Photosynthetic Production of *Ulva rotundata* Bliding Estimated by Oxygen and Inorganic Carbon Exchange Measurements.in the Field

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Abstract

The photosynthetic performance of an estuarine population of. *Ulva rotundata* Bliding was determined under natural radiation during a daily cycle. The primary production was estimated fol1owing two methods, oxygen evolution and dissolved inorganic carbon (DIC) uptake calculated from the pH change and alkalinity in the media after incubation in closed bottles. Maximum values of oxygen evolution (261 µmol O_2 m^{-2} min^{-1}) were reached during midday. The daily course of the DIC uptake rates was different since the maximum rate (248±10 µmol C m^{-2} min^{-1}) was obtained later in the afternoon. In any case, no photoinhibition was detected by either O_2 evolution or DIC uptake. Additionally, the parameters of photosynthesis vs. photon fluence density curves were similar in spite of the technique used. During the daily cycle, significant changes in CA activity and percentage of photosynthetic inhibition following the addition of an external CA inhibitor (acetazo-lamide) were produced. However, these changes appeared to have a limited effect on photosynthesis. On the other hand, chlorophyll *a* and *b* contents decreased drastically in the afternoon and there was not a good correlation between pigment content and photosynthesis rates. Taken into account the results obtained, the daily primary production of *U. rotundata* based on oxygen evolution was 20% higher than-that based on DIC up take (1.8 and 1.5 g C m^{-2} day^{-1}, respedively)·.

Keywords: Carbonic anhydrase; photoinhibition; photosynthetic quotient; photosynthesis; Ulva.

Introduction

The world's coastline accounts for 14-30 % of the oceanic primary production although it only covers 7 % of the surface of the ocean (Mantoura et al. 1991). Currently, several models are being developed to determine the productivity of the algal communities which inhabit coastal areas in order to assess whether they are net sinks or sources of CO_2 for the atmosphere (Pernetta et al.1995, Gattuso et al. 1998).

A problem with these models is the wide range of variation in primary production values available in literature (Heip et al. 1995). This high variability can be attributed to different techniques that are used to estimate the primary production in the field. A number of methods are available, ranging from the demographic approaches (Aranda et al. 1984) to estimations based on measurements of photosynthesis in the short term. Photosynthesis is often estimated by following carbon uptake with the carbon radioisotope technique (Gagné et al. 1979, Harris 1980) or by means of calculations based on pH and alkalinity changes occurring in the surrounding medium during photosynthesis (Axelsson 1985, Rosenberg et al. 1995). Oxygen evolution rates are widely used as estimators of primary production, especially in macroalgae (Kanwisher 1966, Johnston et al. 1981, Fernández and Niell 1984, Aranda et al. 1984, Jiménez et al. 1998).

There is limited literature on direct comparisons of DIC uptake and O_2 evolution in laboratory cultures (Burris 1981, Maclnnis et al. 1993, Iriarte 1999), natural populations of phytoplankton (Laws 1991, Williams et al. 1991, Cajal-Medrano et al. 1992, Robertson et al. 1993) and intertidal macrophytes subjected to natural conditions of irradiance (Rosenberg et al. 1989, 1995). A conclusion from these papers is that the ratio of O_2 release to DIC uptake measured in sin, is often consistently different from 1 - 1.3, the theoretical value expected. The authors have pointed out that changes in photorespiration and uptake of nutrients during the daily cycle could explain these values (Rosenberg et al. 1995). However, the way which acclimation of the photosynthetic mechanisms in response to changes on environmental variables could contribute to this inbalance remains unclear. Thus, it has been well documented that light stress produces inactivation of photosystem II (Osmond 1981, 1994). affecting the O_2 evolution rates (Hader et al. 1996) which decrease between soon and late afternoon for a number of macroalgae (Huppertz et al. 1990, Henley 1993, Hanelt et al.1993, Jiménez et al. 1998). However, there has been comparatively little experimental effort to study the effect of the photoinhibition on the mechanisms implied in DIC uptake and its photosynthetic fixation.

In this paper, we studied the correlation between C; uptake and O_2 evolution in the estuarine green macroalga *Ulva rotundata* Bliding subjected to changing solar radiation during a daily cycle. Other reports have focused on the de termination of photosynthetic performance of *Ulva* species under natural conditions. Misbkind et al. (1979) described large daily oscillations in the in situ O_2 evolution rates in *U. lactuca* L. Also Arnold and Murray (1980) found photoinhibition of O_2 production at midday in *U. rigida* C. Agardh and V. lobato (Kütz.) Harvey, and Hader et al. (1999) described a reduction on photosynthetic quantum yield in *U. rigida* and *U. gigantea* (Kütz.) Bliding, based on photosystem II fluorescence. At present, the only available data comparing O_2 evolution and DIC uptake in *Ulva* are provided by Rosenberg et al. (1995) Who reported values of the ratio of O_2 release to CO_2 uptake from 0.4 - 1.01 for *U. fasciata* Delile, subjected to solar radiation. In our experiments, the utility of the oxygen exchange measurements as an estimation of daily primary production is examined. The changes in chlorophyll content and activity of the inorganic carbon uptake mechanisms were also determined in order to test their possible contribution to modifications of the photosynthetic quotient.

Material and methods

Study area and algal material

The experiments were performed at the Palmones River Estuary during summer. The Estuary is located in Algeciras Bay, southern Spain, at the end of a small catchment area (Clavero et al. 1997). At the estuary, *Ulva rotundata* grows fully exposed to unfiltered solar radiation at low tide, while it floats in water at high tide. The site chosen for the experiments was located at the entrance of the estuary. Epiphyte-free thalli of *Ulva* were placed into open aquaria under full solar irradiance and with a continuous renewal of the medium, resembling the natural conditions of growth. Different samples were collected from the aquaria at discrete time intervals in order to perform incubations for measuring photosynthesis. After the incubations, pieces of the thalli were frozen in liquid nitrogen and transported to the laboratory to determine chlorophyll, soluble protein content and total carbonic anhydrase (CA) activity. Solar photon irradiance was monitored during the daily cycle by using a cosine corrected sensor (LICOR 192 SB) coupled to a LICOR LI-1000 radiometer. The experiments were repeated on two different days during June 1999.

Oxygen exchange and inorganic carbon uptake

Photosynthesis and respiration rates were measured as oxygen exchange under full solar irradiance every 2-3 h starting at 8:00 LT. Pieces of alga (from 0.2-0.5 g) were incubated in cylindrical closed glass bottles of 250 ml volume for 20-30 min. Temperature was controlled by placing the bottles into aquaria pumped with seawater continuously. Mixing of the medium was provided by shaking the bottles several times during incubation. The incubations were performed simultaneously using different samples. Respiration was estimated in triplicate as oxygen depletion in dark bottles. The initial and final (after the incubation) oxygen concentrations and pH were estimated. Oxygen concentrations were measured using a Crison OXI-92 (Alella, Spain) oxygen electrode. The oxygen concentration in the medium within the vessels increased ca. 25-35 % after incubation under solar radiation. The pH electrode (52-08, Crison, Alella, Spain) was calibrated in buffer solutions referred to the NBS scale (Crison). Inorganic carbon uptake rates were determined from the changes in pH and alkalinity produced during the incubations. The initial pH in the medium was ca. 8.2 and it increased by 0.2-0.4

units after incubation under solar radiation. Samples of seawater used for incubations were collected and preserved for determining alkalinity. The alkalinity was measured by potentiometric titration with 100 mM HCl in a closed vessel at 20 °C in the laboratory. DIC concentrations were computed from the alkalinity and pH values using the carbonic acid constants provided by Riley and Chester (1977). The oxygen evolution and carbon uptake rates were expressed on the basis of thallus surface area. For this purpose, a figure of 87.9 g FW per m^2 thallus surface area was used (Mercado et al. 1998). Using the solar radiation change during the experiments, rates of oxygen evolution and carbon uptake obtained at different times were used to construct photosynthesis vs. irradiance curves. The curves were fitted by the function of Henley (1993). The goodness of fit to the model was tested by using least-squares regression analysis. The average R^2 value obtained from the least-squares regression analysis was 0.97. The maximum photosynthetic rate (Pmax) and the light saturation parameter (Ek) were estimated from the model.

Before incubation, seawater was sampled and frozen for determination of the concentrations of nitrate and phosphate in a Technicon Autoanalyser (Traacs 800, Bran Luebbe, Norderstedt, Germany). Nitrate concentration was determined by following Wood et al. (1967). Phosphate concentration was estimated using the malachite green method (Fernández et al. 1985). The concentrations were determined in duplicate.

Determination of the CA activity and effect of acetazolamide

Carbonic anhydrase activity was determined in crude extracts of samples using the method of Wilbur and Anderson (1948). The crude extracts were obtained by grinding 30-50 mg of alga in 2 ml of buffer (100 mol m^{-3} Tris, 5 mol m^{-3} EDTA-Nz, 25 mol m^{-3} ascorbic acid and 2 mol m^{-3} PMSF) at 4 °C. The reaction was started by rapidly introducing 1 ml of icecold CO2-saturated distilled water into the reaction vessel containing the crude extract. Buffer without sample was used as a control. The activity was calculated by measuring the time required for a drop of 0.4 pH units within the pH range 8.4-7.4 at 0-2 °C. One unit of enzyme activity (REA) was defined as $(t_0/t_c)-1$ where t_0 and t_c are the times for pH change of the control and sample, respectively. The activity was expressed on a soluble protein basis.

In order to detect the presence of external CA activity, the effect of AZ (Sigma-Aldrich Química S.A., Madrid, Spain) on oxygen evolution was determined. It was assumed that AZ cannot penetrate into the cell and inhibits only the extracellular CA (Palmqvist et al. 1990, Haglund et al. 1992, Axelsson et al. 1995). The assay of AZ was started by introducing fragments of plant into the cylindrical glass bottles used to measure oxygen evolution. Acetazolamide was added at the beginning of the incubations at 100 mmol m^{-3} final concentration. The exchange rate of oxygen was determined after 20-30 min of incubation under solar radiation. The oxygen evolution rates obtained in the presence of AZ were compared with those obtained in natural seawater (without the inhibitor).

Determination of soluble protein and chlorophyll content

The concentration of proteins in crude extracts was determined in triplicate according to the method of Bradford (1976). Chlorophyll *a* and *b* were extracted in N,N-dimethyl-formamide at 4 °C overnight. Their concentrations were determined spectrophotometrically according to Inskeep and Blom (1982).

Results

The photon fluence density during the experiments ranged from 250 to 2000 μmol photons m^{-2} s^{-1} reached at 8:00 and 15:00 LT, respectively. In addition to the irradiance, large changes on the nutrient concentrations were produced during the day. As mentioned previously, the experiments were performed at the entrance of an estuary. Therefore, the changes in the estuarine water circulation during the daily cycle (due to the tidal regime) produced modifications in the nutrient concentration in the seawater used for the experiments. Thus, the nitrate concentration in the water was roughly constant until 14:00 LT (approx. 1.2 µM). Afterwards, it increased significantly, reaching a maximum value of 80 µM at 19:00 LT. The variation pattern of phosphate was different, with the highest concentration obtained at noon (7.4 pm). The mean value of phosphate was 1 pM.

The net oxygen evolution rate was roughly constant for much of the daily cycle. The highest value was reached at 13:00 LT (Fig. 1). Values 60% and 48 % lower were obtained at 8:00 and 19:00 LT, respectively (the initial and final points). These points coincide with non-saturating values of incident irradiance (250 and 350 µmol photons m^{-2} s^{-1}, respectively). A complete curve of oxygen evolution *vs.* solar irradiance could be constructed using all the data obtained. Maximum photosynthetic rate (Pmax) and half saturation point (Ek) obtained by fit to the model of Henley (1993) were 251 ±7 µmol O_2 m^{-2} min^{-1} and 24 µmol photons s^{-1}, respectively. The mean rate of oxygen consumption in darkness (respiration rate) was 33.7±8.8 µmol O_2 m^{-2} min^{-1} (n = 12). It did not change significantly during the day (data not shown). It is interesting to note that the values of oxygen exchange rates obtained are comparable to those reported by other authors for *Ulva* species maintained in the laboratory (Bjórk et al. 1992, Mercado et al. 1998). The values of light saturation parameter (Ek) are also comparable to those reported for other intertidal macroalgae (Mercado et al. 1999).

Figure 2 shows the DIC uptake rates calculated from the alkalinity and pH change. Lower DIC uptake rates were obtained at 8:00 and 19:00 LT and no reduction was found at midday. A curve of DIC uptake rate vs. solar irradiation was constructed using these values. The values of Pmax and E k were similar to those calculated from the oxygen evolution rates (242±9 µmol O_2 m^{-2} min^{-1} and 32 µmol photons m^{-2} s^{-1}, respectively). However, the daily course of the DIC uptake rate did not coincide fully with the course of O_2 evolution. Thus, a slight stimulation (ca. 30 %) of the DIC uptake rate was produced after midday, reaching the highest rate at 17:30 LT (248 µmol DIC m^{-2} min^{-1}). Accordingly, the ratio of O2 evolution to DIC uptake rate changed significantly during the daily cycle (Fig. 3). In general, values higher than 1.2 mol O_2/mol DIC were obtained during the first part of the cycle. In contrast, the ratio dropped to approximately 1 mol O_2/mol DIC after midday.

In order to determine the dependence of photosynthesis on the external CA activity, incubations were done in the presence of the inhibitor AZ (Fig. 4). Acetazolamide produced a significant inhibition of the oxygen evolution rates at each time of the day in which it was tested, although the strongest effect was found at midday, when AZ inhibited the O_2 evolution rate by 80 %. The total CA activity (i.e. external plus internal) was also determined using crude extracts of alga. Figure 5 shows that the total activity changed during the day. Thus, the lowest activity was found at 13:00 LT, coinciding with the highest photosynthetic inhibition percentage by AZ. Afterwards, the activity increased, reaching values similar to those obtained during the first part of the day.

In addition to changes in gas exchange rates, incubation under full solar radiation produced changes in the characteristics of the thalli. Thus, the chlorophyll *a* content increased four-fold in the morning, reaching its maximum concentration at 13:00 LT (Fig. 6). Afterwards, chlorophyll a content dropped to the initial level and increased slightly at the end of the day. Chlorophyll *b* followed a similar cycle. In fact, no significant differences were found in Chl *a*/*b* ratio during the day. It is worth noting that oxygen evolution rates and pigment content were not correlated. In contrast to chlorophyll, the soluble protein content (Fig. 7) was roughly constant during the first part of the day. After midday, it increased two-fold and declined slightly at the end of the day.

Discussion

Daily changes on the photosynthetic metabolism of U. rotundata

Our data indicate that *U. rotundata* is able to maintain a photosynthetic rate close to P_{max} even under the high values of irradiance reached on the southern coast of Spain at midday (ca. 2000 µmol photons m^{-2} s^{-1}). This result contrasts data previously presented by other authors showing inhibition of oxygen evolution in thalli of *U. lobata*, *U. rigida*, *U. lactuca* L. and *U. curvara* (Kütz.) De Toni adapted to sun conditions (Miskind et al. 1979, Arnold and Murray 1980, Ramus and Rosenberg 1980, Cabello-Pasini et al. 2000). Other authors have also described a reduction of photosynthetic quantum yield as based on photosystem II fluorescence in *U. gigantea* and *U. lactuca* (Häder et al. 1999, Cabello-Pasini et al. 2000). Absence of apparent photoinhibition seems to reflect the adaptation pattern of *U. rotundata* to its natural habitat where it grows exposed to unfiltered solar radiation for much of the tide cycle. It is interesting to note that, in contrast to the oxygen evolution rates, the chlorophyll content decreased drastically at noon. Short-term decrease of the pigment content as an acclimation response to both increasing irradiance in laboratory (Algarra and Niell 1991) and daily changes under field conditions (López-Figueroa 1992, Rüdiger and López-Figueroa 1992) has been described for a number of macroalga species. These changes have been attributed to light damage of the pigments and/or down-regulation of their synthesis.

A few papers have pointed out daily modifications of enzyme activity on the carbon metabolism of algae. In particular, Hwang and Herrin (1994) and Fujiwara et al. (1996) demonstrated circadian rhythms in the expression of genes coding for CA in *Chlamydomonas reinhardtti*, and Flores-Moya et al. (1998) described changes in CA activity in the red alga *Rissoella verruculosa* under solar radiation. Here, daily changes in the inhibition of oxygen evolution by AZ and the total CA activity are described. According to Axelsson et al. (1995), two mechanisms confer a high capacity for using external HCO_3^- by *Ulva sp*: extracellular CA-catalysed HCO_3^- dehydration and HCO_3^- direct uptake. These mechanisms act like a carbon concentrating mechanism that prevents photorespiration. The simultaneous presence of both active mechanisms has been demonstrated in *Ulva sp*. by determining the effect of AZ and DIDS (an inhibitor for the HCO_3^- direct uptake mechanism) on oxygen evolution (Mercado et al. 1998, Axelsson et al.1995). Larsson and Axelsson (1999) demonstrated different degrees of photosynthetic inhibition by AZ in *U. lactuca*. Total inhibition by AZ was found in *Ulva* thalli lacking HCO_3^- direct uptake. Additionally, Axelsson et al. (1995) demonstrated that *Ulva sp*. could modify the activity of both mechanisms in response to environmental changes. The changes in sensitivity to AZ found during the daily cycle in *U. rotundata* could indicate that the degree of activation of both mechanisms varies in the short term during the daily cycle. According to this assertion, most of the HCO_3^- use was dependent on the external CA activity at noon. In contrast, use of HCO_3^- could occur mainly by means of its direct uptake in the afternoon. In any case, these possible changes in the activation degree of the mechanisms for using HCO_3^- in *U. rotundata* were not followed by major changes in the photosynthetic rates estimated by DIC uptake. In fact, the strong drop in the total CA activity which occurred at midday did not produce a significant reduction in the photosynthetic rates. Therefore, it can be postulated that the use of the external HCO_3^- was not a limiting step for DIC fixation at saturating irradiance during the daily cycle.

O2 release vs. DIC uptake

In general, there was a very good agreement between the photosynthesis rates estimated as oxygen evolution and DIC uptake calculated from the measurements of alkalinity and pH change occurred during the incubation. Also, the photosynthetic performances as inferred from parameters of photosynthesis *vs*. irradiance curves were similar in spite of the technique used. Although proton uptake (due to the incorporation of ions other than HCO_3^-, mainly nitrate) can also contribute to changes in pH during, photosynthesis, Atkinson and Smith (1983) showed that about 95 of the ApH value stems from DIC uptake in macroalgae. In the thalli of *U. rotundata* used for our experiments, the overall contribution of the proton uptake to pH increase could be ca. 6 % as calculated by taking into account the mean C/N ratio of the thalli (16:1, data not shown). Therefore, it can be assumed that the mean ratio of O_2 release to CO_2 uptake estimated from the pH increase is representative of the PQ. A decrease in PQ was seen during the daily cycle, in particular, values close to 1.0 were obtained at noon.

Changes of PQ have been attributed to photorespiration and/or changes in nutrient uptake (Rosenberg et al. 1995). As mentioned previously, photorespiration can be discounted in *Ulva rotundata* because it has an inorganic carbon concentration mechanism which appears to be active during the daily cycle. In contrast, strong changes in nitrate and phosphate concentration were found. In particular, nitrate concentration increased by one order of magnitude from noon to the end of the day. These changes could produce changes in uptake rate by the algae, contributing to PQ variations. In this sense, Lopes et al. (1997) and Vergara et al. (1998) described daily changes in nitrate-reductase activity in some alga species. However, according to Axelsson et al. (1985), any increase in nitrate uptake must produce an increase in PQ, in contrast to our results. Therefore, more experimental effort is necessary to clear this point.

In spite of the reasons presented to explain the changes in PQ, it is clear that different values of daily primary production could be obtained depending on the method used for its estimation. From the data shown in this paper, the daily net primary production of *U. rotundata* can be calculated by integrating the curve that describes the daily changes of the photosynthesis rate. Thus, a figure of 1.8 g C m^{-2} day^{-1} is obtained when the O_2 exchange rates are considered in doing the calculations. The daily primary production based on DIC uptake is lower by about 20 % (1.5 g C m^{-2} day^{-1}). According to our data, the difference between the two techniques is not due to technical inconsistencies of the methods used but to changes in the photosynthetic performance of *U. rotundata* produced during the daily cycle. Therefore, we propose that these daily changes must be taken into account to correct values of primary production based on oxygen evolution.

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Figure caption

Fig. 1. Daily variation in the oxygen evolution rates of *Ulva rotundata*. The rates were estimated after incubating pieces of alga in closed transparent bottles under full solar radiation. Data are means of 3-5 independent samples. Vertical lines indicate one SD.

Fig. 2. Daily variation in the dissolved inorganic carbon uptake rates of *Ulva rotundata.* The uptake rates were estimated from the changes in alkalinity and pH produced after incubating pieces of alga into closed transparent bottles under full solar radiation. Data are means of 3-5 independent samples. Vertical lines indicate one SD.

Fig. 3. Daily changes on the ratio of oxygen evolution to DIC uptake. Vertical lines indicate one SO.

Fig. 4. Net photosynthesis by *Ulva rotundata* after addition of acetazolamide at different parts of the daily cycle. Data are means of 4-5 independent experiments. The vertical lines indicate one SD.

Fig. 5. Daily variation on total carbonic anhydrase activity of the thalli of *Ulva rotundata*. The activity was estimated using crude extracts of the alga. Data are means of 5-8 in-dependent samples. Vertical lines indicate one SD.

Fig. 6. Daily variation of chlorophyll *a* and *b* content of the thalli of *Ulva rotundata* incubated under full solar radiation. The thalli were maintained under full solar radiation until pieces were collected at various times of day. Data are means of 5-7 independent samples. Vertical lines indicate one SD.

Fig. 7. Daily variation in soluble protein content of the thalli of *Ulva rotundata* incubated under full solar radia-tion. Data are means of 4-5 independent samples. Vertical lines indicate one SD.

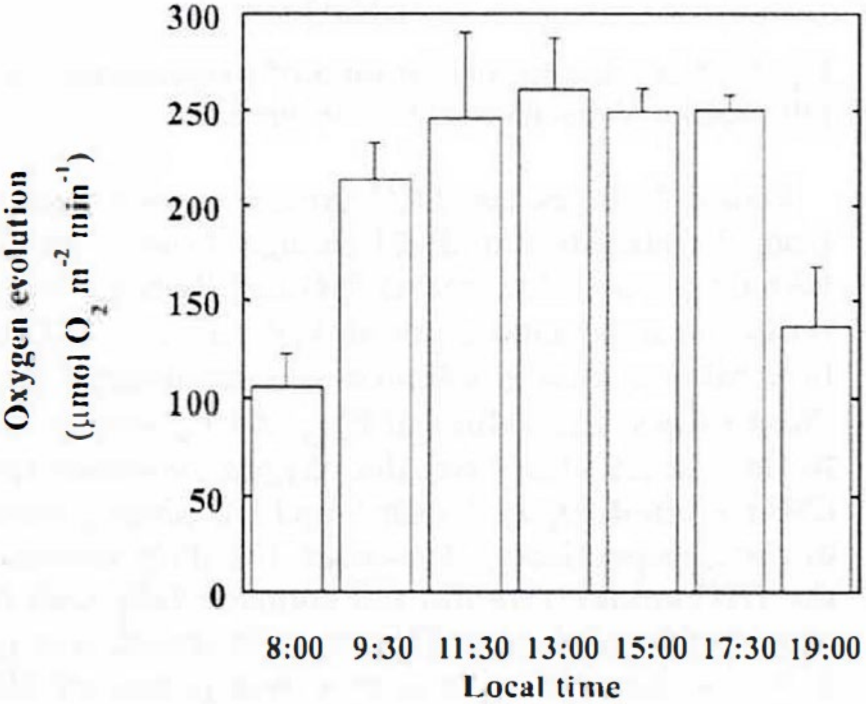

300
250
200
150
100
50
0
Oxygen evolution
(μmol O$_2$ m^{-2} min^{-1})
8:00
9:30
11:30
13:00
15:00
17:30
19:00
Local time

Fig. 1

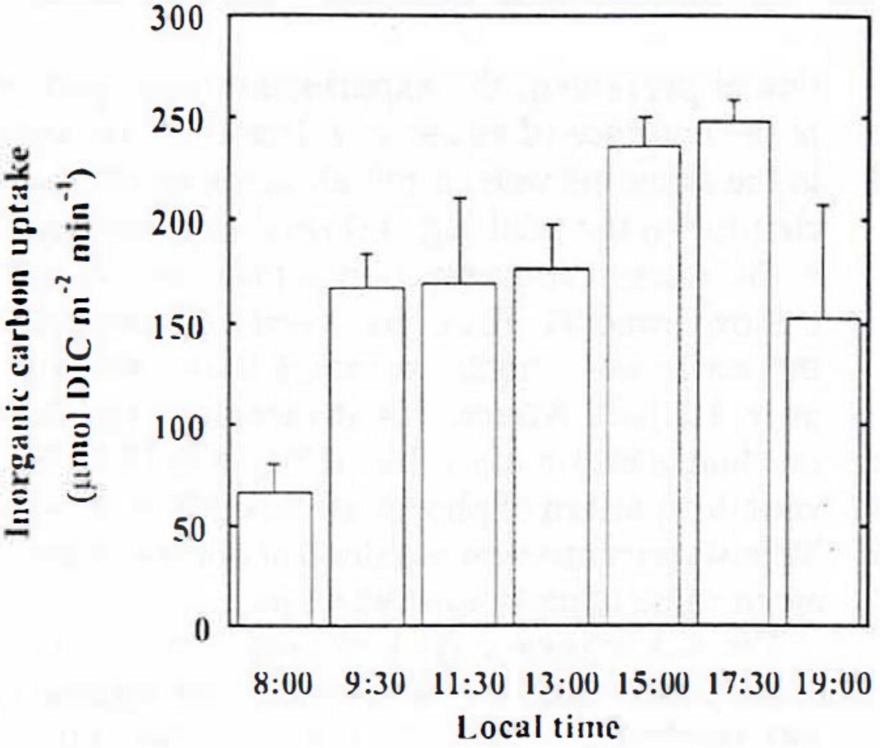

300
250
200
150
100
50
0
Inorganic carbon uptake
(µmol DIC m^{-2} min^{-1})
8:00
9:30
11:30
13:00
15:00
17:30
19:00
Local time

Fig. 2

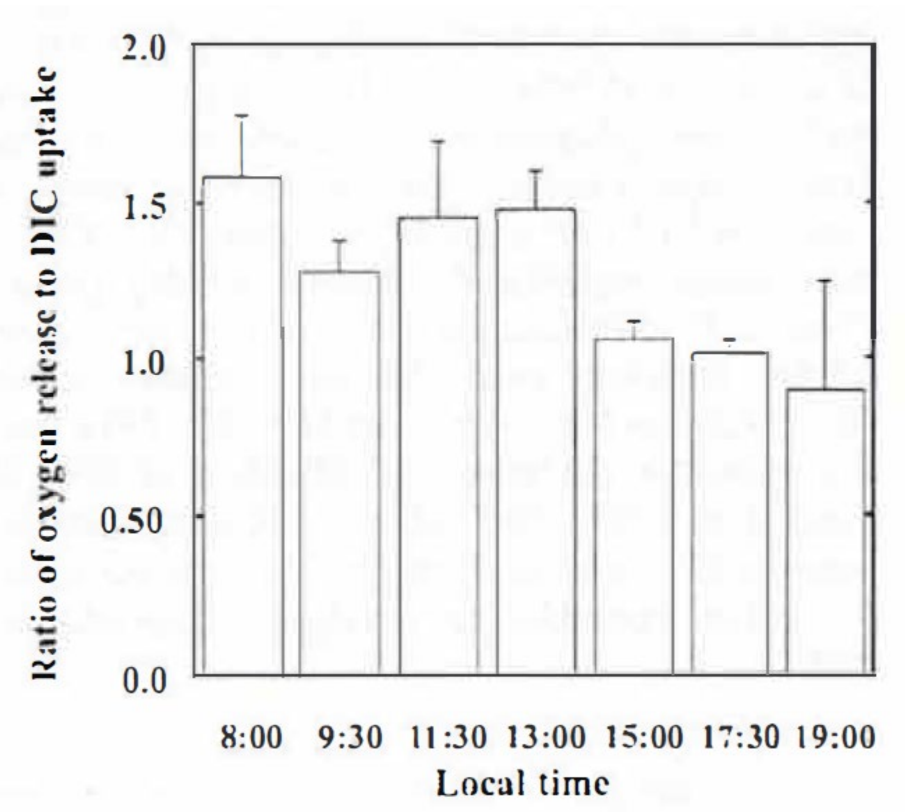
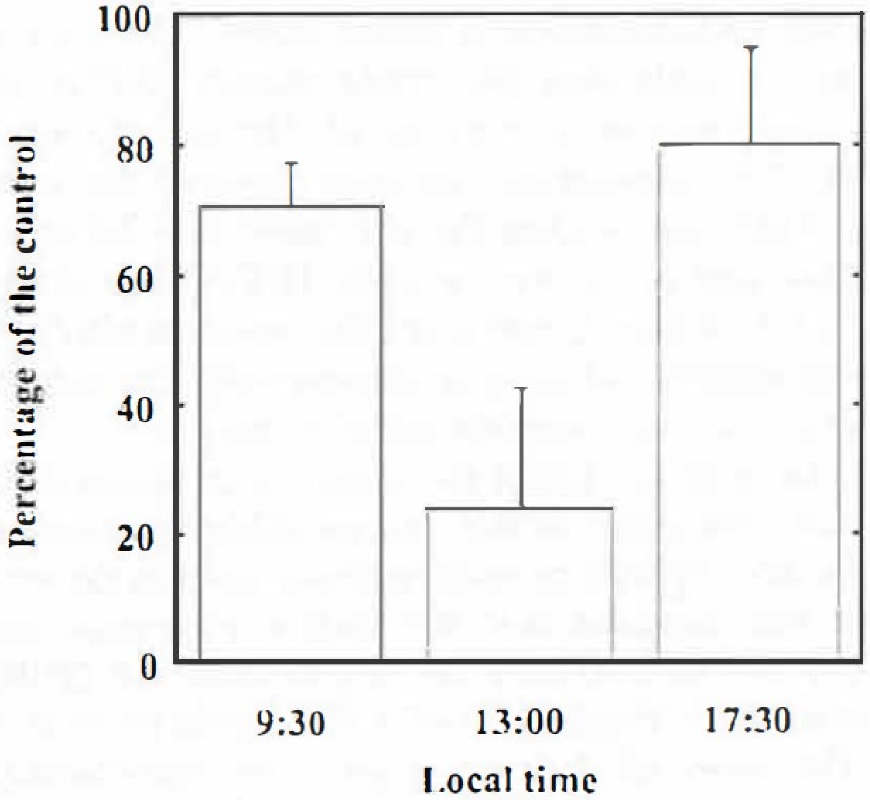

Ratio of oxygen release to DIC uptake
2.0
1.5
1.0
0.50
0.0
8:00
9:30
11:30
13:00
15:00
17:30
19:00
Local time

Fig. 3



100
80
60
40
20
0
Percentage of the control
9:30
13:00
17:30
Local time

Fig. 4

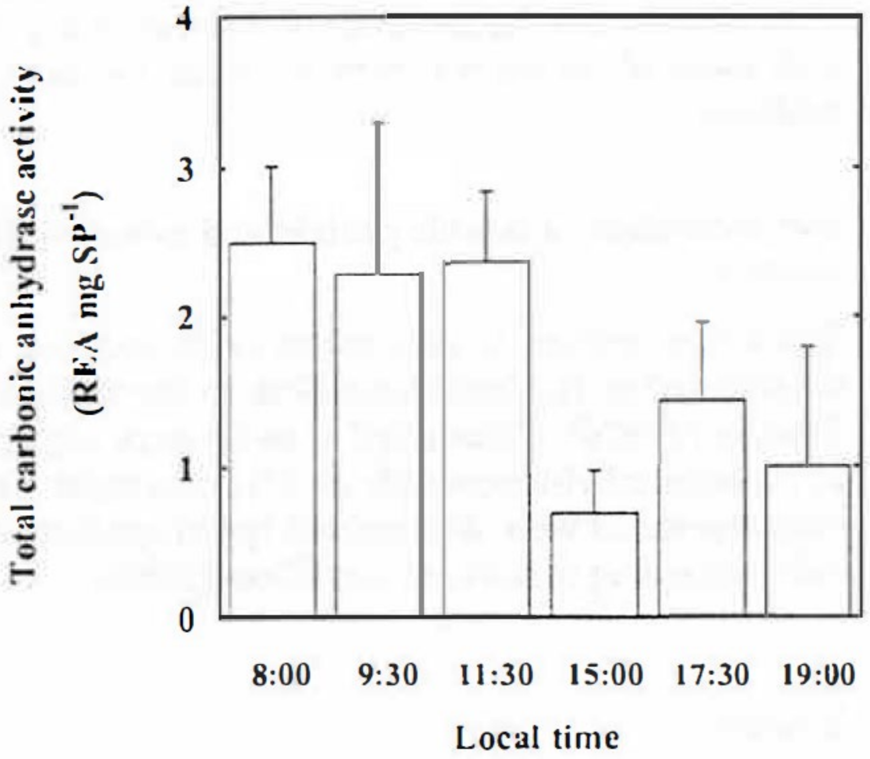

Total carbonic anhydrase activity (REA mg SP^{-1})
4
3
2
1
0
8:00
9:30
11:30
15:00
17:30
19:00
Local time

Fig. 5

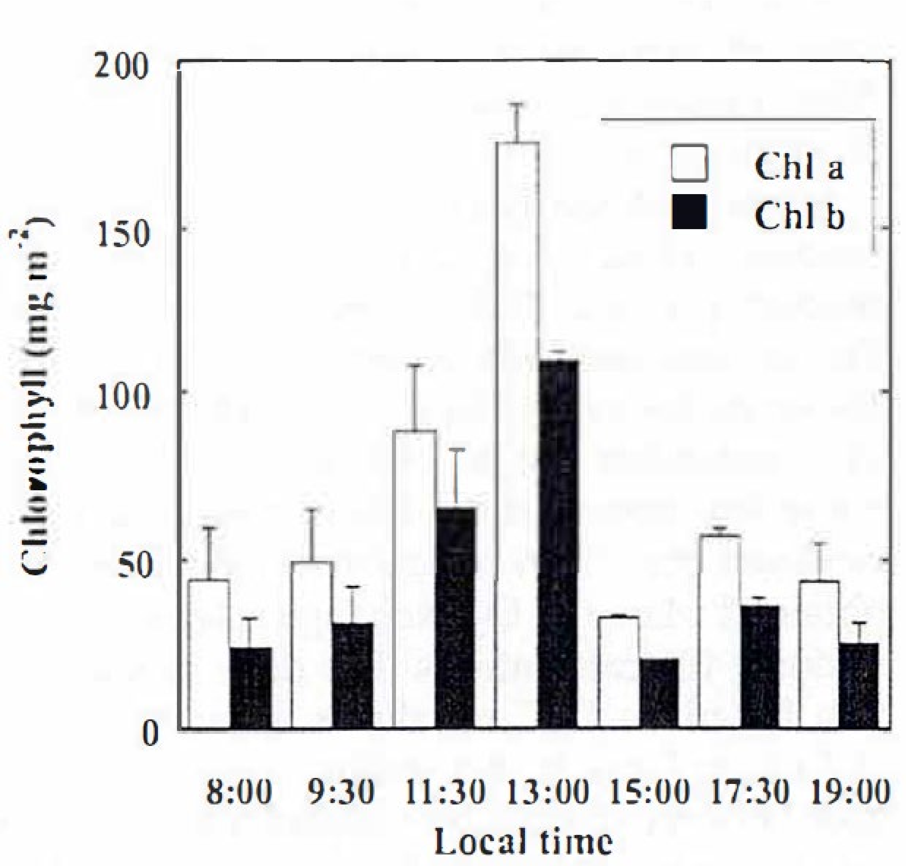

200
150
100
50
0
Chlorophyll (mg m^{-2})
Chl a
Chl b
8:00
9:30
11:30
13:00
15:00
17:30
19:00
Local time

Fig. 6

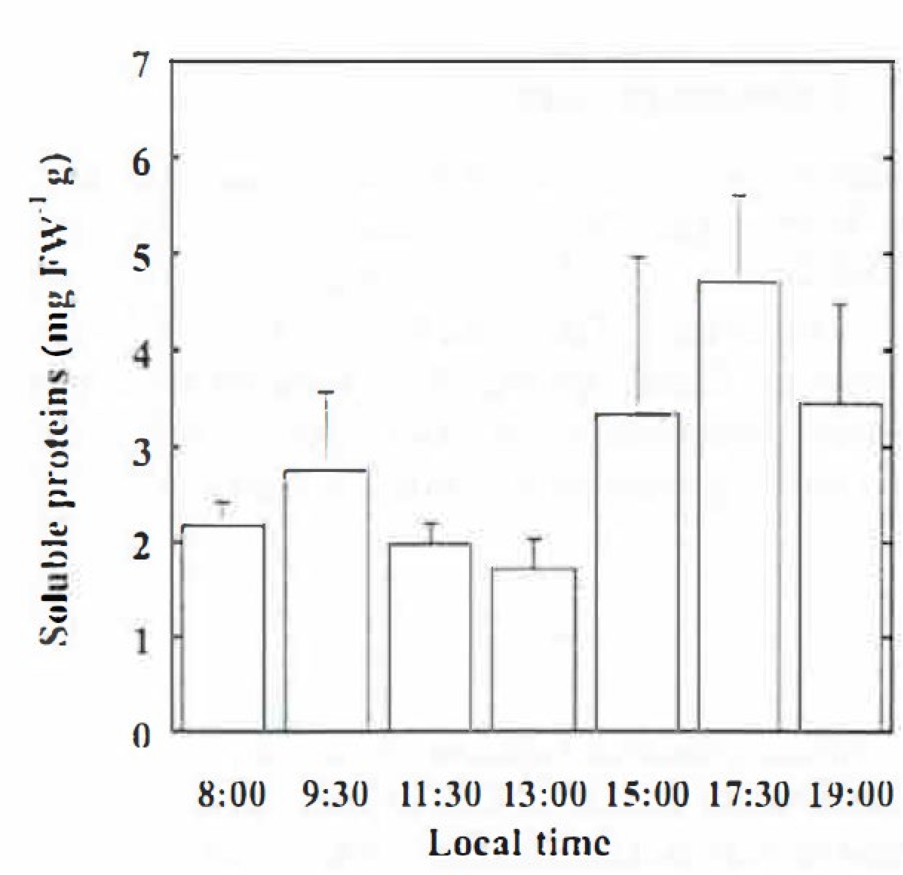

Soluble proteins (mg FW^{-1} g)
0
1
2
3
4
5
6
7
8:00
9:30
11:30
13:00
15:00
17:30
19:00
Local time

Fig. 7