleyi* that requires strong biological control. This study also suggests that one of the function of coccoliths might be to remove excess cytosolic calcium that could be toxic for the cell. Experiments on coccolith-bearing and coccolith-less cells also show that the relationship between light, attached coccoliths and photosynthesis is complicated. *E. huxleyi* seems to be adapted to high light conditions, since attached coccoliths are not shading

the alga at high PFD, while they prevent light penetration at low PFD. The calcite plates also participate in protecting the photochemical apparatus of *E. huxleyi* against the damaging effect of excess light.

# Chapter 6 :

# Calcification and photosynthesis in hermatypic corals

Introduction :

Coral reefs cover 6 x $10^5$ $Km^2$ of the Earth's surface (Smith 1978). Although this is only 0.17 % of the area of the global ocean it represents 15 % of the area of shallow sea. In the past decades, the conservation of coral reefs has become an important topic because of the incidence of mass mortality events in coral reefs. Their conservation is of prime interest not only because of their wide distribution in the tropics (up to a third of the length of tropical coastline), but also because they exhibit the greatest diversity of marine life per unit area (Paulay 1997). A third of all marine fish species, for example, lives in coral reefs (Lieske and Myers 1994). The large number of reef-dwelling species of animals and plants (35000 – 60000) that have been identified might still be underestimated (Reaka-Kudla 1994) because only representatives of the better-known taxa have so far been described. Damage to coral reefs would thus strongly affect the ecology of the coastline in the tropics. In addition, destruction of coral reefs would also have serious financial effects for many less economically developed regions of the tropics. This is the result of the extensive use of coral reef resources for purposes such as food, building materials, medicines, jewellery, curios and tourism (Hodgson 1997). Corals are also important in the global carbon cycle, since half of the world's $CaCO_3$ precipitation takes place in coral reefs (Smith 1978).

Coral reefs are built by hermatypic corals. Corals consist of colonies of polyps that share a common gastrovascular system (see Chapter 1 for details).

The coelenteron is made of two epithelia, the ectoderm in contact with seawater and the endoderm facing the gastrodermic cavity. Ectoderm and endoderm are separated by a thin connecting layer called mesogloea, which is formed from the association of collagen, mucopolysaccharide and cells (Muller-Parker and D'Elia 1997).

Despite their simple morphology, corals carry out two important carbon-consuming processes : photosynthesis and calcification.

Photosynthesis takes place in symbiotic microalgae of 10 to 12 μm in diameter (Muscatine *et al.* 1998), which are localised along the axis of endodermic cells, facing the coelenteric cavity (Furla *et al.* 1998b), as shown in figure 6.1. These symbionts are called zooxanthellae. The term zooxanthellae refers to all golden-coloured symbiotic microalgae (Trench 1992), such as Bacillariophyceae, Cryptophyceae and Dinophyceae. For instance, the zooxanthellae found in benthic foraminifera of tropical reefs are pennate diatoms (Rowan 1998), but the zooxanthellae of reef-building corals are dinoflagellates of the genus *Symbiodinium* (Ralph *et al.* 1999). In nature, only the coccoid, non-motile stage of *Symbiodinium*, has been found inside host tissues (Rowan 1998, Muller-Parker and D'Elia 1997). They also have no known sexual reproduction. It was thought for many years that all corals harboured a genetically homogeneous population of *Symbiodinium*, several recent studies have demonstrated that there is considerable genetic diversity among zooxanthellae living in the same coral species (Santos *et al.* 2001, Darius *et al.* 2000, Goulet and Goffroth 1997).

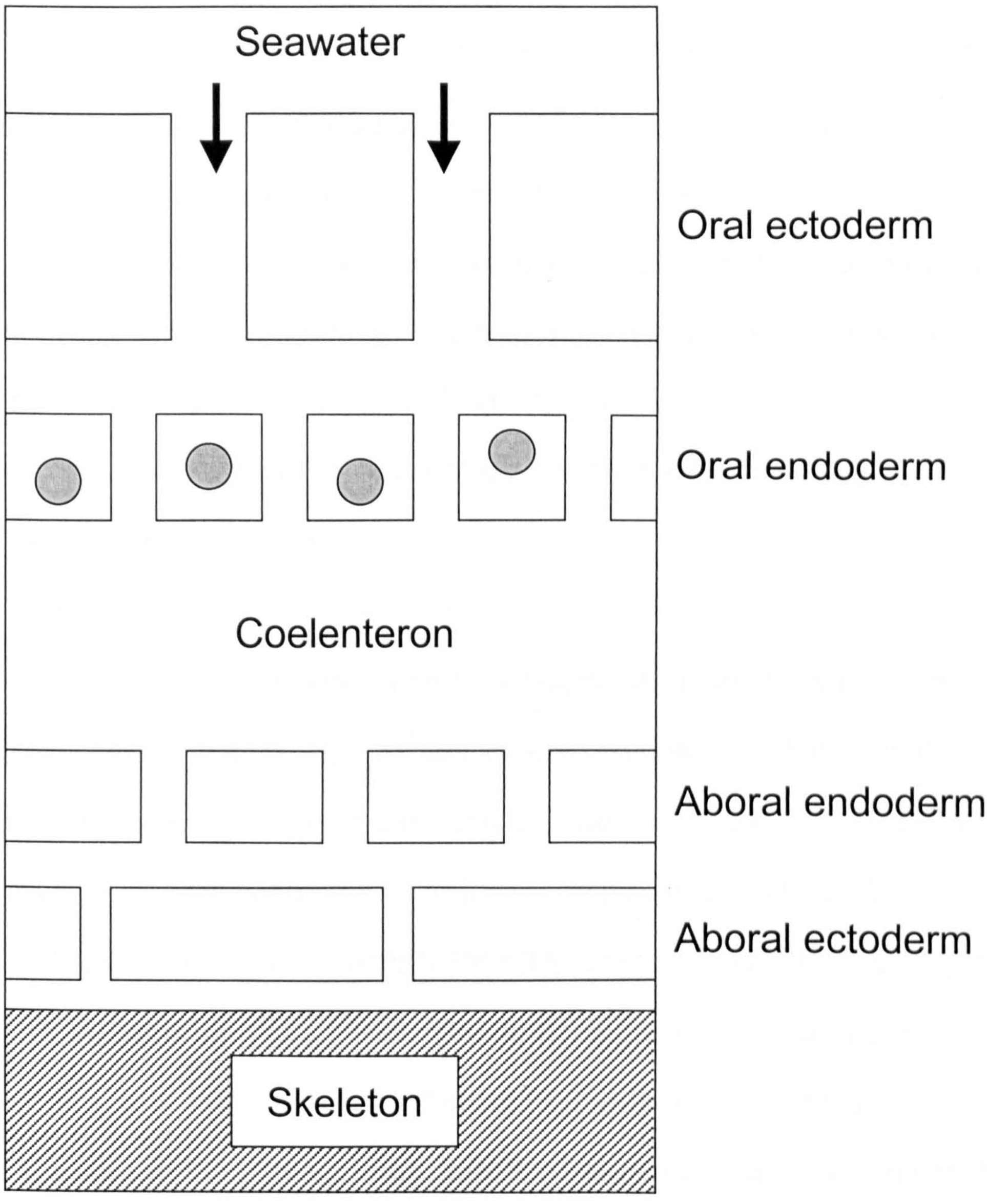



Figure 6.1 : Diagram illustrating the morphology of the epithelium of hermatypic corals (modified from Furla *et al.* 2000 and Tambutté *et al.* 1996).

Muscatine and Pool (1979) have shown that a simple host cell, of 25 μm in length and 10 μm in width, contains 1 to 2 zooxanthellae. However, the host-symbiont association is not fixed since the cell specific density can vary under different environmental conditions. The cell specific density increases in ammonium and nitrate enriched seawater (Muscatine *et al.* 1998, Dubinsky *et al.* 1990). In contrast, under stressful conditions, the cell specific density is reduced (Lesser *et al.* 1989, Ogden and Wicklund 1988, Roberts 1987). Whether the animal expels the algae or the zooxanthellae leave the host remains undetermined (Rowan 1998).

The lost of pigmentation of reef-building corals upon stress is called bleaching (Jones *et al.* 1998). As well as resulting from a diminution in the number of zooxanthellae in the coral, this can also be the consequence of a reduction of pigment concentration inside the symbiotic algae (Kleppel *et al.* 1989, Hoegh-Guldberg and Smith 1989). The precise mechanism leading to coral bleaching is still unknown even though it has been the subject of much recent research. The synergistic effect of the elevation of sea-surface temperature and the increase in UV radiation has been suggested as a possible cause of bleaching events (Van Woesik 2001, Brown 1997a,b , Glynn 1993).

Jones *et al.* (1998) showed that in *Stylophora pistillata* heat stress begins with damage to assimilatory electron flow in the first step of the Calvin cycle, due to the disruption of the Mehler-Ascorbate-Peroxidase (MAP) cycle. This leads, as a secondary effect, to damage in Photosystem II. In fact, recent studies have shown that non-assimilatory oxygen-consuming pathways, such as the MAP cycle, protect the photosynthetic apparatus (Park *et al.* 1996, Osmond and Grace 1995, Schreiber and Neubauer 1990), by consuming excess electrons when the rate of electron transport exceeds the rate of enzymatic reactions of the Calvin cycle. Jones *et al.* (1998) suggest that zooxanthellae are very sensitive to an interruption of the MAP cycle because these symbionts contain a type II Rubisco similar to that of bacteria (Rowan *et al.* 1996, Whitney *et al.* 1995), whereas all other eukaryotes have a type I Rubisco. Type II Rubisco has a lower affinity for $CO_2$ versus $O_2$ than type I (Jorden and Ogren 1981). When the MAP cycle is altered, the disturbance of electron flow around Photosystem I would more easily lead to $O_2$ poisoning of form II Rubisco, because of its limited carboxylation activity. Lesser and Shick (1989) have also related zooxanthellae expulsion to oxygen toxicity.

Bleaching is a natural response to environmental stress from which many corals can recover (Lasker *et al.* 1984). Bleaching events can be followed by the process of 'rebrowning', which is the regaining of zooxanthellae by animal tissues (Muller-Parker and D'Elia 1997). Rowan *et al.* (1997) and Buddemeier and Fautin (1993) have suggested that bleaching is an adaptive strategy that allows corals to recombine with genetically different zooxanthellae. Upon environmental stress, less resistant zooxanthellae are then replaced by

symbionts that are more adapted to the new conditions. This 'adaptive-bleaching hypothesis' is supported by the fact that corals with more genetically variable zooxanthellae population are less prone to bleaching (Loh *et al.* 2001). Furthermore, 'species' of *Symbiodinium* have been shown to differ in their photoacclimation abilities (Rowan 1998). Symbiotic zonation with depth has been observed in Caribbean corals. For example, at shallow to intermediate depths *Acropora cervicornis* bears *Symbiodinium* A, while deeper, *Symbiodinium* C, the low-light adapted 'species', is found (Rowan, 1997). When corals are exposed to high light, most bleaching occurs at intermediate depth with the selective elimination of *Symbiodinium* C, because its physiological limits have been reached (Rowan, 1997). All the above considerations tend to confirm that bleaching is a natural response of corals to survive environmental stress.

Nevertheless, since the beginning of the 1980s, extensive bleaching events have led to mass mortality of corals all over the tropics (Lasker *et al.* 1984). Mumby *et al.* (2001) have demonstrated that this global phenomenon is not exaggerated and, for instance, that the high mortality of *Porites* sp. after the major bleaching event of 1998 is unprecedented in the French Polynesia. Kinsey (1988) has suggested that corals have a high recovery rate when subject to only one stress, but combined stresses (human and natural) will lead to poor regeneration of living coral. Nowadays, coral reefs are exposed to so much human disturbance, such as eutrophication, sedimentation, oil pollution and coral mining, that corals have become extremely sensitive to any additional natural stresses such as storms, cyclones, hurricanes, or the outbreak of the Crown-of-Thorns starfish *Acanthaster planci*.

The widespread coral mortality that follows extreme bleaching events provokes a shift of reef community from a coral dominated benthos to one where macroalgae (Hughes 1994) and bioeroders (Glynn 1996) prevail. Such a reef erodes quickly. Eakin (1993) has calculated that a Panamanian reef, which before 1983 was depositing 24.7 tonnes of $CaCO_3$ per year, is currently eroding at a rate of 5.9 tonnes of $CaCO_3$ per year. So bleaching has a potent negative effect on calcium carbonate deposition on the reef. First, when bleaching only causes a lost of coral pigments, the rate of calcification is reduced (Brown 1997a). When bleaching provokes tissue necrosis, calcification is completely inhibited and finally, in badly damaged reefs, bioeroders consume the calcium carbonate.

Hence, bleaching adversely affects both photosynthesis and calcification. Chave *et al.* (1975) have estimated that 10 Kg of $CaCO_3$ per $m^2$ of coral reef were deposited each year. Calcification is thus a major process of coral reefs.

Even though studies on coral calcification started decades ago with the pioneering work of Goreau and his collaborators (Goreau 1959, Goreau and Goreau 1959, Goreau and Bowen 1955), the physiology and biochemistry of coral biomineralisation remains poorly understood (Allemand 1995).

Calicoblastic cells, located in the aboral ectoderm of corals, are responsible for skeletogenesis (plate 6.1). Calicoblastic cells are 0.5 to 3 μm in thickness and 10 to 100 μm in length and are positioned parallel to the skeleton surface (Gattuso *et al.* 1999). Using confocal laser scanning microscopy on cryofixed undecalcified freeze-substituted polyps of *Galaxea fascicularis* and *Acropora*

*formosa*, Marshall and Wright (1993) showed that these are elongated where rapid calcification occurs. Whilst describing the formation of the density banding, Barnes and Lough (1993) gave an overview of how such a large amount of calcium carbonate can be deposited by corals. The first step is called the extension phase and is characterised by the crystallisation of aragonite on the outer surface of the coral. The thickening of the existing skeleton that remains associated with the animal tissue marks the second stage. Finally, every 30 days, animal tissues become detached from a part of the skeleton. So it is the raising of animal tissue from the skeleton that allows the expansion of coral reefs and consequently enables perpetual calcification by corals.

McConnaugey (1994) has separated calcification into two distinct types, using the terms 'cis' or 'trans'. The difference between these two processes is the sites at which calcification and photosynthetic carbon uptake occur. In the 'cis-calcification' model, external calcification takes place on the same side of the organism as photosynthetic carbon uptake. One the other hand, in the 'trans-calcification' model, $CaCO_3$ deposition and carbon uptake take place at distinct and separate surfaces. Corals as well as coccolithophorids are trans-calcifying organisms.

In coccolithophores, photosynthesis and calcification occur in the same cell, but in corals, photosynthesis and calcification are more spatially separated processes (Vandermeuler and Muscatine 1974). Photosynthesis takes place in the zooxanthellae located in the polyps, whereas calcification occurs at the base of the coral, in the animal tissues. However, as for coccolithophorids,

calcification and photosynthesis seem to be linked, but the nature of this association is not clearly established (Gattuso *et al.* 1999, Barnes and Chalker 1990).

Muscatine *et al.* (1984) showed that the amount of carbon translocated in *S. pistillata* represents 143 % of the carbon required for respiration. The authors therefore argue that the spare carbon could be used for other metabolic pathways such as calcification. Excess translocated carbon was also found in *Pocillopora eydouxi* by Davies (1984) and in *Porites porites* by Edmunds and Davies (1986). So, enough excess carbon is produced by corals to at least partially support calcification.

The fact that calcification proceeds faster in the light than in the dark has led to the long-established view that photosynthesis and calcification are closely coupled (see Gattuso *et al.* 1999 for review). Gattuso et al. (1999) reviewed 108 data sets from 26 publications and concluded that the average ratio of light/dark is about 3. This discrepancy has led many scientists to think that calcification is light-enhanced (Gattuso *et al.* 1999, Carlon 1996, Goreau *et al.* 1996, Barnes and Chalker 1990).

Marshall (1996a) disagrees with this concept and argues that calcification is dark-repressed rather than light-stimulated. He found that in the light, the rates of $^{45}Ca$ incorporation per mass of skeleton of symbiotic (*Galaxea fiscularis*) and non-symbiotic (*Tubastrea faulkeneri*) corals were the same, but that in the dark or with DCMU addition, the calcification rates were reduced in *G. fiscularis*,

but remained constant in *T. faulkeneri*. Marshall therefore concluded that while calcification in non-symbiotic corals was light independent, in symbiotic corals, mineralisation was composed of two phases, one being light independent. He also suggested that calcification should be considered dark-repressed rather than light-enhanced. The validity of his findings were subsequently questioned by Carlon (1996) and Goreau *et al.* (1996). The former pointed out that Marshall used only one of each type of coral, while it is well known that the variation in calcification rates between hermatypic coral species is large (up to 10-fold). Similarly, Goreau *et al.* (1996) were unconvinced by his conclusions because of the "inappropriate normalization of his $^{45}Ca$ uptake data by the weight of skeleton". Marshall (1996b) has however responded to both criticisms. He called attention to the fact that although Carlon was right about the extensive range in the rates of calcification, his study did indeed demonstrate that those of hermatypic and ahermatypic can overlap. He also disputed the use of "tissue biomass protein, or nitrogen content" for the normalisation of his data that Goreau *et al.* (1996) proposed and instead argued that skeleton weight was the best choice since the two coral tested had similar skeletal mico-architecture. Many doubts remain concerning Marshall's experimental design and his conclusions (Gattuso *et al.* 2000), and calcification is still commonly considered light-enhanced.

As described above, the link between photosynthesis and calcification has often been suggested to be correlated with carbon use. Several studies have shown that the bicarbonate derived from seawater was the source of carbon for photosynthesis in anthozoans (reviewed in Gattuso *et al.* 1999, Allemand *et al.*

1998a). For example, Benazet-Tambutté *et al.* (1996b) demonstrated that photosynthesis is completely inhibited when tentacles of *Anemonia viridis* are incubated in $HCO_3^-$-free seawater. In addition, Al Moghrabi *et al.* (1996) showed that bicarbonate ions withdrawn from seawater support photosynthesis in *Galaxea fascicularis*, and that animal cells transport these ions to the zooxanthellae.

There are limited data on the source and the mode of transport of carbon for calcification in corals (Gattuso *et al.* 1999). The scientific community still argues about whether carbon is supplied to the calcification site by direct seawater uptake or by metabolic $CO_2$ production. Furla *et al.* (2000) showed that metabolic $CO_2$ is the major source of carbon for calcification in *S. pistillata*, with only 25-30 % originating from seawater and that the calcification rate is saturated in ambient seawater. In contrast, Marubini and Thake (1999) established that the calcification rate of *P. porites* doubles when seawater is enriched with 2 mM DIC. Measurements of $O_2$ production in *Goniopora* sp. showed that photosynthesis is also enhanced by DIC addition (B. Thake, personal communication 2000). The aim of the current study was to determine if the increase in calcification rate following DIC addition observed in *P. porites* was correlated with higher photosynthetic rates and to assess the closeness of coupling between the two processes.

Dose responses of photosynthesis and DIC and of calcification and DIC in the light were compared in two hermatypic corals originating from widely different geographical regions (Caribbean and Indo-Pacific respectively). A comparison

between light and dark incubation of the rates of these two processes was also performed. In addition, the susceptibility of photosynthesis of these two coral species to different carbon transport inhibitors was examined.

## Materials and methods :

### *Materials :*

#### ***Porites porites* :**

Branches of *P. porites* were collected in September 2000 from a 3 m depth reef situated on the West Coast of Barbados. These were promptly returned to the wet-bench of the Bellairs Research Institute of McGill University, Holetown, Barbados, and made into nubbins (Davies 1995). The seawater of this wet-bench was actively aerated, had a temperature of 30 °C, a salinity of 35 ppt, and was illuminated with a light / dark cycle of 12 hours by metal halide lamps, which generated a photon flux density of 200 μmol $m^{-2}$ $s^{-1}$ at the surface of the nubbins.

Nubbins were carefully scraped to remove any incrusting algae before being replaced in the wet-bench for at least 3 days to allow acclimation before experiments were initiated.

#### ***Acropora* sp. :**

Several colonies of *Acropora* sp. of Indo-Pacific origin were supplied by the Tropical Marine Centre (Chorleywood, UK) between February and May 2001. These were acclimatised and maintained in synthetic seawater (Tropic Marin, Wartenberg, Germany). Before experiments colonies were made into nubbins.

Experiments were carried out directly at the Tropical Marine Centre or shortly after transfer to our laboratory at Queen Mary University of London.

*Photosynthesis measurements :*

During $O_2$ evolution measurements, nubbins or polyps of *P. porites* and *Acropora* sp. were incubated in simple Harrison's medium (appendix 3) with a salinity of 35 ppt and a pH of 8.2.

After the completion of the experiment, nubbins and polyps were frozen at –25 $^0$C.

***Porites porites* :** NUBBINS :

Strathkelvin $O_2$ electrodes were used to measure $O_2$ evolution of nubbins of *P. porites*. Chapter 2 provides more details on the Strathkelvin $O_2$ electrodes. The control box was linked to a chart recorder. The $O_2$ probe was placed inside a transparent respirometer consisting of an inner incubation chamber (100 ml) surrounded by an outer water jacket connected to a recirculating water-bath. Temperature was fixed at 27 °C and magnetic stirring bars maintained water movement inside the chamber. White fluorescent lights provided a downwelling photon flux density of 500 μmol $m^{-2}$ $s^{-1}$.

The $O_2$ evolution of nubbins of *P. porites* was recorded for 30 minutes. The dark respiration rate was obtained by switching the light off and covering the chamber with aluminium foil.

***Porites porites* : POLYPS :**

Illuminated nubbins of *P. porites* were anaesthetised by slowly transfusing magnesium chloride (30 ppt) into seawater (plate 6.1). Anaesthetised polyps were removed from the nubbin with surgical scissors and placed in seawater.

$O_2$ evolution measurements were carried out with Rank Brothers $O_2$ electrodes (Cambridge, UK) and $O_2$ stratification was avoided using magnetic stirrer bars. Temperature was maintained at 27 °C using a recirculating water-bath. Slide projectors provided a photon flux density of 500 μmol $m^{-2}$ $s^{-1}$ to each electrode. Control boxes were linked to a two-channel chart recorder. This set up is described in more detail in Chapter 2.

***Acropora* sp. : NUBBINS :**

Nubbins of *Acropora* sp. were incubated in 30 ml polycarbonate vials completely filled with seawater medium. Vials were placed in a water-bath, at 27 °C. White fluorescent lights provided a downwelling photon flux density of 500 μmol $m^{-2}$ $s^{-1}$.

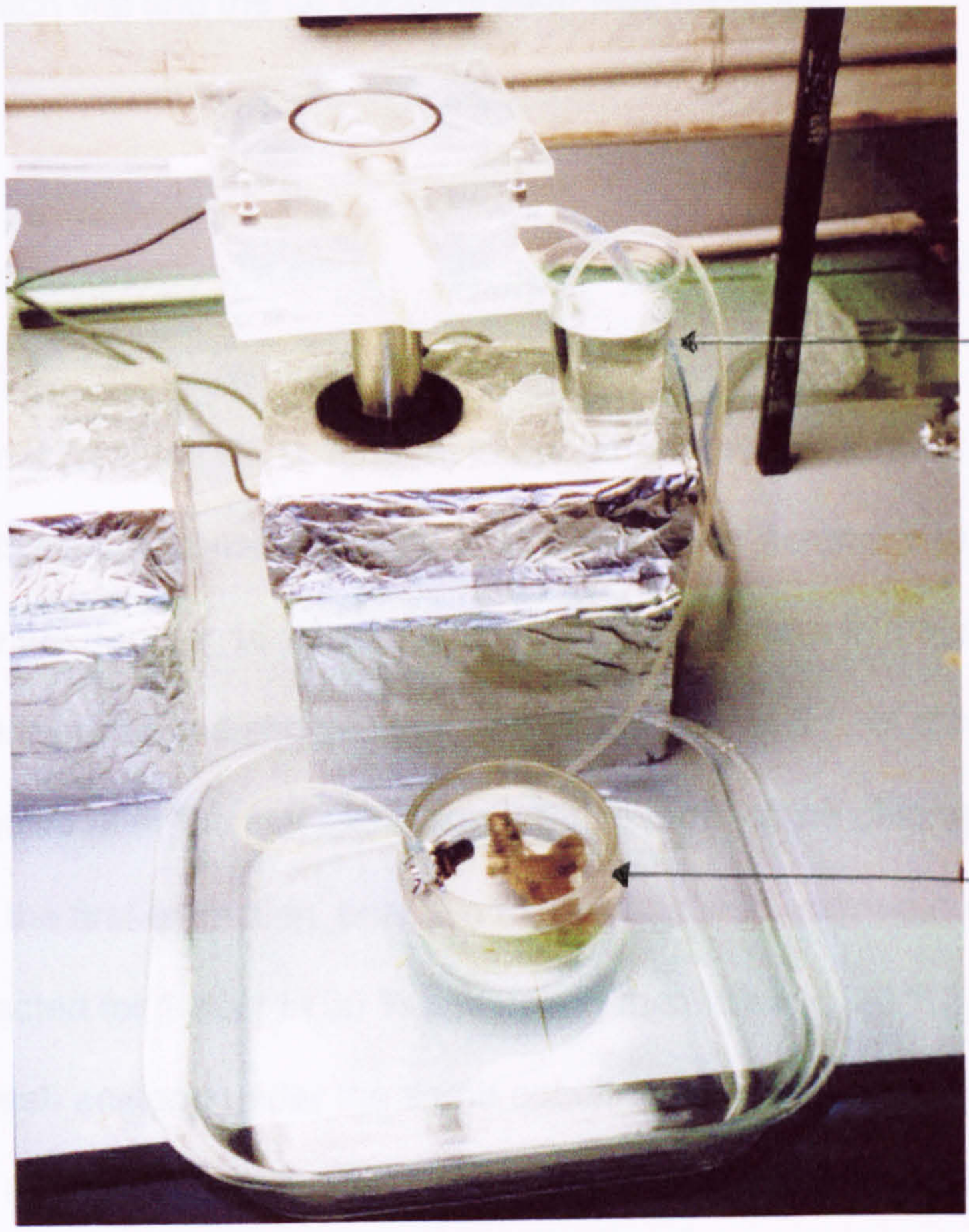



Plate 6.1 : Photograph illustrating the method used for anaesthetising nubbins of *P. porites*.

At the end of the incubation period, a Strathkelvin $O_2$ electrode was inserted into each vial and the $O_2$ concentration was recorded.

*Chlorophyll extraction :*

I. Taubner (another Ph.D. student in our laboratory) performed almost all of the chlorophyll extractions. Nubbins of *P. porites* and *Acropora* sp. were freeze-dried in the dark for 16 hours. Pilot experiment where five successive extractions were performed showed that only an additional 5 % of chlorophyll could be extracted after the first extraction. Since most of the chlorophyll was gained during the first extraction, only two extractions were carried out. Nubbins were first extracted for 1 hour in 90 % acetone in the dark at 6-10 °C, and then for 2.5 hours in fresh acetone under the same conditions. Beakers were covered with cling film to reduce evaporation. Absorbance was measured using a spectrophotometer (JENWAY 6405 v/vis).

The following equation (Jeffrey and Humphrey 1975) was used to calculate the chlorophyll contained in each nubbin :

Chlorophyll a in μg = ((11.85 X Abs. 664nm) – (1.54 X Abs. 647nm) –

(0.08 X Abs 630nm)) X Vol. acetone in ml

*Measurements of Photosystem II activity :*

PSII activity was monitored with a Hansatech Fluorescence Monitoring System. More details concerning the FMS are given in Chapter 2. PSII activity was monitored in nubbins of *Acropora* sp. placed in polycarbonate vials.

Figure 6.2 shows the conversion of actinic light values to photon flux densities when calibrated through a piece of polycarbonate vial. Actinic lights of 12 and 24 are equivalent to photon flux densities of 110 and 541 μmol $m^{-2}$ $s^{-1}$ respectively.

Nubbins of *Acropora* sp. were incubated in 30 ml polycarbonate vials, under white fluorescent lights providing a downwelling photon flux density of 100 μmol $m^{-2}$ $s^{-1}$, in a temperature controlled bath, at 27 °C. Harrison's medium contained 2 mM DIC and was changed once during the incubation period. After 24 hrs, PSII activity was measured. The same nubbins of *Acropora* sp. were then incubated under the same conditions for 1 hour in Harrison's medium in which DIC concentration was raised to 8 mM. PSII activity of the nubbins was then remeasured.

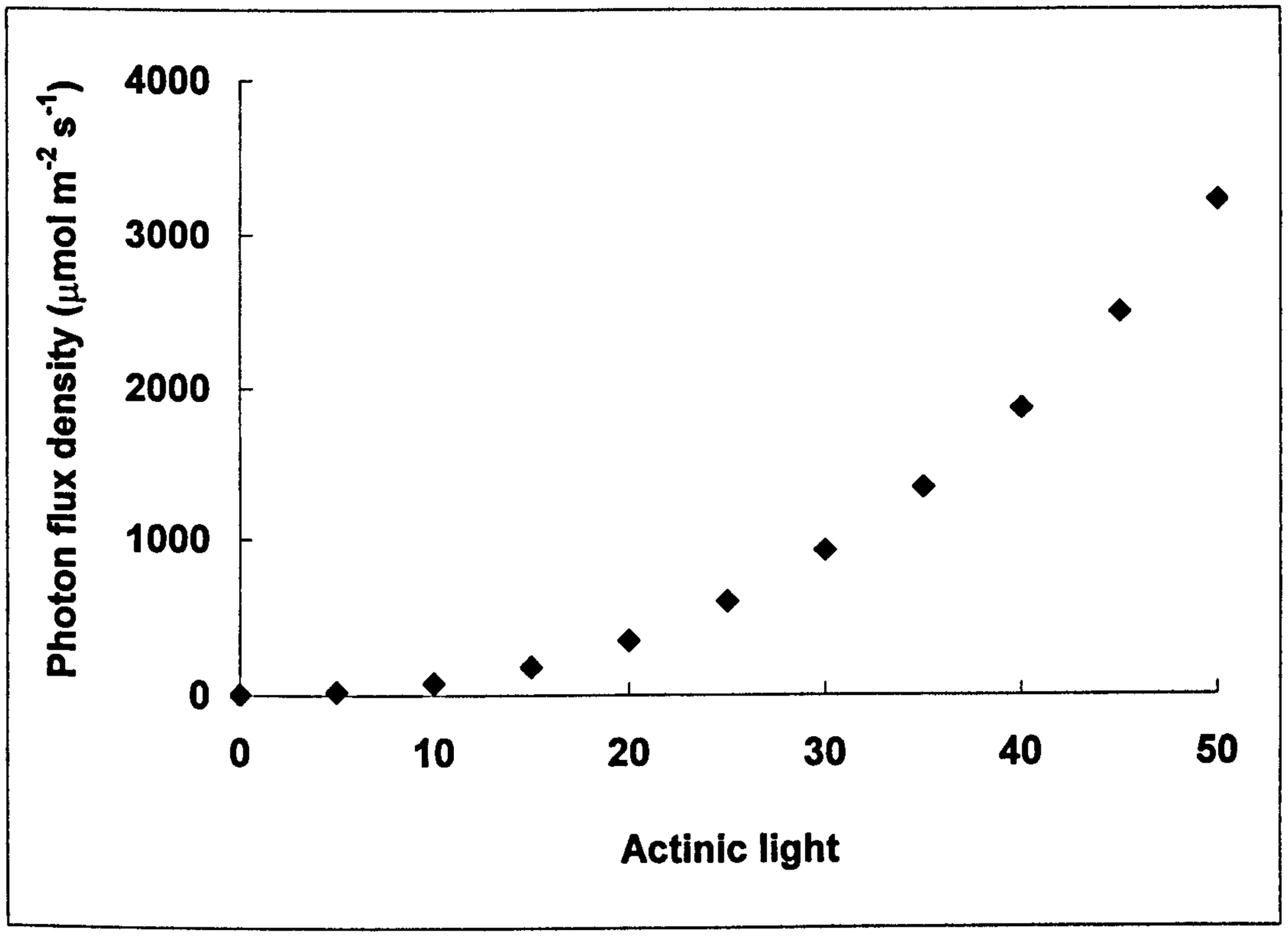



Figure 6.2 : The conversion of actinic light values to photon flux densities when calibrated through a piece of polycarbonate vial.

*Calcification measurements :*

**Theory :**

Calcification rates were measured using the alkalinity anomaly technique, a non-destructive method, whereby the total alkalinity (TA) of seawater is determined from potentiometric titration data. This method for the determination of the calcification rate of corals has been validated by Chisholm and Gattuso (1991). Dickson (1981) describes the total alkalinity of seawater as 'a measure of the proton deficit of the solution relative to an arbitrary defined zero level of proton'. In other words, TA, which is an excess of base over acid in seawater, can be represented as followed :

$$TA = [HCO_3^-] + 2\,[CO_3^{2-}] + [B(OH)_4^-] + [OH^-] - [H^+] \qquad \text{equation 6.1}$$

Although three biological processes (calcification, photosynthesis and respiration) affect the total amount of DIC in seawater, only calcification (or calcium carbonate dissolution) changes the TA of seawater (Chisholm and Gattuso 1991). This is due to the fact that, as shown in equation 6.1, alkalinity is associated with charge balance, so that the addition or removal of neutral $CO_2$ by photosynthesis and respiration do not generate alkalinity changes (Brewer and Goldman 1976). Furthermore, when bicarbonate is the form of DIC taken up by organisms as the source of carbon for photosynthesis, because $CO_2$ is the DIC species fixed by Rubisco, ultimately this bicarbonate uptake is equivalent to the removal of $CO_2$ from seawater. So, in these conditions, the TA of seawater

remains unaffected by photosynthesis (Chisholm and Gattuso 1991). On the other hand, when one mole of $CaCO_3$ is precipitated, TA is reduced by two molar equivalents (equation 1.4). Calcification rates can therefore be determined from measured changes in TA.

TA is estimated by titrating a known volume of seawater with a strong acid until reaching the endpoint corresponding to the formation of carbonic acid from bicarbonate (Dickson 1981). The amount of acid used is proportional to the total amount of hydrogen ions required to neutralise the negative charges of the solution. Gran (1952) proposed a step-wise procedure whereby only a few pHs are recorded between 4.4 and 3.7. The pH readings in this acid range are then used to calculate the Gran function $F_2$ given by :

$$F_2 = [\text{antilog } (5 - pH)] \text{ X } (Vs + v)$$

where $V_s$ and v are the volume (ml) of the sample and of the titrant respectively. The Gran function $F_2$ yields a linear plot against the volume of titrant v, so that, by extrapolation, the point of intersection with the horizontal axis, denoted $v_2$, can be determined. This value $v_2$ marks the alkalinity equivalence end-point. Finally, the TA of the sample is obtained using the following equation :

$$TA \text{ (mEq } l^{-1}) = v_2 \text{ X } (1000 / V_s) \text{ X } n$$

where n is the concentration of the titrant.

**Procedure :**

Nubbins were first rinsed in $NaHCO_3$-free medium. A 20 ml aliquot of seawater was withdrawn from the stock solutions (each containing different DIC concentrations) prior to incubation and constituted the 'before sample', which was kept at 4 °C.

Nubbins *of P. porites* and *Acropora* sp. were incubated in 100 ml plastic beakers and 30 ml polycarbonate vials respectively, in simple Harrison's medium, under white fluorescent lights that provided a photon flux density of 500 $\mu mol\ m^{-2}\ s^{-1}$. Beakers and vials were placed in a water-bath to maintain a constant temperature of 27 °C.

At the end of the incubation period, 20 ml of medium were withdrawn to constitute the 'after sample' and store at 4 °C.

Before TA measurements, samples were allowed to warm up to room temperature. 10 ml of each sample were titrated with 0.01 M HCl using a titrator (EDP Plus, RAININ instrument co, inc., USA). TA was computed as previously explained.

The difference in TA was obtained by subtracting the TA values of the 'after sample' from those of the 'before sample'. TA was calculated using the stoichiometric relationship between $CaCO_3$ precipitation and the TA depletion of seawater.

*Preparation of inhibitors of carbon transport mechanisms:*

Acetazolamide (AZ) was prepared in distilled water in which the pH was raised to 11 for dissolution and then lowered back to 8.2 for the experiment (Beer and Rehnberg 1997).

Ethoxyzolamide (EZ) was also dissolved in distilled water by raising the pH to 11. The pH could not be lowered back to 8.2 because the inhibitor then reprecipitated.

The inhibitor of the anion exchanger 4-Acetamido-, 4'-isothiocyanostilbene-2,2'-disulfonic acid (SITS) was dissolved in distilled water and kept in the dark.

Results :

*Rate of photosynthesis with different DIC concentrations :*

Figure 6.3 shows the rate of $O_2$ evolution of nubbins of *P. porites* with different DIC concentrations incubated for 30 minutes at a PFD of 500 $\mu mol\ m^{-2}\ s^{-1}$. The photosynthetic rate was saturated at 4 mM DIC with values of $O_2$ production of 1.4, 1.9 and 2.0 $\mu mol\ h^{-1}\ \mu g\ chl.\ a^{-1}$ for 2, 4 and 6 mM DIC respectively.

The effect of different DIC concentrations on the photosynthetic rate of excised polyps of *P. porites*, incubated at a PFD of 500 $\mu mol\ m^{-2}\ s^{-1}$, can be seen in figure 6.4. The rate of $O_2$ production increased from 6.0 to 7.7 $\mu mol\ h^{-1}\ polyp^{-1}$ when incubated in 2 and 4 mM DIC respectively.

Figure 6.5 illustrates that the photosynthetic rate of nubbins of *Acropora* sp. is limited at the DIC concentration of normal seawater and only saturates at 6 mM DIC, when incubated for 6 hours at PFD of 500 $\mu mol\ m^{-2}\ s^{-1}$. The rate of $O_2$ production increases from 0.42 to 0.54 $\mu mol\ h^{-1}\ \mu g\ chl.\ a^{-1}$ between 4 and 6 mM DIC.

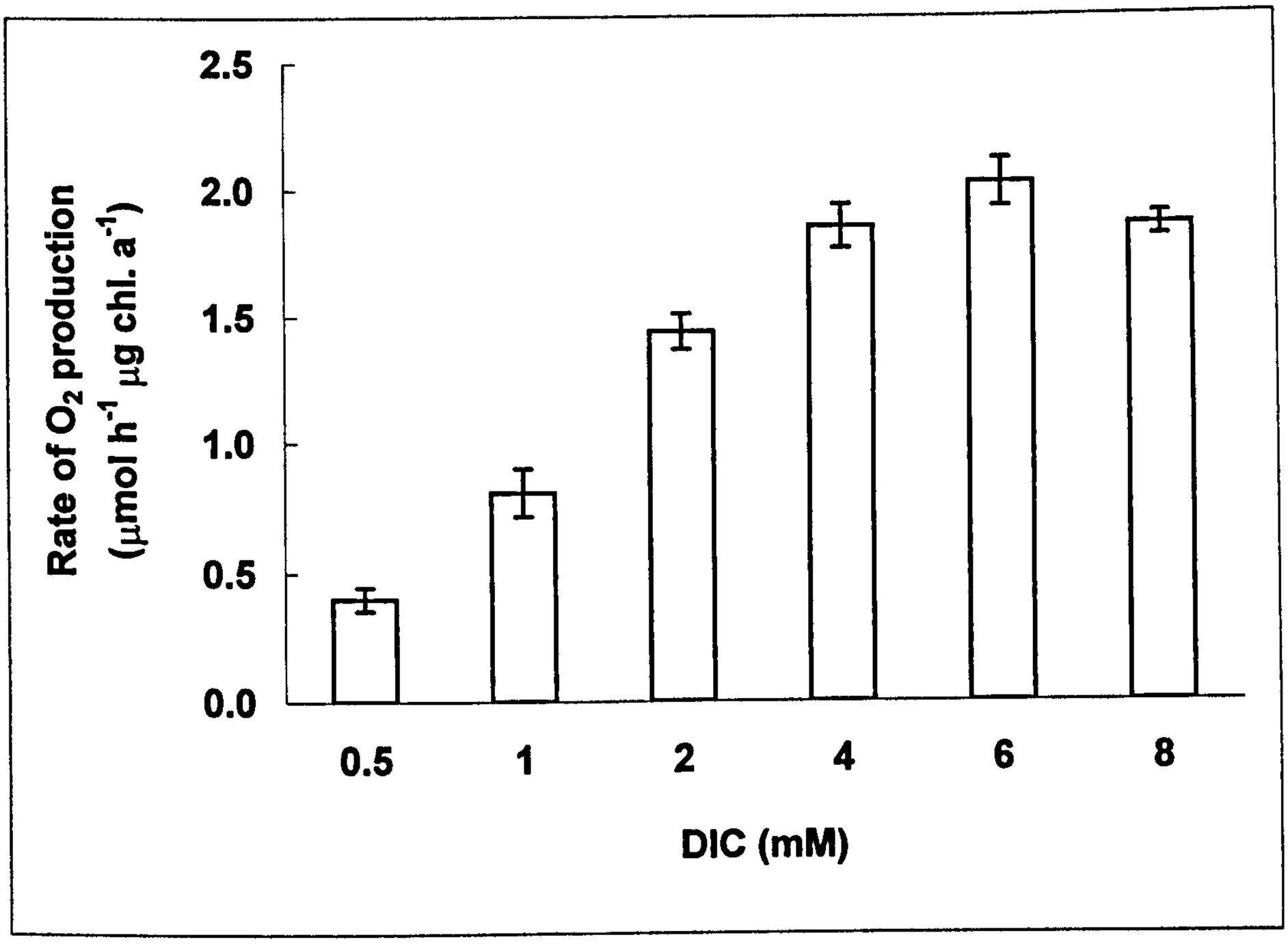



Figure 6.3 : The effect of different DIC concentrations on the photosynthetic rate of nubbins of *P. porites* incubated for 30 minutes at a PFD of 500 $\mu$mol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 4-6.

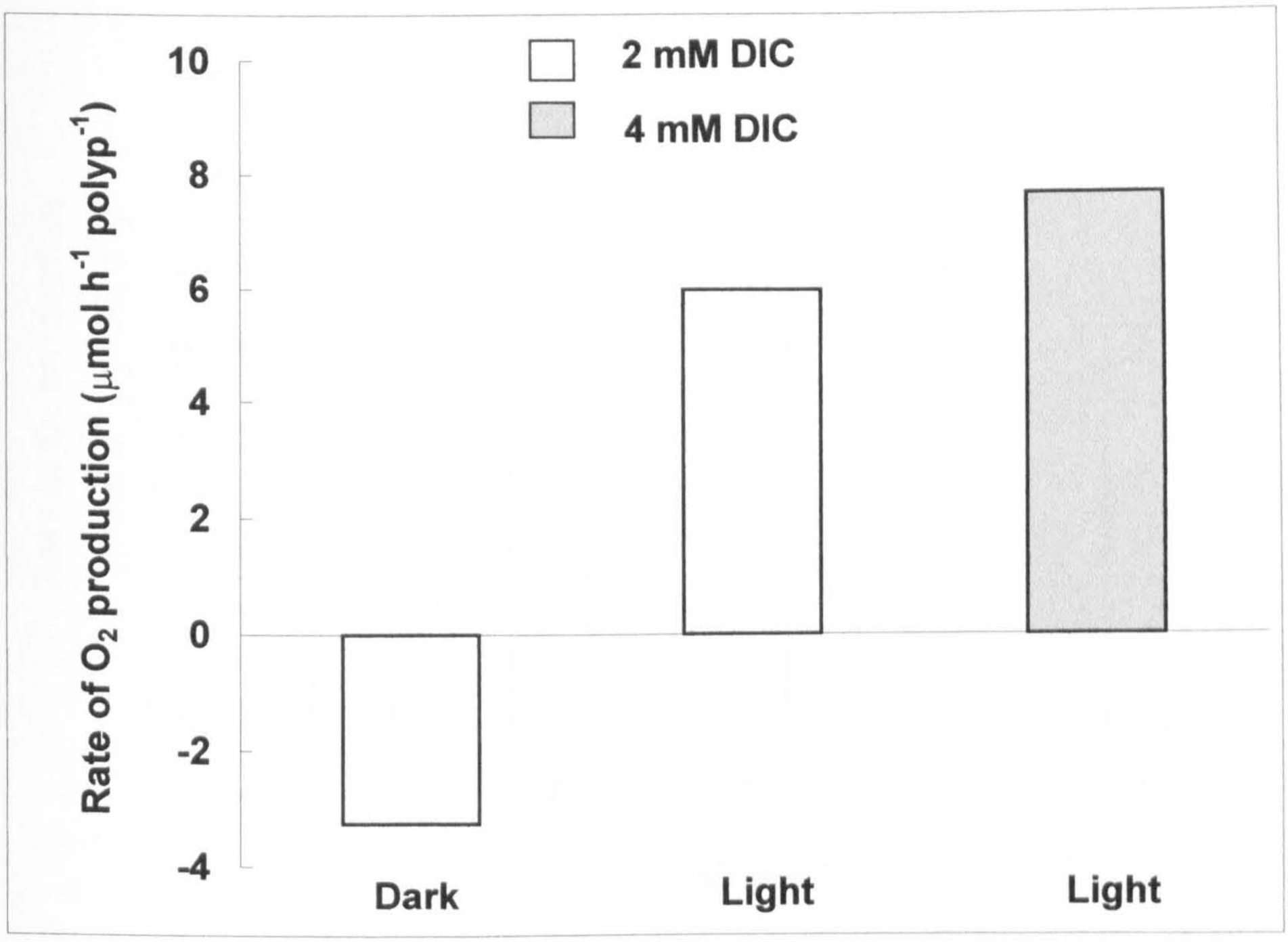



Figure 6.4 : The effect of different DIC concentrations on the photosynthetic rate of excised polyps of *P. porites* incubated at a PFD of 500 $\mu$mol $m^{-2}$ $s^{-1}$.

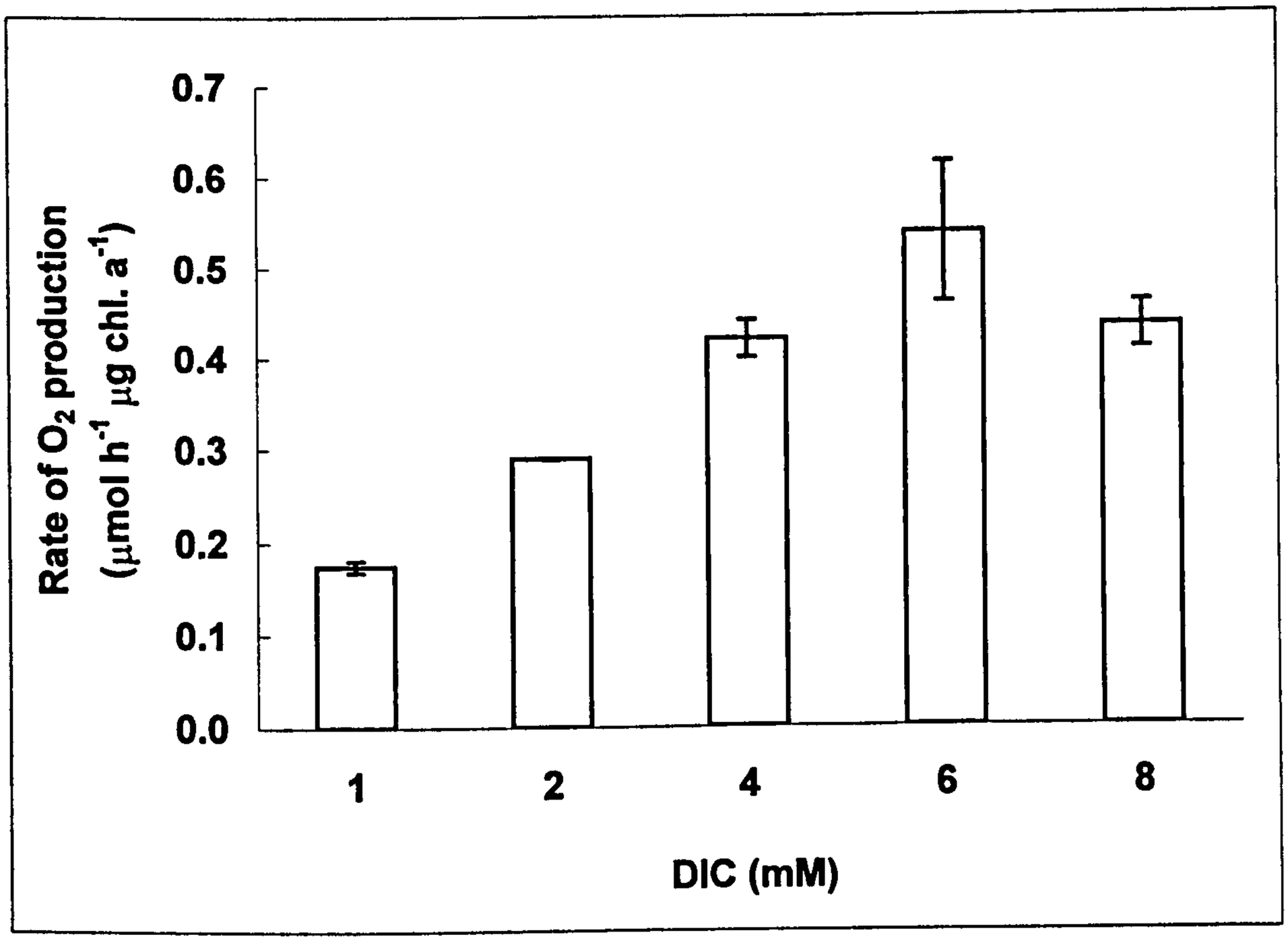



Figure 6.5 : The effect of different DIC concentrations on the photosynthetic rate of nubbins of *Acropora* sp. incubated for 6 hours at a PFD of 500 μmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 3.

*Respiration rate with different DIC concentrations :*

Figure 6.6 shows that the respiration rate of *Acropora* sp. is unaltered by increase in DIC concentration, since the rates of $O_2$ consumption measured after 6 hours of incubation in the dark are not significantly different with different DIC concentrations (1-8 mM).

*Photosystem II activity with different DIC concentrations :*

Figure 6.7 displays the PI curve of nubbins of *Acropora* sp. using induced fluorescence

In subsequent experiments PFD of 110 and 540 μmol $m^{-2}$ $s^{-1}$ were used as limiting and saturating light respectively.

Figure 6.8 shows that at both limiting and saturating light (110 and 540 μmol $m^{-2}$ $s^{-1}$) the quantum efficiency of PSII is enhanced by adding DIC. ΦPSII increases from 0.27 to 0.39 at limiting light and from 0.07 to 0.11 at saturating light with 2 and 8 mM DIC respectively.

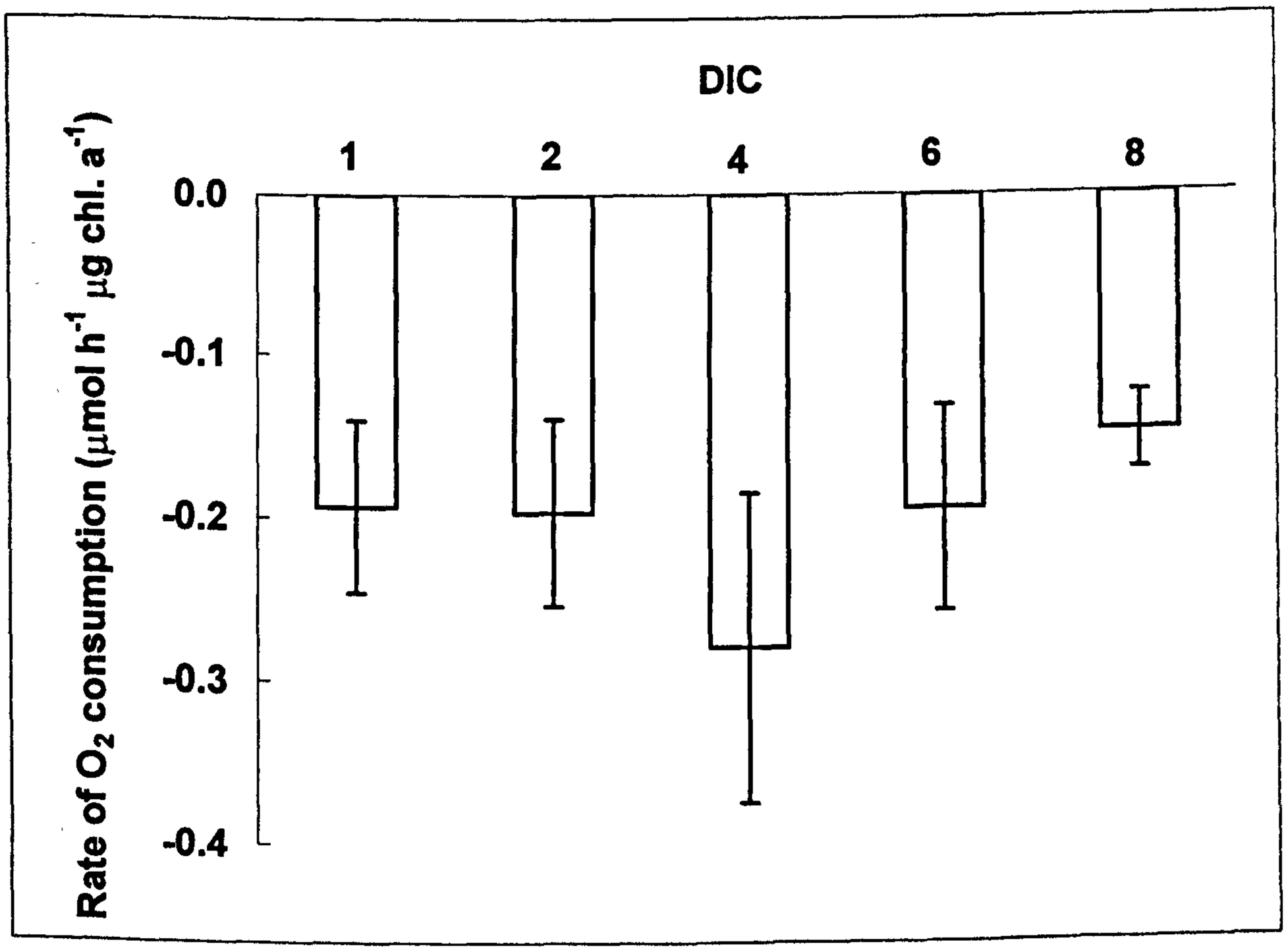



Figure 6.6 : The effect of different DIC concentrations on the respiration rate of nubbins of *Acropora* sp. incubated for 6 hours in the dark.

Data represent means ± SE, n = 3.

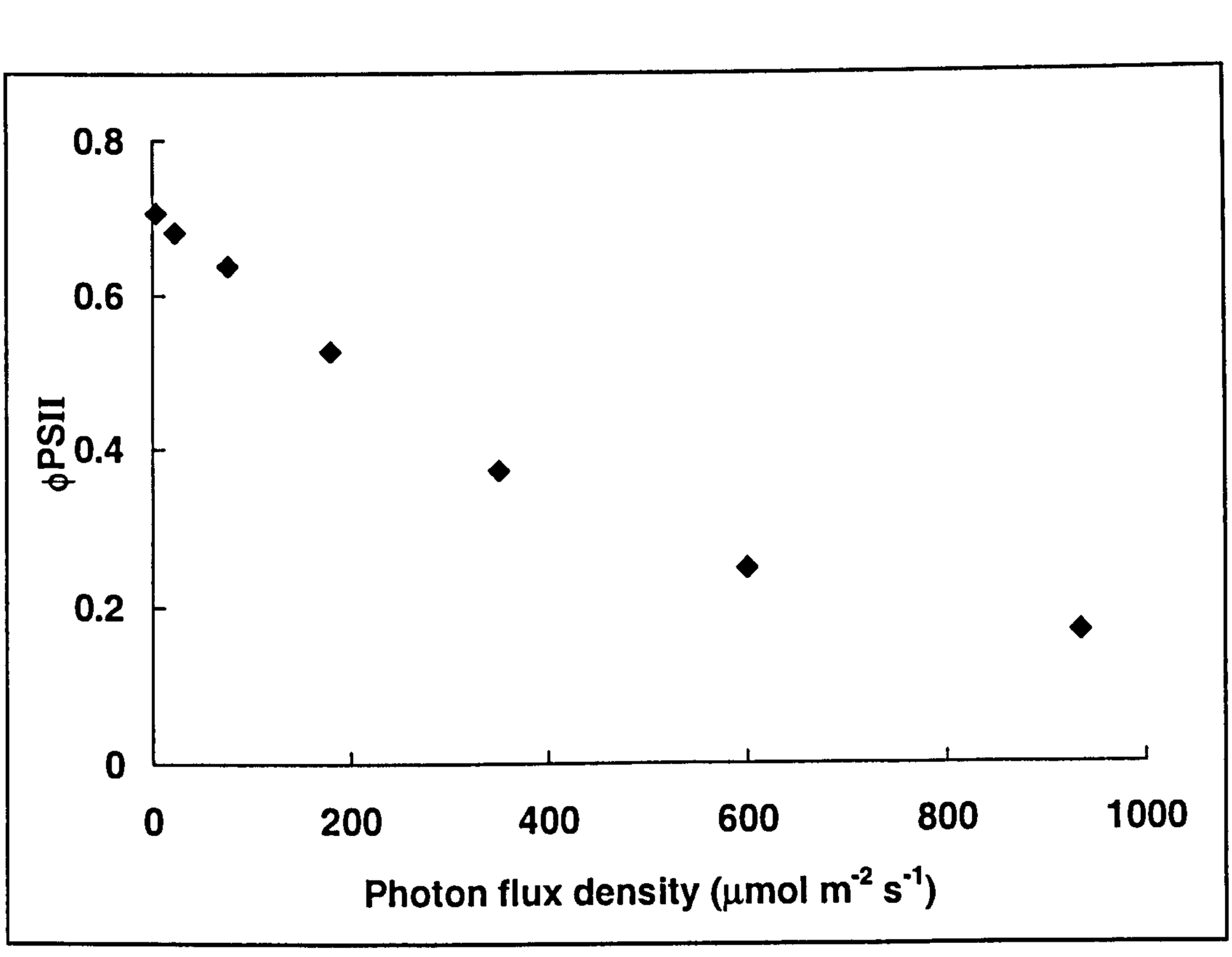



Figure 6.7 : PI curve of nubbins of *Acropora* sp.

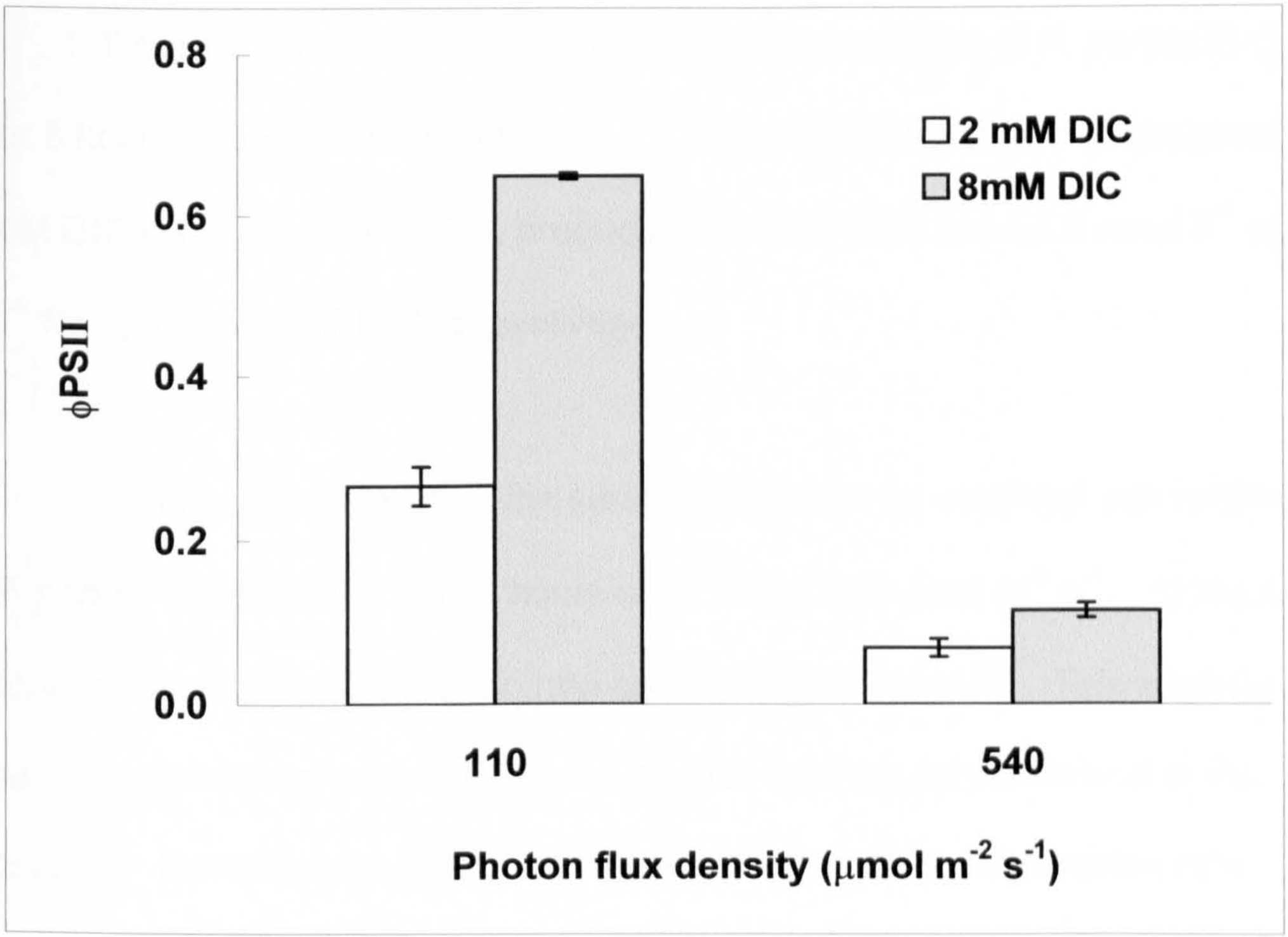



Figure 6.8 : The effect of different DIC concentrations on quantum efficiency of PSII in *Acropora* sp. nubbins measured at a PFD of 110 and 540 $\mu$mol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 3.

*Calcification rate with different DIC concentrations :*

Figure 6.9 shows the rate of calcification of nubbins of *P. porites* incubated for 8 hours at a PFD of 500 μmol $m^{-2}$ $s^{-1}$. The calcification rate saturates at 6 mM DIC with values of $CaCO_3$ production of 17.4, 33.6 and 54.8 nmol $h^{-1}$ μg chl. $a^{-1}$ for 2, 4 and 6 mM DIC respectively.

Figure 6.10 shows that the same pattern can be obtained with nubbins of *P. porites* incubated for only 3 hours at a PFD of 500 μmol $m^{-2}$ $s^{-1}$. At low DIC concentrations, the calcification rate could not be measured. This might be because the difference in TA may be smaller than the detection limit of the titration. Nevertheless, figure 6.10 again shows that the calcification rate increases with added DIC concentrations.

As illustrated on figure 6.11, the calcification rate of nubbins of *Acropora* sp. does not saturate over the range of DIC concentrations used (1-8 mM), when incubated for 6 hours at a photon flux density of 500 μmol $m^{-2}$ $s^{-1}$. The rate of $CaCO_3$ production increases from 2.7 to 4.5 nmol $h^{-1}$ μg chl $a^{-1}$ between 6 and 8 mM DIC.

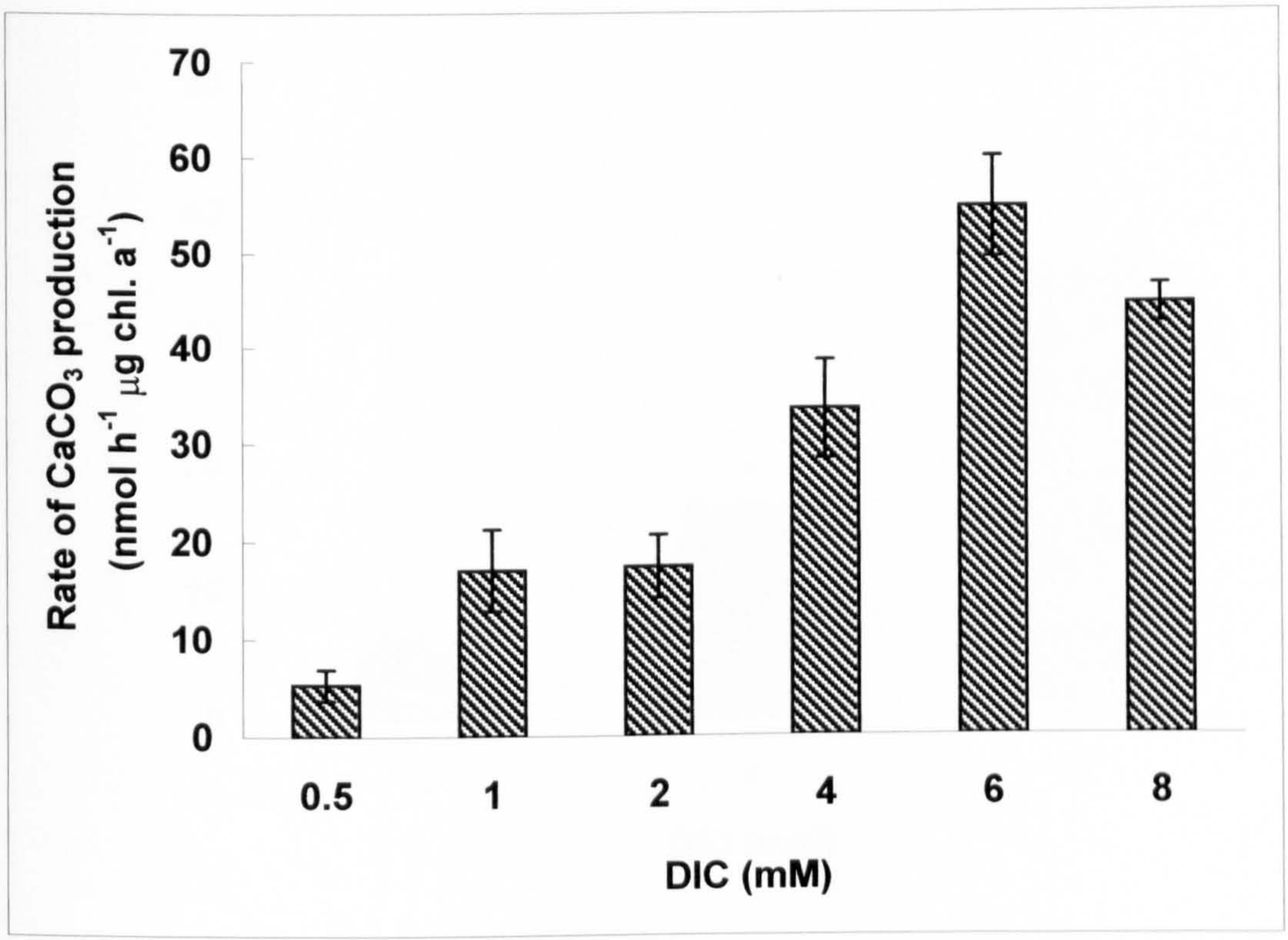



Figure 6.9 : The effect of different DIC concentrations on the calcification rate of nubbins of *P. porites* incubated for 8 hours at a PFD of 500 µmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 3.

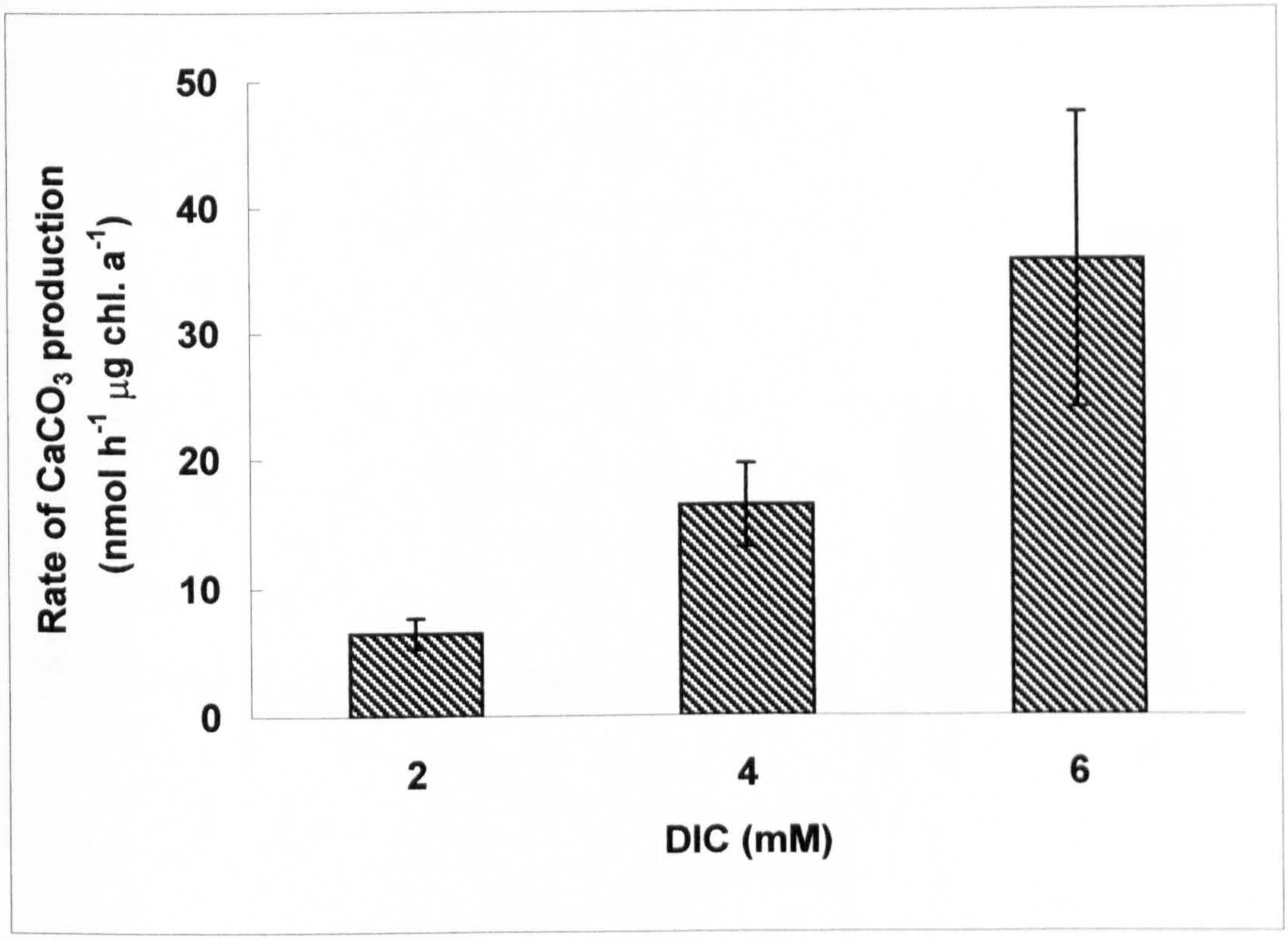



Figure 6.10 : The effect of different DIC concentrations on the calcification rate of nubbins of *P. porites* incubated for 3 hours at a PFD of 500 µmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 3.

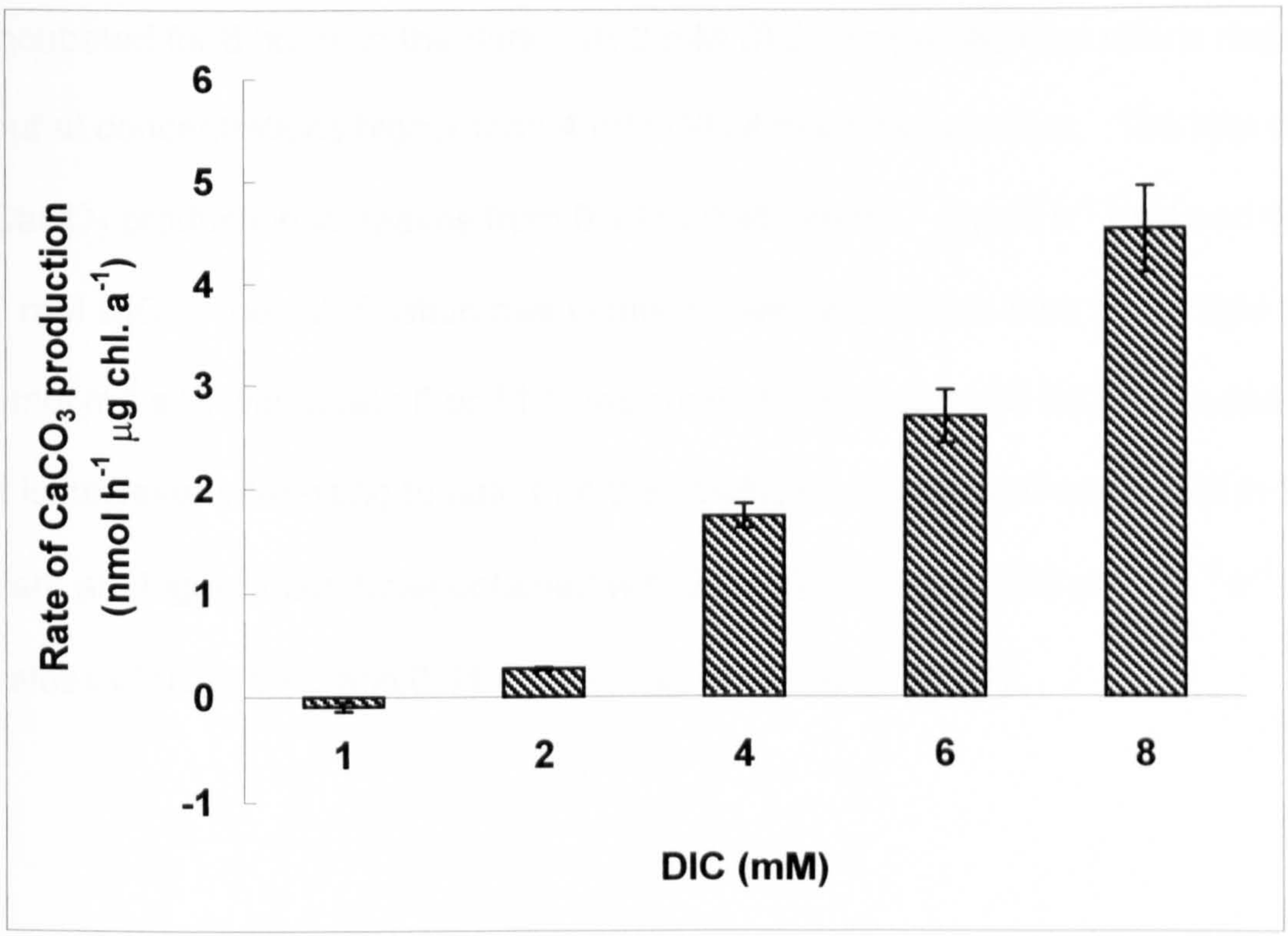



Figure 6.11 : The effect of different DIC concentrations on the calcification rate of nubbins of *Acropora* sp. incubated for 6 hours at a PFD of 500 µmol $m^{-2}$ $s^{-1}$. Data represent means ± SE, n = 3.

Rather unexpectedly, Figure 6.12 also shows that increased DIC concentrations enhance the calcification rate of nubbins of *Acropora* sp. incubated for 6 hours in the dark. At 2 mM DIC, the calcification rate is negative, but at concentrations higher than 4 mM DIC it becomes positive. The rate of $CaCO_3$ production increases from 0.13 to 0.46 nmol $h^{-1}$ μg chl $a^{-1}$ between 4 and 6 mM DIC. The calcification rate is much lower in the dark than in the light (500 μmol $m^{-2}$ $s^{-1}$), with a rate 6 or 11 times smaller at 6 and 8 mM DIC respectively. It is however interesting to note that the calcification rates at 6 and 8 mM in the dark are higher than those obtained with 2 mM in the light (500 μmol $m^{-2}$ $s^{-1}$), with values of 0.28, 0.46 and 0.41 nmol $h^{-1}$ μg chl $a^{-1}$ respectively.

*Identification of carbon transport mechanisms :*

Figure 6.13 shows the effect of adding 100 μM AZ and 100 μM EZ on photosynthesis in nubbins of *P. porites*. AZ is a membrane-impermeable specific inhibitor of carbonic anhydrase (CA), which therefore only stops external CA activity. EZ is capable of penetrating the membrane and thus inhibits both internal and external CA. The percentage inhibition of the rate of $O_2$ evolution is high with both chemical probes, with total inhibition of the photosynthetic rate with EZ and 77 % inhibition with AZ. The chemicals and not the rise of pH causes this inhibition since when 40 μl of distilled water of pH 8.2 or 11 were introduced in the respirometer, 0 or only 4 % of inhibition were recorded respectively.

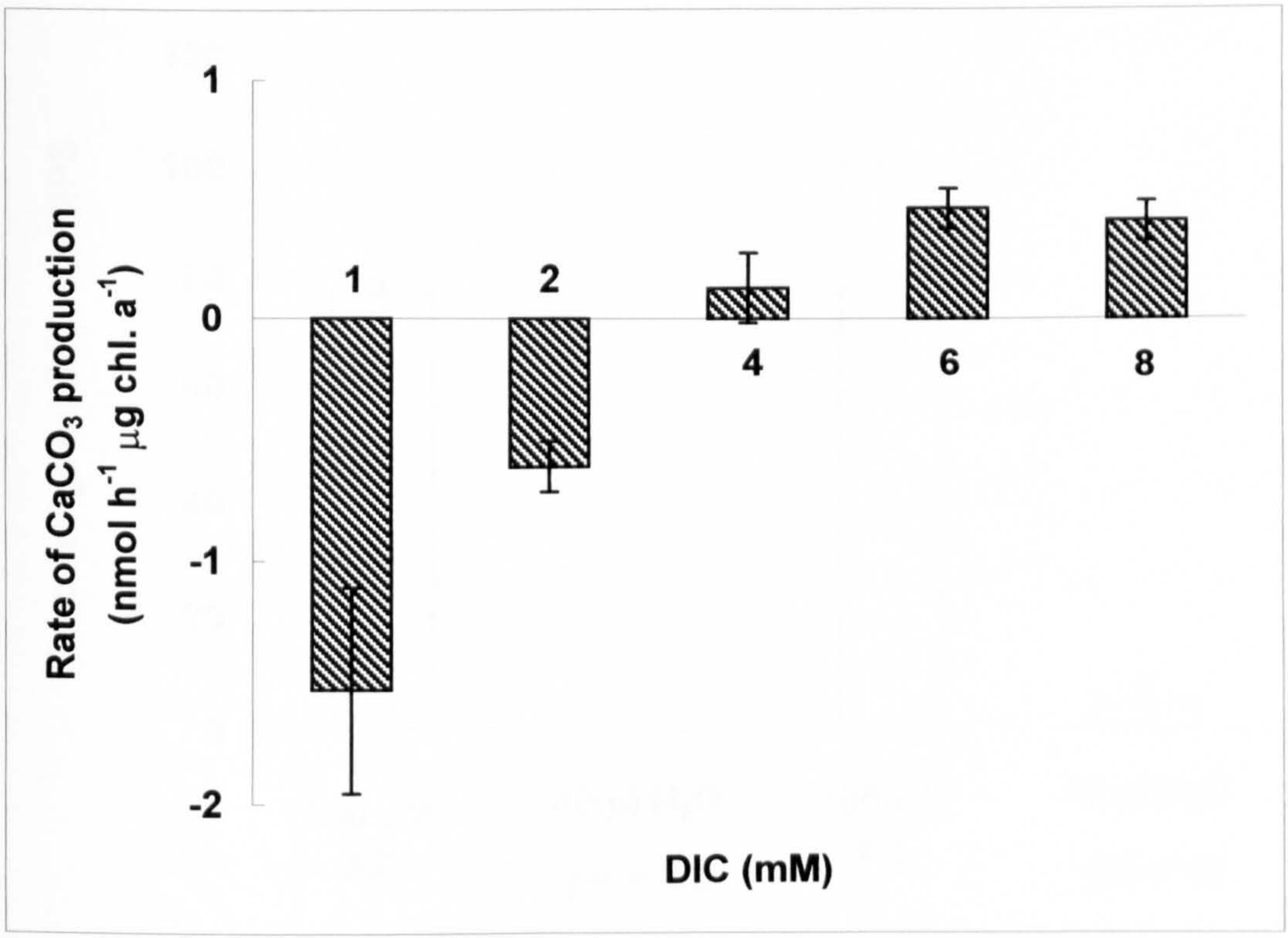



Figure 6.12 : The effect of different DIC concentrations on the calcification rate of nubbins of *Acropora* sp. incubated for 6 hours in the dark.

Data represent means ± SE, n = 3.

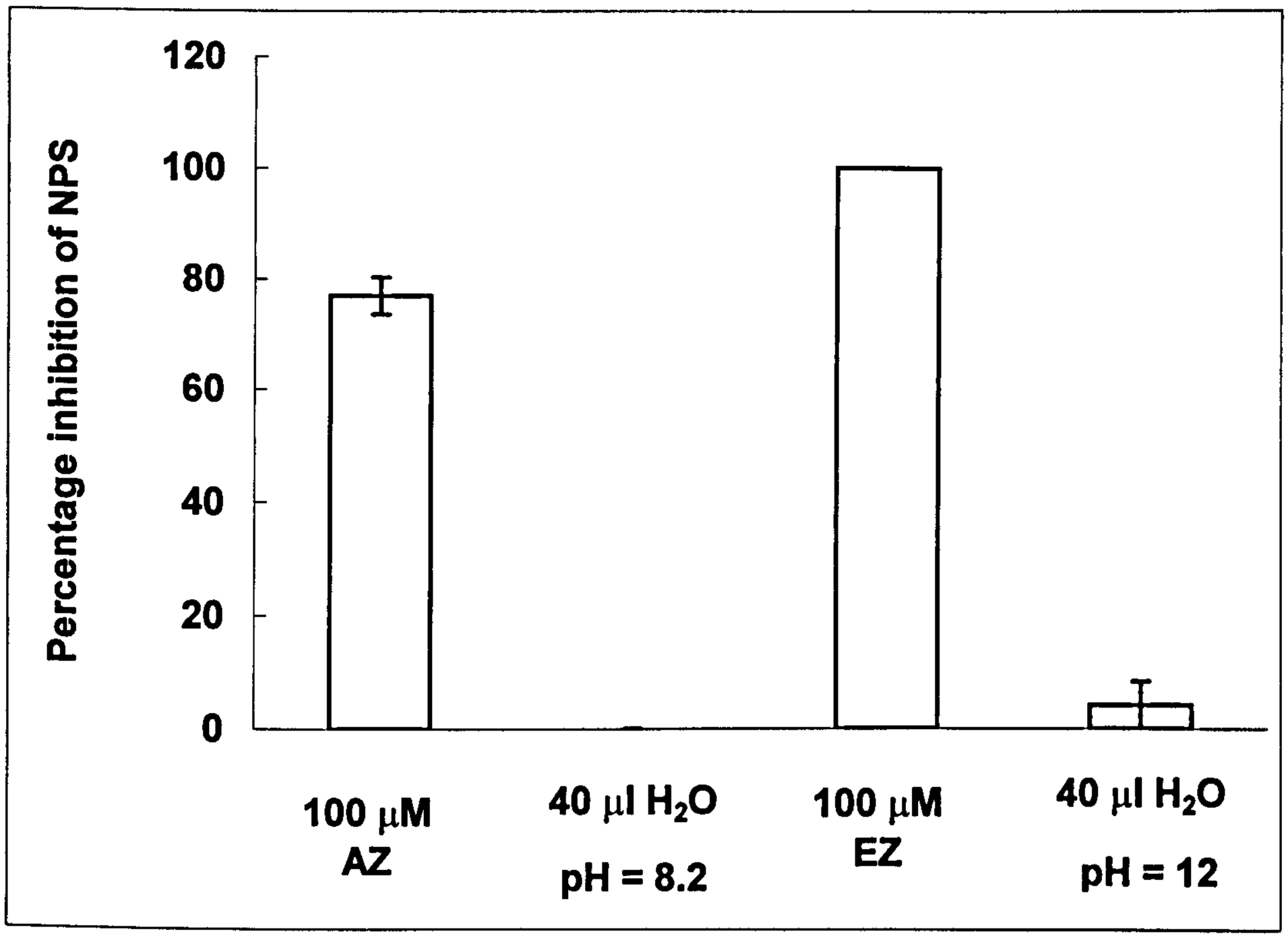



Figure 6.13 : The percentage inhibition of photosynthesis in nubbins of *P. porites* by 100 μM AZ and EZ and 40 μl of distilled water of pH 8.2 and 11. Data represent means ± SE and n = 3.

Figure 6.14 represents the percentage inhibition of photosynthesis of nubbins and excised polyps of *P. porites* at two different concentrations of AZ, 50 and 100 μM. The photosynthetic rate is much lower in nubbins than in polyps at both concentrations of inhibitors. Moreover, 50 μM is sufficient for total inhibition of photosynthesis in nubbins, but not in polyps.

Figure 6.15 displays the percentage inhibition of photosynthesis of excised polyps of *P. porites* with 3 different SITS concentrations (0.5, 1 and 1.5 mM). Photosynthetic inhibition becomes larger with increasing SITS concentrations.

Figure 6.16 shows the effect of 50 μM AZ and 1.5 mM SITS on the photosynthetic rate of nubbins of *Acropora* sp. incubated for 2 hours at a PFD of 500 μmol $m^{-2} s^{-1}$. Both inhibitors seem to reduce photosynthesis, but the statistical analysis demonstrates that this is not true, since both t-tests give a p-value higher than 0.05 with value of 0.51 and 0.06 respectively.

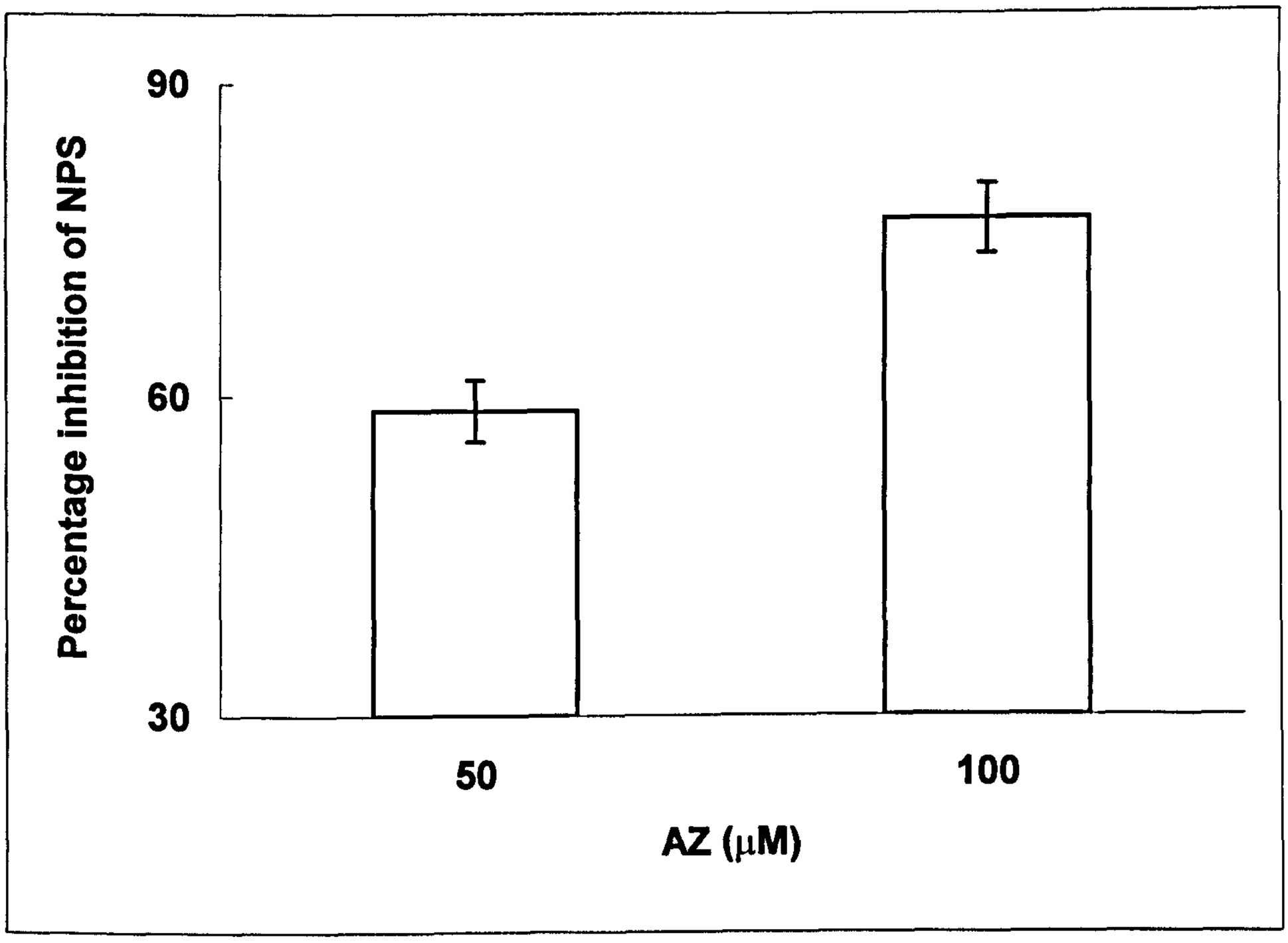



Figure 6.14 : The percentage inhibition of photosynthesis of nubbins and excised polyps of *P. porites* at two different concentrations of AZ, 50 and 100 μM. Data represent means ± SE and n = 3.

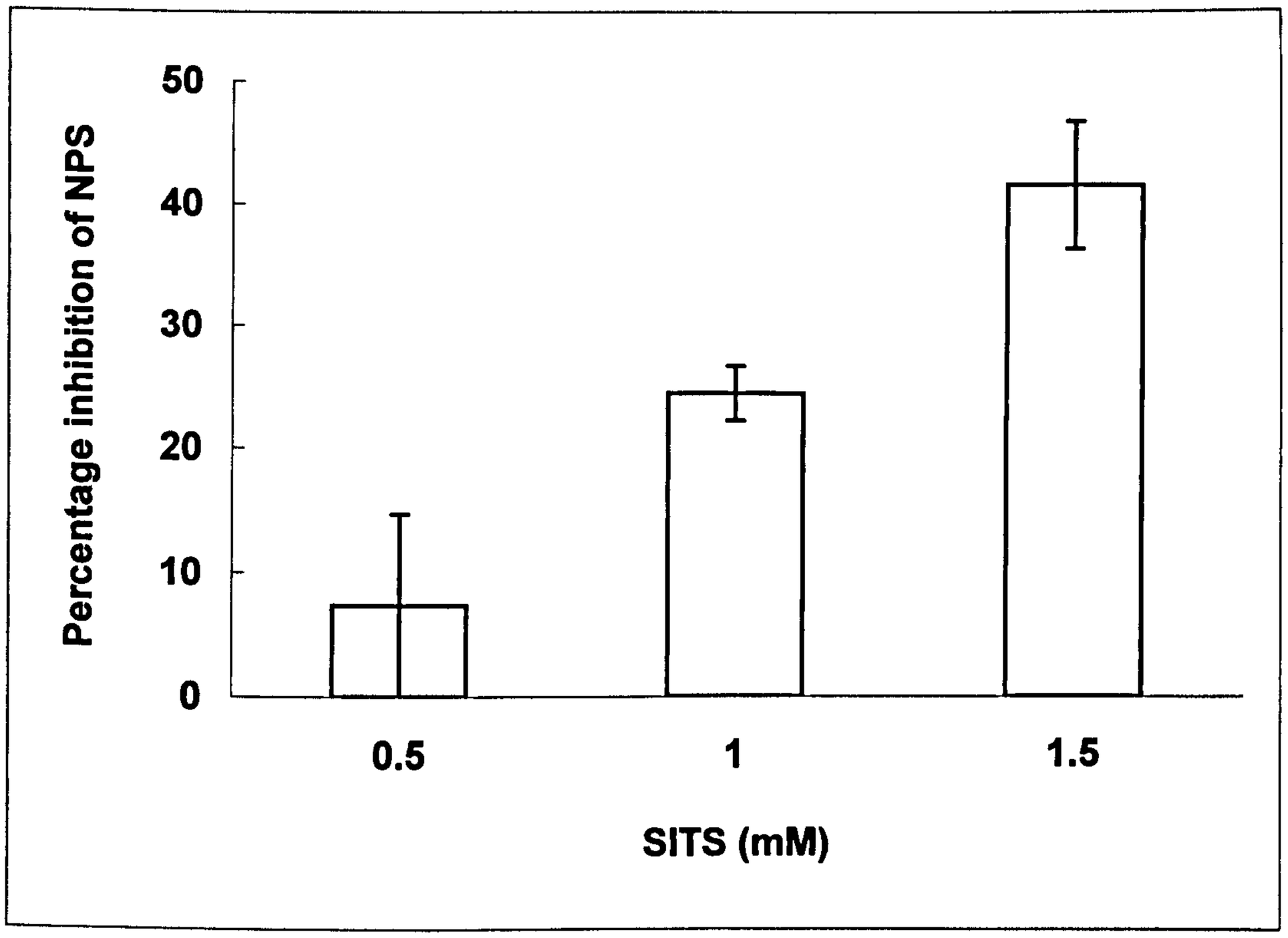



Figure 6.15 : The percentage inhibition of photosynthesis of excised polyps of *P. porites* with 3 different SITS concentrations (0.5, 1 and 1.5 mM). Data represent means ± SE and n = 4.

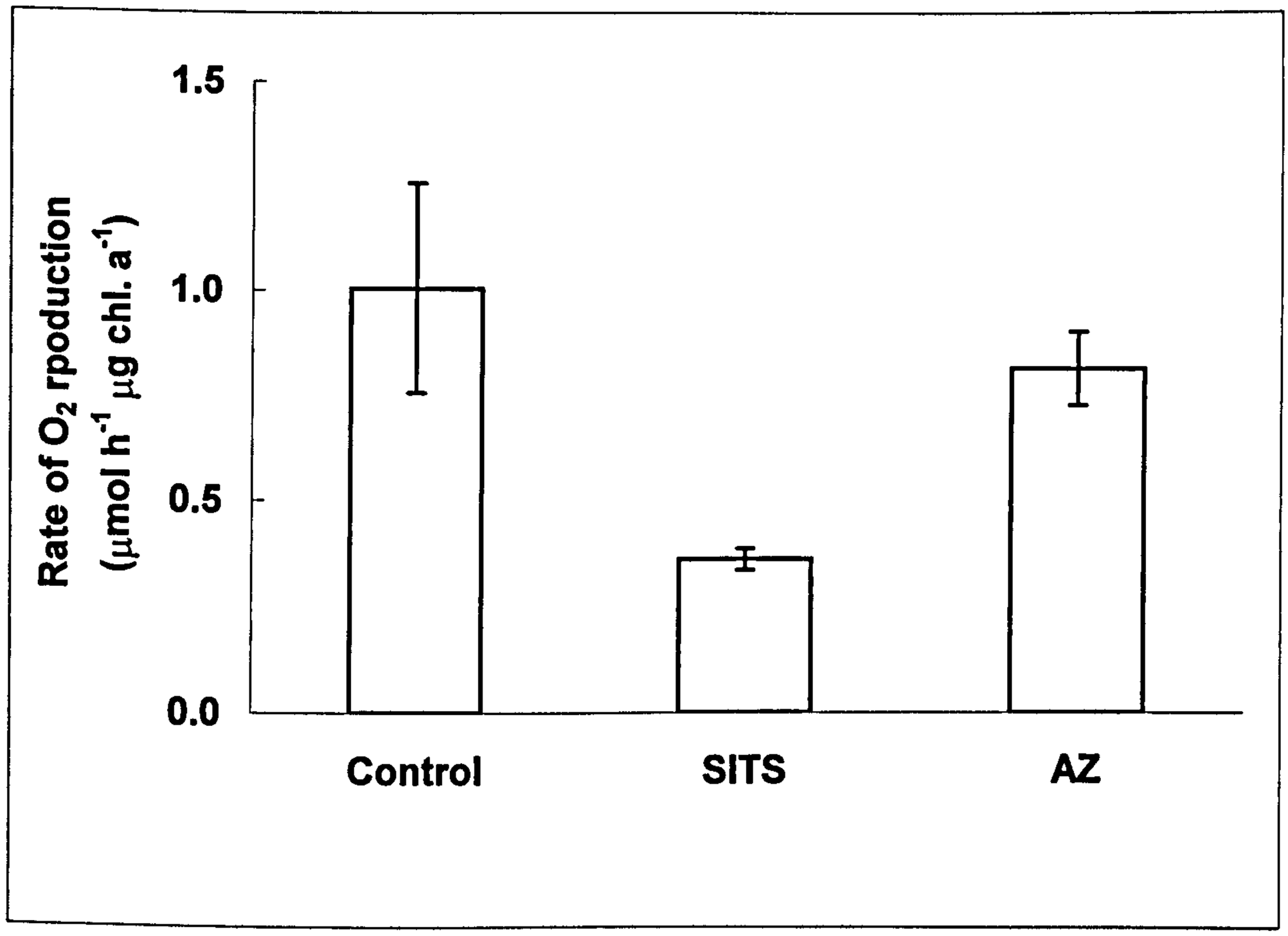



Figure 6.16 : The effect of 50 μM AZ and 1.5 mM SITS on the photosynthetic rate of nubbins of *Acropora* sp. incubated for 2 hours at a PFD of 500 μmol $m^{-2}$ $s^{-1}$. Data represent means ± SE and n = 3.

Discussion :

Hermatypic corals are reef-building anthozoans that bear within their endodermic cells symbiotic photosynthetic microalgae called zooxanthellae. Photosynthesis and calcification, which occur in the oral gastroderm and the aboral epiderm respectively, are spatially separated (Vandermeuler and Muscatine 1974), with at least 25 μm between each site. Calcium carbonate deposition, in the form of the structurally strong crystal, aragonite, takes place in the vicinity of calicoblastic cells.

Several studies have established that calcium uptake was necessary for calcification (Tambutté *et al.* 1996, Krishnaveni *et al.* 1989 and Chalker 1976), but there is no agreement on the method of transepithelial calcium transport. Wright and Marshall (1991) provided evidence of active oral and aboral mediation of calcium, while Tambutté *et al.* (1995), who measured $^{45}Ca$ efflux in *S. pistillata*, have established the occurrence of passive oral uptake of calcium. Although calcium uptake mechanisms remain somewhat unclear, the calcium pathway to the site of calcification is better understood (Gattuso *et al.* 1999). Marshall (1996a) and Tambutté *et al.* (1996) have demonstrated the presence of transcellular transport of calcium to the calicoblastic epithelium, which is performed by L-type voltage-dependent calcium channels. In addition, Allemand *et al.* (1998b) have shown that calcium is supplied to the calcification site by the exocytosis of vesicles, rather than by an intracellular transport. This is in agreement with the findings of Isa (1986) and Isa and Okasaki (1987), who have

shown that the skeletal organic matrix of corals is rich in calcium binding substances. Specialised transport proteins therefore achieve the mediation of calcium to the calcifying site in corals.

Data concerning the source and transport of carbon for calcification in corals are also limited (Gattuso *et al.* 1999). Contradictory results arising from methodological problems have caused arguments about whether the carbon used for calcification originates predominantly from direct seawater uptake (Al Moghrabi *et al.* 1996) or from metabolic $CO_2$ (Furla *et al.* 2000, Muscatine *et al.* 1984, Erez 1978).

Marubini and Thake (1999) established that the calcification rate of *P. porites* doubles with the addition of 2 mM DIC to normal seawater. They monitored calcification by determining the skeletal weight using the buoyant weight technique (Davies 1989). This method requires at least a few days of incubation and preferably weeks to gain results. In the present study, the alkalinity anomaly technique (Chisholm and Gattuso 1991) was employed to measure the calcification rate of coral nubbins in short term incubations of only a few hours. Also in this work, corals were incubated in synthetic seawater, while Marubini and Thake (1999) used natural seawater. Despite these methodological differences, the two methods cross-calibrate well since data presented in this report confirm the doubling of the calcification rate of *P. porites* nubbins when incubated from 2 to 4 mM DIC.

Calcification rates of two scleractinian corals, *P. porites* and *Acropora* sp., incubated in seawater containing different DIC concentrations (0.5-8 mM), were measured. Dose-responses for DIC and calcification show that saturation occurs at different concentrations in these two coral species. This questions the importance of the saturation state theory (see Gattuso *et al.* 1999 for review), whereby biogenic calcification is regarded as a consequence of the aragonite saturation state ($\Omega_{aragonite}$). Stumm and Morgan (1981) described the saturation state of calcium carbonate as being proportional to the activity of $Ca^{2+}$ and $CO_3^{2-}$ ions in seawater (see Chapter 1 for details). Several authors argue that $\Omega_{aragonite}$ alone controls calcification in corals (Marubini *et al.* 2001, Langdon *et al.* 2000, Gattuso *et al.* 1998). If mineralisation merely took place as the result of the chemical activity of calcium and carbonate ions in seawater, calcification rates of all coral species would saturate at the same DIC concentration. Clearly, the difference in the DIC concentration at which the calcification rates of *P. porites* and *Acropora* sp. saturate implies that the aragonite production of those corals is under strong biological control and that factors other than $\Omega_{aragonite}$ alone exert control over the process of skeletogenesis.

It could be argued that by changing the bicarbonate concentration of seawater, carbonate concentration was increased and $\Omega_{aragonite}$ was thus manipulated. pH was however always adjusted to 8.2 after bicarbonate addition and although the carbonate concentration increased at this pH, over 80% of the DIC is in the form of bicarbonate (Stumm and Morgan 1996). Also, in corals, no carbonate diffusion or transport mechanisms have been identified (Gattuso et al. 1999), whereas bicarbonate transport mechanisms are well-characterised. This

suggests that in addition to the well-established influence of the saturation state of aragonite, calcification in corals is also affected by the bicarbonate concentration of seawater.

Furthermore, as seen in detail in Chapter 5, calcium carbonate only precipitates in supersaturated conditions, but metals behave differently in inorganic and organic environments (Westbroek, 1985). When external crystallisation takes place, i.e. outside the organism and in direct contact with seawater, it is possible that calcification rates could be explained by the saturation state theory. It is, however, not easy to ascertain whether simple mineralisation or biomineralisation is happening. For instance, although alternative macroscopic calcified and non-calcified zones been have identified at the external surface of the giant cell of the freshwater alga, *Chara* (McConnaughey 1991, McConnaughey and Falk 1991), mineralisation is biologically controlled by a series of cis- and trans-mechanisms (McConnaughey 1994). In fact, in *Chara*, the carbon derived from water enters the cell through the non-calcified regions of the surface and is transported to the calcification sites (McConnaughey 1994). In this example, carbon used for calcification is dependent on different carbon transport mechanisms and not simply by $\Omega_{aragonite}$.

Since carbonate-supersaturated conditions are required at the calcification site, carbon transport mechanisms are likely to play a major role in the biological control of the calcification of corals. Sea anemones host zooxanthellae similar to those of corals. As they do not calcify, their bodies resemble a simple bag made of a three layers-wall (ectoderm, endoderm and mesogloea). Their tentacles can

be easily manipulated, which facilitates transepithelial flux experiments, such as the kinetic measurements using an Ussing chamber carried out by Furla *et al.* (1998a). Sea anemones have been widely used as a model to investigate the carbon transport mechanisms of symbiotic anthozoans. Several studies on *Anemonia viridis* and *Aiptasia pulchella* have established that the epithelium of sea anemones has a functional polarity, which is linked to the presence of many carbon transport mechanisms (Furla *et al.* 2000, Furla *et al.* 1998a, Benazet-Tambutté *et al.* 1996a and b, Weis 1993, Weis 1991). These studies have demonstrated that many carbonic anhydrases (CA), a $Cl^-$ / $HCO_3^-$ exchanger and an $H^+ATP_{ase}$ take part in the carbon concentrating mechanism (CCM) that maintains $CO_2$ supply to Rubisco. The following paragraph summarises the different steps leading to the DIC supply of zooxanthellae in *A. viridis*, as shown by Furla *et al.* (2000). Protons are secreted by an $H^+$ / $ATP_{ase}$, which promotes the formation of $CO_3^-$ in the seawater surrounding the sea anemone. An external CA localised at the surface of the epithelium performs the conversion of $HCO_3^-$ into $CO_2$, as well as the uptake of carbon. An internal CA rehydrates $CO_2$ into $HCO_3^-$ to limit the loss of $CO_2$. A $Cl^-$ / $HCO_3^-$ exchanger accomplishes the transport of carbon to the zooxanthellae. Finally, another internal CA situated in the immediate vicinity of the symbiots retransforms $HCO_3^-$ into $CO_2$. The pathway of DIC from seawater to zooxanthellae has been identified in great details in sea anemones, but the mechanisms of carbon transport from ectoderm to endoderm remain unknown, as well as the mode of regulation of the whole carbon system (Furla *et al.* 2000).

Carbon uptake and transport mechanisms of corals are likely to resemble those of sea anemones, but since calcification renders their identification more difficult, the detailed localisation of the different carbon transport mechanisms of corals has not yet been achieved. Nevertheless, both carbonic anhydrase and anion transporter activity has been detected in various coral species. Al-Moghrabi *et al.* (1996) and Goiran *et al.* (1996) have shown that a $Cl^-$ / $HCO_3^-$ exchanger and a $Na^+$ / $HCO_3^-$ cotransporter participate in carbon transport in *G. fascicularis*. Al-Moghrabi *et al.* (1996) have also identified the presence of CA in the animal tissues of *Galaxea fascicularis*. Furla *et al.* (2000) have shown that CA and an anion exchanger also contribute to the DIC supply of photosynthesis in *S. pistillata*. In addition, the activity of CA in animal tissues is directly correlated with the presence of zooxanthellae in the coral. Indeed, after examining 29 different species of cnidarians, Weis *et al.* (1989) have concluded that CA was more active in the animal tissues of symbiotic than non-symbiotic species.

The involvement of a CA and an anion exchanger in the bicarbonate supply to calcification has also been characterised in corals. Indeed, the calcification rate of *S. pistillata* is greatly inhibited by the addition of 400 μM DIDS (Furla *et al.* 2000). So, in *S. pistillata*, $CaCO_3$ precipitation requires the mediation of carbon transport by an anion carrier protein. Tambutté *et al.* (1996) and Isa and Yamazato (1984) have also established that CA was localised in the calicoblastic epithelium of *S. pistillata* and *Acropora hebes* respectively. When Kingsley and Watabe (1987) studied the ahermatypic gorgonian, *Leptogorgia virgulata*, they discovered that CA activity was localised on the vacuole

surrounding $CaCO_3$ spicules, in the axial epithelium and scleroblast. The authors have therefore concluded that CA was strongly associated with the mechanisms leading to calcification in this coral.

Data from the current study provide further evidence that carbonic anhydrase and anion exchanger both participate in DIC uptake in corals. Inhibitor experiments show that these two carbon transport mechanisms were active in *P. porites*. Similarly, in *Acropora* sp., results presented here suggest that a CA and an anion carrier protein may take part in carbon uptake mechanisms. The latter result cannot however be considered conclusive because the effects on photosynthesis of the specific inhibitors of CA and of anion exchanger were not statistically different from the control, eventhough the pattern indicates a diminution of the rates. Future work should carried out experiments with more replicates to establish statistically if CA and anion exchanger are active in *Acropora* sp..

Interestingly, an unexpected result emerged from the inhibitor study on excised polyps of *P. porites*. Photosynthesis was inhibited by 24 and 77 % with the addition of 1 mM SITS and 100 μM AZ respectively. The combined effect of the inhibitors reduces photosynthesis by more than 100 %. This result implies that bicarbonate uptake is not carried out by one or the other transport mechanism, but that it involves both. A sequential model of DIC transport is therefore proposed to explain those data, whereby the anion exchanger depends on CA to remove bicarbonate from seawater. Recent studies have demonstrated that a carbonic anhydrase II binds to the cytoplasmic terminal

domain of the anion exchange 1 protein of human erythrocytes and that this association allows maximal bicarbonate transport by the anion-exchanger (Sterling *et al.* 2001, Reithmeier 2001, Vince *et al.* 2000, Vince and Reithmeier 2000). As with humans, corals may posses a metabolon (i.e. 'a weakly associated complex of sequential metabolic enzymes', Reithmeier 2001), whose components are carbonic anhydrase and anion exchange 1 protein. Although the picture is becoming clearer in humans, in corals, the characterisation of the nature of the association of these two carbon uptake mechanisms remains necessary. Nevertheless, the data presented in this report, suggest a strong interaction between the carbon transporters in corals.

This study has also established that a higher concentration of AZ is required to reach high percentages inhibition of photosynthesis in excised polyps than in nubbins of *P. porites*. When observed under a microscope, excised polyps appear highly curled, which indicates that a smaller area of the surface area is exposed to seawater. This suggests that the absorption of inhibitors, and also of bicarbonate ions, depends on the exposed surface area of tentacles. If the inhibitors were absorbed through the mouth, the percentage inhibition of photosynthesis would be independent from the tentacle exposed surface area. Data presented here show that this is not the case, since inhibition is higher when more surface epithelium is exposed to seawater. So, in corals, bicarbonate uptake occurs mainly by transepithelial transport mechanism and not by water exchanged through the mouth. This contradicts the finding of Bénazet-Tambutté *et al.* (1996a) that, in *A. viridis*, bicarbonate uptake was mostly performed through the mouth and not through the epithelium.

Zooxanthellae have a high rate of photosynthesis (Ralph *et al.* 1999, Falkowski *et al.* 1984). Burris *et al.* (1983) and Goiran *et al.* (1996) have determined that the ambient DIC concentration of seawater saturates photosynthesis in corals. In contrast, measurements of $O_2$ production in *Goniopora* sp. showed that photosynthesis is immediately enhanced when extra DIC is added to normal seawater (B. Thake, personal communication 2000). Moreover, for a decade, many authors (Lesser *et al.* 1994, Weis 1993, Dubinski *et al.* 1990, Weis *et al.* 1989, and Muscatine *et al.* 1989) have speculated about DIC limitation in corals. The present study has demonstrated clearly that the photosynthetic rates of two species of hermatypic corals, *P. porites* and *Acropora* sp., are limited by the ambient DIC concentration of seawater of 2 mM. The data presented here establish that DIC also stimulates the photochemistry of corals directly. PSII activity of *Acropora* sp. was increased after one hour of incubation at 8 mM DIC. DIC has therefore a dual effect on photosynthesis in corals; $O_2$ production is not only increased when more substrate is available for Rubisco, but bicarbonate ion also stimulates the photochemistry. Although net photosynthesis is enhanced by DIC addition, experiments in which nubbins of *Acropora* sp. were incubated in the dark have established that the respiration rate remains constant with different DIC concentrations.

Since DIC promotes photosynthesis, it was possible that the increases in calcification rate with added DIC concentrations was caused by the enhancement of the photosynthetic rate rather than by independent and direct DIC stimulation. However, a concomitant increase of the rate of both processes following DIC enrichment of seawater does not automatically imply a link between

photosynthesis and calcification. In fact, the difference in the kinetics of calcification and photosynthetic rates observed in both coral tested suggests that these two processes are more independent than commonly thought. In *P. porites*, at 4 mM DIC, calcification remains carbon-limited, while the photosynthetic rate has already reached saturation. This difference is even more obvious in *Acropora* sp. since the photosynthetic rate reaches a maximum with 6 mM DIC, whereas calcification rate does not saturate within the range of DIC concentrations used (0.5-8 mM). If calcification stimulated photosynthesis, the kinetics of the two processes would be similar. The fact that the calcification rates increase at high DIC concentrations without any augmentation of the photosynthetic rate strongly suggests that calcification does not promote photosynthesis in corals.

This result agrees with other studies carried out on *S. pistillata*. Yamashiro (1995) has established that the 99% inhibition of calcification caused by the addition of 0.5 mM of 1-hydroxyethylidene-1, 1-bisphosphonic acid (HEBP), a specific inhibitor of mineral deposition photosynthetic, did not cause a reduction in the photosynthetic rate of *S. pistillata*. Gattuso *et al.* (2000) have also shown that calcification does not stimulate photosynthesis in corals. These authors established that, while the $CaCO_3$ production rate of *S. pistillata* decreased by 2 to 2.4-fold when incubated in low calcium medium, photosynthesis was unaffected by this treatment. This result contradicts the finding of Al-Moghrabi *et al.* (1996), who showed that low calcium concentrations cause a diminution in rates of both calcification and photosynthesis in

*G. fascicularis*. However, their experiment was carried out with 0.2 mM calcium, which might be too low to support necessary vital cellular functions. Corals were incubated in seawater containing 2.85 mM calcium in the study of Gattuso *et al.* (2000), which allowed the authors to examine the effect of reduced calcification rates on photosynthesis, without the added variable of very low calcium concentrations.

The data of Yamashiro (1995) and Gattuso *et al.* (2000), as well as those presented in this report, reject part of the trans-calcification hypothesis, which states that 'calcification functionally precedes the photosynthetic utilisation of bicarbonate' (McConnaughey and Whelan 1997, McConnaughey 1995, McConnaughey 1994). McConnaughey argues that protons released by $CaCO_3$ precipitation contribute to the dehydration of bicarbonate into carbon dioxide, which in turn is used by photosynthesis. According to this model, the principal product of calcification is not $CaCO_3$, but protons (McConnaughey and Falk 1991). Since data presented in this thesis show a lack of stimulation of photosynthesis by high DIC concentrations when calcification rate remains limited, it is clear that calcification does not 'functionally precede the photosynthetic utilisation of bicarbonate' in corals.

Nevertheless, the fact that calcification proceeds faster in the light than in the dark has led to the long-established view that photosynthesis and calcification are closely coupled. Several suggestion have been formulated to explain how photosynthesis can enhance calcification. Crossland and Barnes (1974) suggested that through photosynthesis, zooxanthellae remove the metabolic

wastes of their hosts, and thus stimulate calcification. Translocation of photosynthetates, which are required for active transport mechanisms (Chalker and Taylor 1975) and for organic matrix synthesis (Wainwright 1963) also occurs in the light. Goreau (1959) argued that photosynthesis stimulates calcification by removing the $CO_2$ produced during skeletogenesis from the coelenteron. More recently, McConnaughey (1994) refined this idea and proposed as part of the trans-calcification model that photosynthesis promotes calcification by removing the $H^+$ derived from the precipitation of $CaCO_3$ which involves a $Ca^{2+}/2H^+$ exchanger. In all these models calcification is considered to be light-enhanced because it is stimulated by photosynthesis.

The data presented in this thesis support this idea. Calcification rates of *Acropora* sp. measured at 2 mM DIC and in the dark were indeed much lower than those obtained in the light. This difference between dark and light rates implies a strong dependence of calcification on light and suggests an interaction with photosynthesis. Light is however, not the only variable which enables high calcification rates to be achieved. The addition of $HCO_3^-$ to seawater can raise calcification rates in the dark to higher values than those measured in the light in normal seawater. Bicarbonate addition in the dark can thus compensate for the lack of photosynthesis.

Both $HCO_3^-$ and photosynthesis affect the alkalinity of the cytosol. Since it is well established that high calcification rates are positively correlated with elevated pH (Marubini and Atkinson 1999), the interaction between calcification and photosynthesis in corals may result from the photosynthesis-derived

alkalisation of the cytosol. In the dark, alkaline conditions can be achieved simply by increasing the bicarbonate concentration of seawater. High bicarbonate may also buffer the $H_2CO_3$ generated in calcification. In addition to their possible regulatory functions, bicarbonate ions act as a direct source of carbon for $CaCO_3$ precipitation. Our data show that in both coral species incubated in the light, photosynthesis and calcification became uncoupled at high DIC concentrations. Both *P. porites* and *Acropora* sp. exhibited $HCO_3^-$-saturated rates of photosynthesis and $HCO_3^-$-limited calcification rates, thus demonstrating that bicarbonate can enhance calcification directly and not only by stimulating photosynthesis.

This suggests that in hermatypic corals incubated in the light, high rates of calcification are achieved by the synergistic action of photosynthesis (which maintains a high cytosolic pH) and bicarbonate ions (which act as a direct source of carbon for skeletogenesis). In the dark however, $HCO_3^-$ alone can support these two functions when present in sufficiently high concentrations. The potency of this ion in calcification in hermatypic corals is not surprising considering that high oceanic bicarbonate concentration is thought to have been the main driving force for the emergence of biomineralisation (Kempe and Kaźmierczak 1994).

So this study indicates that a better understanding of the physiology of hermatypic corals and in particular of the interaction between photosynthesis and calcification can contribute to the preservation of coral reefs of tropical oceans. Indeed, since bicarbonate addition can enhance calcification rates in both light

and dark conditions, this atavistic physiological role of bicarbonate has important implications for coral propagation in aquaria, for the restoration of damaged reefs, and hence for coral conservation. This would prevent the destruction of coral reefs for commercial purposes, and also enable the recolonisation of damage reefs with cultured corals.

# Chapter 7 :

# Conclusions

The sun emits radiation that is composed of ultraviolet, visible and near infrared wavelengths. Only 70 % of the solar radiation that reaches the Earth penetrates the atmosphere. The rest of the energy is reflected back by clouds and dust particles. In addition, the ozone layer absorbs most of the UV radiation. However, the planet does not absorb all the radiation that arrives at its surface. In fact, the albedo (defined as the percentage of solar radiation reaching the Earth's surface that is reflected back into the atmosphere) can be as high as 90 % in the case of snow (Bearman 1995). One the other hand, calm water has an albedo of only 2 %.

Furthermore, if there was no atmosphere, the black body temperature of the earth would be 255 K (- 18°C), while the actual mean temperature of the planet is 286 K (13 °C) (Falkowski and Raven 1997). This difference of 31 K is due to the reradiation of some of the long wavelength radiation back to the Earth's surface. Solar radiation that is actually absorbed by the Earth's surface warms up the planet, which in turn causes the radiation of infrared and longer wavelengths from the planet towards the atmosphere. Since atmospheric water vapour and $CO_2$ efficiently absorb infrared wavelengths, they act as an insulating blanket around the planet. This phenomenon is called the greenhouse effect. The heat released by the Earth would be lost into space were it not for these greenhouse gases. Although atmospheric water vapour and $CO_2$ are the main greenhouse gases, they are not the only ones. In fact, methane, nitrous oxide and artificially produced chlorofluorocarbons (CFCs), such as $CCl_3F$, also participate in the greenhouse effect. The use of CFCs as refrigerants and

aerosol propellants has been reduced in recent years as it became clear that they were a major cause of the ozone depletion in the upper atmosphere.

Greenhouse gases greatly influence how much solar radiation reaches the planet. The surface temperature of the Earth, which is determined by the amount of absorbed radiation, has not been constant since the formation of the planet. In fact, there have been at least four major glaciation periods. Those were followed by inter-glaciatial periods, which are characterised by the warming up of the Earth's surface. Deglaciation events, i.e. increased surface temperature, seem to always be positively correlated with high atmospheric $CO_2$ concentrations. Although the exact mechanisms leading to the transition between glaciation to inter-glaciation periods remain controversial, it is clear that rising $CO_2$ concentrations play an active role during this process.

It is commonly accepted that in the 10,000 to 250 years before the Industrial Revolution the global carbon cycle was in steady state, with equal amounts of carbon being released by processes such as terrestrial, atmospheric, soil or oceanic respiration as being sequestered by terrestrial and oceanic photosynthesis, and calcification. Biological fluxes of carbon are centrally important in the global carbon cycle as they are much larger than geochemical fluxes, although changes in the latter can yield significant long term effects on atmospheric $CO_2$. Raven and Falkowski (1999) gave a complete account of the carbon fluxes for this period and showed that the system was well balanced. The Industrial Revolution has however resulted in the release of $CO_2$ into the atmosphere as a consequence of fossil fuel burning and high rates of

deforestation with 2.9 and 5.4 Pg C produced each year by these anthropogenic activities (Raven and Falkowski 1999). So far, the planet has not been able to balance the effects of human industry, since only part of this recent input has been used by carbon sequestering processes. In fact, terrestrial gross primary production has significantly increased from 100.7 to 102 Pg C $year^{-1}$ (Raven and Falkowski 1999), but this extra 1.3 Pg C $year^{-1}$ atmospheric absorption is not enough to counterbalance the extra 8.3 Pg C $year^{-1}$ recently produced. Most predictions indicate that the increase of atmospheric $CO_2$ will persist in the future. However, over geological time, it is the inorganic carbon of the ocean that controls the atmospheric concentration of $CO_2$. Marine photosynthesis is the major carbon using process in the sea and its net primary production is comparable to the terrestrial one, with 45 and 56 Pg C $year^{-1}$ respectively (Raven and Falkowski 1999), but no change in the overall rate of photosynthesis has been noted in the sea, despite the numerous chemical and physical alterations that have already occurred on the planet.

The increase in greenhouse gases in the atmosphere has caused global warming of the Earth's surface. The speed of this process is particularly problematic. The last glaciation period ended with a rate of increase in surface temperature of 1 °C per 1000 years (with the exception of localised rapid short periods of warming up), whereas a 0.5 °C rise has already been recorded, for just the last century (Pittock 1999).

Global warming also strongly affects the hydrological cycle. Rising temperatures have led to more evaporation, hence disturbing the global moisture balance (Pittock 1999). This causes an increase both in rainfall, resulting in localised diminution of coastal water salinity due to large freshwater input, and in turbidity and pollution, because of enhanced frequency of erosional flood-flow events. Increase in ocean water temperature; tectonic effects; variation of water volume due to the melting or growing of mountain glaciers; changes in the volume of the Greenland and Antarctica ice-sheets; and finally (to a lesser extent) human activities, such as dams or groundwater depletion, have all led to a rise in sea-level (Pittock 1999). The mean sea-level is expected to increase by 15 to 95 cm by 2100 (Pittock 1999). So, in addition to chemical variations, physical changes are also expected.

Since the beginning of the Industrial Revolution, all theses physical and chemical changes on Earth have affected and perturbed ecosystem structure and function. Moreover, these adverse effects are expected to persist. As discussed in Chapter 6, as a result of increased human disturbance and global warming, extensive coral bleaching events have occurred and have greatly damaged whole coral reef ecosystems. Similarly, in temperate regions, as a consequence of human activities and global warming, an increase in the outbreaks of large-scale blooms of the coccolithophore *Emiliania huxleyi* has been observed in recent years. Aggravating the problem of algal blooms may lead to the perturbation and eventually to the degradation of coastal ecosystems (Stumm and Morgan 1981). The physical and chemical changes on Earth

provoked by human activities could thus have a devastating effect on marine ecosystems and maybe also on the rest of the biosphere.

At the start of this work, prediction on biological responses remained uncertain, because of our limited understanding of the physiology of calcification and photosynthesis and of the exact nature of the relationship between these two processes (Gattuso and Buddemeier 2000).

Using examples of the largest photosynthetic producers of calcium carbonate (coccolithophores and hermatypic corals), the present study has established that calcification does not promote photosynthesis in short-term measurements. In fact, the primary function of calcification in *E. huxleyi* may be to remove excess toxic calcium molecules. Coccoliths may also protect the photochemical apparatus against the damaging effects of excess light.

In *E. huxleyi* and hermatypic corals, both photosynthesis and calcification were strongly stimulated by DIC addition. This work has determined that in *Acropora* sp., bicarbonate addition can compensate for the lack of photosynthesis. This study also provides evidence that in addition to supplying $CO_2$ to Rubisco, bicarbonate ions also directly stimulate PSII activity in *E. huxleyi*, thereby promoting photosynthesis both at limiting and saturating light. Bicarbonate ions are therefore central to several aspects of physiological function in *E. huxleyi* and hermatypic corals.

The involvement of bicarbonate ions at various levels of physiological processes in these organisms may be related to the high concentration of bicarbonate ions in the early ocean. Dismukes *et al.* (2001) have linked the estimated high bicarbonate concentration of the Archaean ocean (30 to 30,000 times more than at present) with the essential role of this ion in the efficient functioning of the magnesium cluster of the water splitting complex in the photochemical apparatus. Furthermore, these authors argue that oxygenic photosynthesis as we known it might have not occurred without bicarbonate ions. In addition, with their 'soda ocean' hypothesis, Kempe and Degens (1985) argue that the early ocean was characterised by low calcium and high bicarbonate concentrations. The widespread occurrence of stromalites in the Precambrian rock records, together with the fact that these organisms only develop in waters containing a high level of $CaCO_3$ supersaturation and alkaline conditions provide support for the hypothesis. Kempe and Kazmierczak (1994) have associated the sudden and concomitant emergence of mineral skeletal structures in different groups of organisms around the Precambrian / Cambrian boundary with an increase in seawater calcium concentration. Bicarbonate was then in ample supply to be used for calcification and provided a mechanism for $Ca^{2+}$ detoxification (Kazmierczak *et al.* 1985). So, the present-day strong influence of bicarbonate ions in photosynthesis and calcification may have its origin in the early ocean chemistry and the central role played by bicarbonate ions may be an atavistic one.

The present study agrees with this essential role of bicarbonate ions since although experiments were all short-term, the important feature of marine calcification and photosynthesis does not appear to reside much in their interaction, but more in their relationship with bicarbonate ions. What is now important to consider is whether or not the bicarbonate concentration of seawater will be modified in the future and what consequences this would have on photosynthesis and calcification.

The predicted rise in $_pCO_2$ will not alter the fact that seawater is saturated with respect to aragonite and calcite (Stumm and Morgan 1981). Hence, alkalinity will remain constant (Stumm and Morgan 1981). However, by the end of next century, the pH of seawater will decrease by 0.35 units, which according to figure 1.1, will lead to a 50 % diminution of the concentration of $CO_3^{2-}$ ions (Riebesell *et al.* 2000). Since alkalinity will remain constant, the decrease in concentration of $CO_3^{2-}$ ions will be compensated for by an increase in bicarbonate ion concentration. So, rising atmospheric $CO_2$ concentration will eventually lead to a 7% increase in concentration of bicarbonate ions in seawater. Since it is only a few percent, this increase in bicarbonate concentration is considered negligible in some studies (Marubini *et al.* 2001). However, bicarbonate remains the main carbon ion species in seawater (even at the predicted lower pH) and this small percentage actually corresponds to 149 μM of extra bicarbonate (calculated from Stumm and Morgan 1996). This is a considerable increase as compared with the expected 68 μM decrease of carbonate. There will thus be twice as much bicarbonate produced than carbonate lost.

This study has established that both *E. huxleyi* and hermatypic corals have developed bicarbonate transport mechanisms, which can supply carbon for calcification and photosynthesis. So, these marine organisms are already adapted to take up and use increased bicarbonate concentrations. In the long-term, because of the central physiological role of bicarbonate ions in *E. huxleyi* and hermatypic corals, the predicted chemical and physical changes on Earth might not have such a catastrophic impact on the calcification and photosynthetic rate of these marine organisms. Marubini *et al.* (2001) have however shown that low pH inhibits the calcification rate of hermatypic corals, although in their experiment bicarbonate concentration was increased to its predicted end of the century value (from 1718 μM to 1896 μM). In their study, these authors only measured calcification. The present work monitored both photosynthesis and calcification and has established that these two processes were not saturated by the present DIC concentration of the ocean; that increased bicarbonate concentrations directly promote calcification; that calcification was light-enhanced and was positively correlated with increasing bicarbonate concentrations. It could thus be argued that it may be possible that over a long *period of acclimatisation, this strong* link between the two processes and the potent role of bicarbonate could counterbalance *the negative effect* of a low pH.

Calcification was shown to be strongly biologically controlled in both *E. huxleyi* and hermatypic corals. This finding questions the importance of the saturation state theory, whereby calcification rates are simply determined by the saturation state of calcium carbonate in seawater (Marubini *et al.* 2001). So, the predicted chemical changes derived from human activities and global warming will not have a straightforward effect on calcification, but will depend on the way organisms cope with these alterations of their environment. Since calcification is strongly biologically controlled, adaptation to new chemical and physical conditions is possible. In fact, for example, coral reefs have already survived climate fluctuations for millions of years over glacial-interglacial cycles. However, this is the first time in history that they are subject to both natural and human disturbance. It is possible that this dual interference has a greater impact now than it would have had in the past, because the DIC concentration of seawater has decreased so dramatically that organisms such as corals are now reaching their physiological limits. At present it seems that the level of stress is too great, but their large capacity of adaptation may overcome the effect of global warming and human disturbance. This optimistic view is supported by the evidence presented in this thesis which shows that the physiology of two of their main processes is greatly enhanced by increased bicarbonate concentrations.

In conclusion, the importance of bicarbonate ions has been disregarded for decades, since because of its present-day mM concentration in seawater it was not considered as a limiting factor for aquatic photosynthesis and calcification (Redfield 1958). The present study has however confirmed that photosynthesis and calcification are indeed limited by the ambient DIC concentration of seawater in both *E. huxleyi* and hermatypic corals.

Furthermore, contrary to their being unimportant, bicarbonate ions may play a crucial role in the future because of their strong relationship with two of the major processes of global carbon cycling : photosynthesis and calcification. When predictions are made on the effect of global warming on aquatic photosynthesis and calcification, factors such as $CO_2$ concentration, pH and temperature are considered (Riebesell *et al.* 2000), but the bicarbonate concentration of seawater is never mentioned. This failure to appreciate the central position occupied by bicarbonate may have led to unnecessarily alarmist predictions of the consequences of rising atmospheric $CO_2$.

# Appendices

## Appendix 1

Composition of artificial seawater, with a salinity of 30 ppt (from Harrison *et al.* 1980).

| Anhydrous salts | mmol $l^{-1}$ |
|---|---|
| NaCl | 362.66 |
| $Na_2SO_4$ | 24.99 |
| KCl | 8.04 |
| $NaHCO_3$ | 2.06 |
| KBr | 0.0724 |
| $H_3BO_3$ | 0.037 |
| NaF | 0.0657 |

| Hydrated salts | mmol $l^{-1}$ |
|---|---|
| $MgCl_2 \cdot 6H_2O$ | 47.18 |
| $CaCl_2 \cdot 6H_2O$ | 9.14 |
| $SrCl_2 \cdot 6H_2O$ | 0.0082 |

| Nutrient and Trace metals | µmol $l^{-1}$ |
|---|---|
| $NaNO_3$ | 549.09 |
| $Na_2SiO_3 \cdot 9H_2O$ | 105.60 |
| $Na_2glyceroPO_4$ | 21.79 |
| $Na_2EDTA$ | 14.86 |
| $H_3BO_3$ | 61.46 |
| $FeCl_3 \cdot 6H_2O$ | 0.065 |
| $MnSO_4$ | 2.42 |
| $ZnSO_4$ | 0.254 |
| $CoSO_4$ | 0.0057 |

| Vitamins | µmol $l^{-1}$ |
|---|---|
| Thiamine HCl | 0.0297 |
| Vitamin $B_{12}$ | 0.0015 |
| Biotin | 0.00409 |

Appendix 2

Composition of simple artificial seawater with a salinity of 30 ppt

| Anhydrous salts | mmol $l^{-1}$ |
|---|---|
| NaCl | 355.23 |
| $Na_2SO_4$ | 24.5 |
| KCl | 7.914 |
| $NaHCO_3$ | 2.02 |

| Hydrated salts | mmol $l^{-1}$ |
|---|---|
| $MgCl_2 \cdot 6H_2O$ | 46.23 |
| $CaCl_2 \cdot 6H_2O$ | 8.97 |

Appendix 3

Composition of simple artificial seawater with a salinity of 35 ppt

| Anhydrous salts | mmol $l^{-1}$ |
|---|---|
| NaCl | 409.16 |
| $Na_2SO_4$ | 28.33 |
| KCl | 9.032 |
| $NaHCO_3$ | 2.02 |

| Hydrated salts | mmol $l^{-1}$ |
|---|---|
| $MgCl_2 \cdot 6H_2O$ | 59.59 |
| $CaCl_2 \cdot 6H_2O$ | 10.21 |

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