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Seasonal and tidal variability of environmental carbon related physico-chemical variables and inorganic C acquisition in *Gracilariopsis longissima* and *Enteromorpha intestinalis* from Los Toruños salt marsh (Cádiz Bay, Spain)

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Abstract

The seasonal and tidal variability of inorganic C acquisition mechanisms, photosynthesis, internal composition and growth were studied in two co-occurring macroalgae in Los Toruños salt marsh (Cádiz Bay), *Gracilariopsis longissima* and *Enteromorpha intestinalis*. This variability was monitored together with physico-chemical variables affecting carbon availability, photosynthesis, and growth. The environmental variables, such as light, temperature, pH, salinity, oxygen, alkalinity, dissolved inorganic carbon (DIC) and CO_2, displayed not only an expected seasonal cycle but also a daily (tidal) variability, with abrupt and rapid changes influenced by biological activities, physical variables, tidal state and tidal timing. In contrast to environmental variables, photosynthesis, pigments and C:N composition were affected by seasonal changes but not by tidal regimes, as organisms integrated these short-term fluctuations in physico-chemical variables. Photosynthesis, pigments and internal N composition were maximal in autumn and minimal in summer for both species. Growth showed a seasonal trend, displaying a summer drop with negative values. This response can be the result of extreme values of environmental variables (temperature, light, pH, nutrients, and the shortage of DIC) in summer, in comparison with higher growth rates in September onwards. The use of inhibitors of carbon acquisition in situ at normal DIC concentrations (2.2. mM) revealed species-dependent differences. While the external carbonic anhydrase (CA) activity showed a constitutive character in *G. longissima*, it showed little effect in *E. intestinalis*, which relies on internal CA activity. The 4, 4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS)-sensitive bicarbonate

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transport in *G. longissima* was effective in winter. In contrast, DIDS stimulated photosynthesis in summer, and relieved AZ inhibition. This response could suggest a stimulation of a H^+ extrusion mediated-CO_2 transport in periods of low CO_2 availability.

Keywords: Carbonic anhydrase; DIC acquisition; *Enteromorpha*; *Gracilariopsis*; Photosynthesis; Stress

1. Introduction

Dissolved inorganic carbon (DIC) is a fundamental resource for photosynthesis. Since its availability is three orders of magnitude higher than nitrogen (N) and phosphorus (P) in seawater, photosynthesis and growth of marine algae are more likely to be N- or P-limited, especially in HCO_3^- users (Raven and Johnston, 1991). By contrast, in certain temperate, shallow coastal habitats where N and P levels are relatively elevated due to cultural eutrophication, and light availability is high, DIC concentration may constrain photosynthesis and growth (Raven, 1997).

The Rhodophyte *Gracilariopsis longissima* (Stackhouse), Irvine, Steentoft and Farnham and the Chlorophyte *Enteromorpha intestinalis* (L.) Ness are among the most abundant macroalgal species in the temperate eutrophic salt marsh of Los Toruños (Cádiz Bay, Southern Spain). Both macroalgae occur in mixed populations in shallow intertidal pools, although *E. intestinalis* thrives also in the uppermost intertidal locations, being able to withstand long periods exposed to air. In these shallow environments, the DIC concentration is expected to vary on daily and seasonal basis as a result of biological activities (photosynthesis and/or respiration) and changes in physico-chemical variables (light, salinity, pH and temperature) driven by biological, atmospheric, sedimentary and/or hydrological events (e.g. tides) (Maberly, 1990).

Different DIC utilization mechanisms (mostly HCO_3^- uptake) have been suggested in macroalgae: (1) external carbonic anhydrase (CA; 4.2.2.1); (2) ATPase-dependent HCO_3^- transport; (3) H^+/HCO_3^- co-transport; (4) OH^-/HCO_3^- antiport systems (Lucas, 1983). Previous laboratory studies on carbon acquisition in *E. intestinalis* and *G. longissima* indicated that both species possess external CA and a direct uptake of HCO_3^- (a mechanism sensitive to the inhibitor 4, 4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS)), which, in turn, are modulated by the external DIC levels (Larsson et al., 1997; Andría et al., 1999, 2000, 2001). Despite the relatively large amount of studies on C-acquisition in macroalgae, only a limited number deals with the relationship between the habitat and the ability of species to use HCO_3^- (Giordano and Maberly, 1989; Maberly, 1990; Johnston et al., 1992; Larsson et al., 1997; Mercado et al., 1998). Moreover, monitoring of the main environmental variables affecting DIC availability, combined with in situ measurements of photosynthetic C acquisition and growth in marine macrophytes are rarely documented (Larsson et al., 1997). Thus, the aims of this work were 1) to monitor on seasonal and daily (i.e. tidal cycles) basis the main physico-chemical variables affecting DIC availability and 2) to assess the photosynthetic physiology (oxygen evolution, carbon acquisition mechanisms, growth and internal constituents) of two co-occurring macroalgae species in Los Toruños salt marsh.

2. Material and methods

2.1. Environmental variables

Field surveys following daily cycles were carried out seasonally (in January, April, July, November [1999] and February [2000]) in a shallow creek of Los Toruños salt marsh (Cádiz Bay, Southern Spain), where populations of the Rhodophyte *G. longissima* (Stackhouse), Irvine, Steentoft and Farnham and the Chlorophyte *E. intestinalis* (L.) Ness thrive. The Rhodophyte species has been named as *Gracilaria* sp. in previous studies (e.g. Andría et al., 1999, 2000, 2001) due to the lack of a definitive identification. In a recent study based on molecular identification of plastid *rcb*L DNA, our specimen was assigned as *Gracilariopsis longissima* (Gurgel et al., 2003). Within each month, two different sampling dates (coinciding with spring tides) were chosen to detect if there was any effect of the tidal state (i.e. low tide [LT] *versus* high tide [HT]) and timing (i. e. morning rising tide *versus* afternoon rising tide) on environmental variables and physiological performance. Dissolved oxygen (O_2), pH, temperature (T) and salinity (S) were measured using a multiparametric probe (Grant-YSI 3815 Sonde, Grant Instruments, Cambridge) connected to a data logger (Water quality logger 3800) at 10 min intervals. Underwater photon fluence density (PFD) was measured with a quantum spherical PAR sensor (LiCor LI-193SA) connected to a data logger (LiCor LI-1000). Seawater samples were further taken hourly to determine alkalinity. To avoid gradual changes in the alkaline reserves of the samples until analysis, they were stored at 4 °C (ice-refrigerated in the field) in 125 ml borosilicate glass bottles without headspace, after addition of 500 μL of 50% saturated $HgCl_2$ solution as a preservative (Burkhardt and Riebesell, 1997). Alkalinity was calculated from linear Gran-plots (Gran, 1952) after potentiometric titration of 100 ml sample with 0.05 N HCl (Bradshaw et al., 1981). Dissolved inorganic carbon (DIC) and CO_2 concentrations, for a given alkalinity, salinity, temperature and pH, were calculated according to Merhbach et al. (1973), taking into consideration the apparent dissociation constants of carbonic acid in seawater, according to Dickson and Millero (1987). In addition, samples of seawater were taken for nutrient determinations (Bran and Luebbe Technicon Traacs 800). Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations were measured according to Wood et al. (1967) and Shinn (1941), respectively. Ammonium (NH_4^+) and phosphate (PO_4^{3-}) concentrations were determined following Grasshoff et al. (1983).

2.2. Effect of inhibitors of carbon acquisition mechanisms on oxygen evolution rates

The CA inhibitors acetazolamide (AZ) and 6-ethoxyzolamide (EZ) (Sigma-Aldrich Química) were used. It is generally assumed that AZ does not penetrate the cell and therefore only acts on surface-accessible CA (Moroney et al., 1985), although this assumption has been questioned (Williams and Turpin, 1987). The EZ penetrates into the cell, inhibiting external and internal CA. The presence of an alternative HCO_3^- utilization mechanism (a direct uptake by an anion exchanger), was checked by using 4, 4′-diisothiocyanatostilbene-2,2′-disulfonate (DIDS) (Sigma-Aldrich Química). Both AZ and EZ were dissolved in 0.05 N NaOH at a concentration of 50 mM (100 μM final

concentration) while DIDS was dissolved in Milli-Q distilled deionised water at a concentration of 50 mM (300 μM final concentration).

The effect of these inhibitors on O_2 evolution rates was tested at several dates (see above) and under different tidal states (i.e. HT and LT) in the field at ambient temperature. All incubations started nearly at same time of the day (i.e. around 15:00 h GMT). Samples of algae (0.5–1 gFW) were incubated in 60-ml transparent polystyrene flasks filled with artificial seawater [modified "Marine Culture Medium", (Woelkerling et al., 1983) by omitting boric acid] at pH 8.2 and 2.2 mM DIC, without headspace. Triplicate flasks covered with aluminium foil were used to measure respiration rates in the dark. Rubber septum caps were fitted to the screw tops of the polystyrene flasks to inject aliquots of inhibitor solutions through the cap by a polypropylene sterile syringe. Triplicate measurements of the photosynthetic O_2 evolution without inhibitors (control), and in the presence of AZ, EZ and DIDS, were carried out by an oxygen probe (Crison Oxi-92) after 30 min of incubation. Net photosynthesis rates (NPS) were calculated as the difference between O_2 concentration in the seawater before and after incubation, per unit of FW and time.

2.3. Analytical methods

The analytical measurements were performed from thalli fragments harvested after each sampling event, kept in an ice chest and transported to the laboratory. Triplicate samples (0.1 gFW) per treatment were frozen in liquid nitrogen and maintained at −80 °C until analysis. Samples of *G. longissima* were ground in phosphate buffer 0.1 M at pH 6.5 at 4 °C, extracted overnight and centrifuged at 19 000×*g* for 25 min (SIGMA Laborzentrifugen 2K15, Germany). The content of the phycobiliproteins (PBP) *r*-phycoerythrin (RPE) and *r*-phycocyanin (RPC), were determined spectrophotometrically (UNICAM UV/Vis Spectrometer UV2) from the supernatant fraction by using the chromatic equations of Beer and Eshel (1985). The pellet fraction was resuspended in 90% (v:v) acetone, extracted overnight at 4 °C and spun down at 19 000×*g* for 25 min. Chlorophyll *a* (chl *a*) concentration was determined spectrophotometrically according to Talling and Driver (1963). Samples of *E. intestinalis* were also taken to determine the lipid soluble pigment content by grounding in 90% (v/v) acetone, extracted overnight at 4 °C and filtered. Chlorophyll *a* and chl *b* concentrations were determined following Jeffrey and Humphrey (1975). Parallel samples of both macroalgae were oven dried at 60 °C for 48 h to determine total C and N content (Perkin-Elmer 240-C elemental autoanalyzer).

2.4. Growth rates

Net growth rates were measured monthly from February 1999 to March 2000 for both species under field conditions. Previous weighted samples of *G. longissima* (n=10) and *E. intestinalis* (n=5) were maintained in cages for 7 days. The cages attenuated less than 10% of the incident light. The cages were then placed over the sediment and linked to large plastic rods that were sunk into the sediment. To avoid possible fouling, the cages were replaced every sampling day (see Hernández et al., 1997 for further details). The mean relative growth rate (μ), expressed as % day^{-1}, was calculated according to the

Table 1
Seasonal variation of physico-chemical variables in a tidal creek of Los Toruños salt marsh where *E. intestinalis* and *G. longissima* thrive

Date	January-99		April-99		July-99		November-99		February-00	
Variable	LT	HT	LT	HT	LT	HT	LT	HT	LT	HT
PFD ($\mu mol\ m^{-2}\ s^{-1}$)	529 (130–659)	346 (39–591)	588 (341–1038)	380 (303–590)	684 (252–1068)	304 (183–878)	588 (85–806)	292 (96–906)	635 (28–855)	389 (64–782)
pH	8.09 (7.93–8.33)	7.85 (7.63–8.27)	8.16 (7.51–8.25)	7.99 (7.93–8.23)	8.47 (8.16–8.95)	8.71 (8.41–8.87)	8.08 (7.6–8.86)	8.04 (7.76–8.33)	8.82 (8.15–9.67)	8.37 (7.65–8.64)
Salinity	39 (34–43)	39 (36–42)	40 (37–41)	39 (37–40)	41 (39–42)	39 (38–42)	37 (35–38)	37 (36–37)	37 (36–37)	37 (37–38)
Temperature (°C)	10.6 (6.4–17.3)	11.0 (8.5–13.9)	18.3 (14.2–26.6)	19.1 (16.6–22.3)	29.2 (23.1–33.0)	25.4 (20.9–30.6)	13.6 (8.1–17.2)	13.9 (12.3–15.8)	18.3 (13.1–21.0)	14.95 (10.8–17.4)
Dissolved oxygen ($mg\ l^{-1}$)	8.1 (4.2–13.8)	6.7 (4.6–11.7)	9.3 (3.0–15.8)	9.6 (7.1–13.0)	6.4 (4.7–7.5)	6.0 (5.2–6.6)	9.9 (7.2–12.8)	10.3 (9.2–11.5)	16.7 (8.4–22.0)	10.1 (1.4–16.7)
Alkalinity (mM)	2.93 (2.71–3.30)	2.74 (2.63–2.84)	3.18 (2.81–4.16)	2.65 (2.56–2.73)	3.21 (2.64–3.58)	2.75 (2.37–3.48)	3.57 (3.22–3.77)	2.70 (2.51–3.17)	2.86 (2.78–2.93)	2.83 (2.69–3.1)
DIC (mM)	2.67 (2.46–3.07)	2.61 (2.48–2.77)	2.71 (2.53–3.12)	2.42 (2.34–2.47)	2.41 (1.6–3.04)	1.91 (1.45–2.78)	3.21 (2.56–3.74)	2.49 (2.36–2.82)	2.11 (1.61–2.65)	2.42 (2.19–3.08)
CO_2 (μM)	22.08 (14.08–34.21)	41.86 (18.4–70.73)	19.11 (4.98–34.56)	19.25 (15.11–22.04)	8.63 (0.79–17.86)	3.10 (1.18–7.99)	49.41 (2.62–105.73)	26.67 (11.47–40.98)	6.45 (0.61–18.64)	17.67 (5.9–79.07)
NO_3^- (μM)	0.23	1.7	0.75	0.05	0.36	0.87	1.41	6.8	0.09	2.45
NO_2^- (μM)	0.39	0.1	0.52	0.41	0.11	0.13	0.19	3.52	0.13	0.36
NH_4^+ (μM)	3.59	5.56	5.64	4.48	3.81	8.3	3.78	10.13	2.24	4.05
PO_4^{3-} (μM)	0.6	0.14	0.5	0.49	0.25	0.46	0.28	0.56	0.21	0.41

Data are means of daily surveys (see Fig. 1 as an example). Range of variation in parenthesis. (LT=low tide; HT=high tide).

exponential model: $\mu=[\ln(w_2/w_1)/(t_2-t_1)]\cdot 100$, where w_2 and w_1 are FW at the beginning (t_1) (t_1) and the end (t_2) of the period.

2.5. Statistics

The results were expressed as the mean values ± S.E. We used three-way ANOVA [time (sampling date)×tidal state (TS, HT-LT)×tidal timing (TT, morning rising tide versus afternoon rising tide)] for environmental parameters. Photosynthesis, respiration, tissue nutrients, the effect of inhibitors on O_2 evolution and chlorophyll *a* were tested using

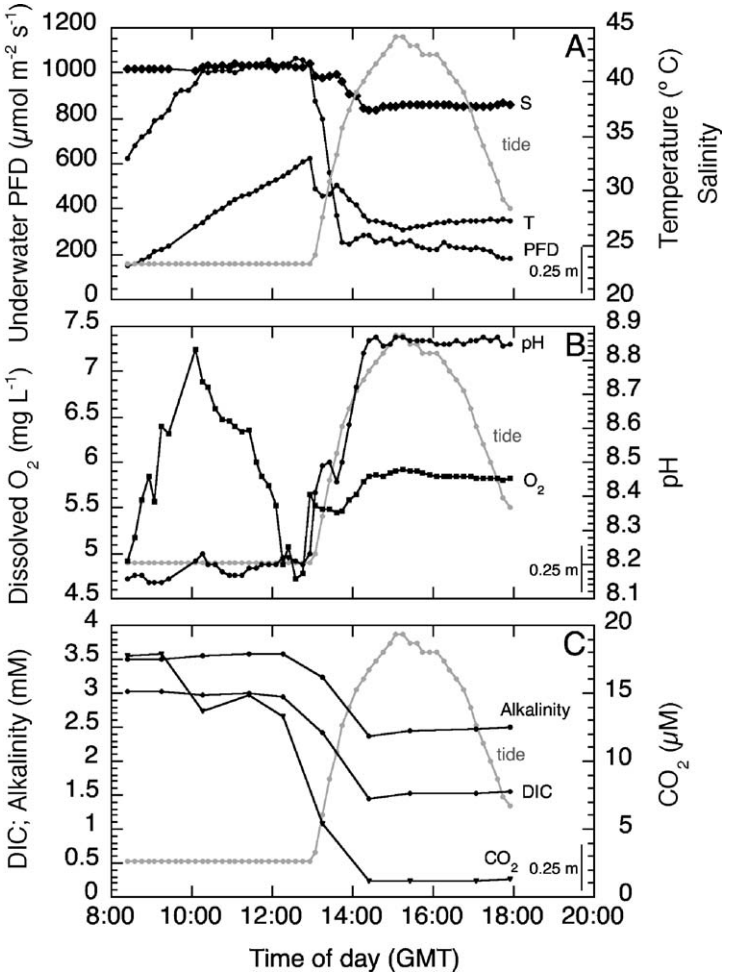

Fig. 1. Summer (July 17th) survey of daily (tidal) changes of some physico-chemical variables in a tidal creek of Los Toruños salt marsh where *E. intestinalis* and *G. longissima* thrive. (A) Underwater photon flux density (PFD), temperature and salinity; (B) dissolved oxygen and pH; (C) dissolved inorganic carbon (DIC), alkalinity and CO_2. Tidal height is depicted as a grey background line. Scale is in the right bottom corners.

Table 2
Three-way ANOVA on the effect of time (T), tidal timing (TT, morning–afternoon) and tidal state (TS, high–low tide) on physico-chemical variables in Los Toruños salt marsh

Variable	Source of variation	*df*	MS	*F*	%Explained
pH	T	4	0.09	294.7***	35.6
	TT	1	0.05	146.9***	19.8
	TS	1	0.02	56.95***	7.9
	T×TT	4	0.02	57.81***	7.9
	T×TS	4	0.02	54.00***	7.9
	TT×TS	1	0.05	174.9***	19.8
	T×TT×TS	4	0.003	8.60***	1.2
	Error	403	0.0003		
Salinity	T	4	0.10	220.9***	51.5
	TT	1	0.004	8.19**	2.1
	TS	1	0.02	33.70***	10.3
	T×TT	4	0.03	57.27***	15.5
	T×TS	4	0.02	41.28***	10.3
	TT×TS	1	0.01	13.79***	5.2
	T×TT×TS	4	0.01	18.36***	5.2
	Error	403	0.0005		
Temperature	T	4	9.97	1400***	58.5
	TT	1	1.33	187.1***	7.8
	TS	1	0.09	13.21***	0.5
	T×TT	4	0.45	62.93***	2.6
	T×TS	4	0.27	38.18***	1.6
	TT×TS	1	4.63	650.5***	27.2
	T×TT×TS	4	0.30	41.65***	1.8
	Error	403	0.007		0.1
Oxygen	T	4	5.71	160.5***	43.4
	TT	1	1.04	29.29***	8.0
	TS	1	1.52	42.75***	11.6
	T×TT	4	0.12	3.24**	0.9
	T×TS	4	1.01	28.53***	7.7
	TT×TS	1	3.10	87.17***	23.6
	T×TT×TS	4	0.61	17.08***	4.6
	Error	403	0.036		0.3
Alkalinity	T	4	0.02	6.07***	5.5
	TT	1	0.02	7.18**	5.5
	TS	1	0.21	82.45***	57.9
	T×TT	4	0.03	9.56***	8.3
	T×TS	4	0.03	9.63***	8.3
	TT×TS	1	0.03	10.07**	8.3
	T×TT×TS	4	0.02	5.64**	5.5
	Error	72	0.003		0.8
DIC	T	4	0.13	30.74***	20.8
	TT	1	0.02	5.03*	3.2
	TS	1	0.10	22.50***	16.0
	T×TT	4	0.02	4.52**	3.2
	T×TS	4	0.06	13.31***	9.6
	TT×TS	1	0.25	59.56***	40.1
	T×TT×TS	4	0.04	9.07***	6.4
	Error	72	0.004		0.6

(continued on next page)

Table 2 (*continued*)

Variable	Source of variation	*df*	MS	*F*	%Explained
CO_2	T	4	11.02	52.15***	30.0
	TT	1	7.07	33.44***	19.2
	TS	1	1.08	5.12*	2.9
	T×TT	4	1.85	8.75***	5.0
	T×TS	4	1.65	7.82***	4.4
	TT×TS	1	13.49	63.83***	36.6
	T×TT×TS	4	0.49	2.33^{ns}	1.3
	Error	71	0.21		0.5

ns: non-significant.
* ($P<0.05$).
** ($P<0.005$).
*** ($P<0.001$).

three-way ANOVA (species×time×tidal state). Two-way ANOVA was used for chlorophyll *b*, *r*-phycoerythrin (RPE), *r*-phycocyanin (RPC) (time×tidal state) and growth rates (time×species). Before statistical analyses, all data were checked for normality and homocedasticity. Physico-chemical variables and the effect of inhibitors were log transformed since they violated the assumption of homocedasticity. After ANOVA, statistical differences were tested using Tukey's HSD test. In all cases, the statistical significance was set at 5% probability (Zar, 1984). Statistical analyses were conducted using the STATISTICA© computing programme.

3. Results

3.1. Environmental variables

Average values and range of variation of the main environmental variables from a seasonal and daily (tidal) sampling programme are shown in Table 1. As an example of the 10 seasonal surveys, Fig. 1 shows the daily fluctuations in variables recorded during the 17th July-99 sampling. In general, seasonality explained the highest percentage of the variance for physical and chemical parameters, although tidal state and tidal timing, contributed significantly to the overall variance (Table 2).

Underwater PFD peaked in July (mean of 684 μmol m^{-2} s^{-1}, at LT), with the highest values at LT irrespective of the season. The light attenuation coefficients caused by water (K_w) were higher at LT than at HT (annual integrated values of 3.3 ± 0.8 and 1.7 ± 0.1 m^{-1}, respectively) as a consequence of the sediment resuspension by the tidal currents. A broad seasonal variation in temperature was recorded. Like PFD, temperature peaked in July (33 °C at LT) with minimum values in January (6.4 °C). Both tidal state and tidal timing further modulated temperature variability. A gradual increase in temperature was recorded at LT irrespective of the season (slopes of 2.5 °C h^{-1} in July and <1 °C h^{-1} in January), resulting in daily increments up to 13 °C (July). On the rising tide, the flood of coastal waters into the creek decreased (July, Fig. 1) or increased (January, April) the temperatures reached at LT. Significant fluctuations were also recorded in salinity (Tables 1 and 2) that

varied between 34 (January) and 42 (July). In general, pH increased during afternoon LT (values≥8.8) being rather conservative during HT, irrespective of the season. The inflowing of coastal water during the afternoon rising tide decreased the pH values,

Table 3
Three-way ANOVA on the effect of species (S), time (T), and tidal state (TS, high–low tide) on internal composition, photosynthesis and respiration in *G. longissima* and *E. intestinalis* from Los Toruños salt marsh

Variable	Source of variation	*df*	MS	*F*	%Explained
Internal C	S	1	528.7	90.44***	70.1
	T	3	55.16	9.44***	7.3
	TS	1	11.08	1.90^{ns}	1.5
	S×T	3	10.62	1.82^{ns}	0.2
	S×TS	1	21.77	3.73^{ns}	2.9
	T×TS	3	79.79	13.64***	10.6
	S×T×TS	3	40.98	7.01***	5.4
	Error	32	5.85		0.8
Internal N	S	1	7.07	74.36***	46.5
	T	3	3.31	34.83***	21.8
	TS	1	0.37	3.86^{ns}	2.3
	S×T	3	1.22	12.78***	8.0
	S×TS	1	1.31	13.81***	8.6
	T×TS	3	1.46	15.34***	9.6
	S×T×TS	3	0.36	3.79*	2.4
	Error	32	0.10		0.6
Chl *a*	S	1	154.5	309.6***	88.3
	T	3	8.79	17.61***	5.0
	TS	1	1.22	2.45^{ns}	0.7
	S×T	3	5.57	11.15***	3.1
	S×TS	1	1.68	3.37^{ns}	1.0
	T×TS	3	1.13	2.27^{ns}	0.6
	S×T×TS	3	1.92	3.84*	1.1
	Error	32	0.10		0.1
Photosynthesis	S	1	12.46	237.0***	83.0
	T	3	0.32	5.99**	2.1
	TS	1	0.02	0.35^{ns}	0.1
	S×T	3	1.24	23.60***	8.3
	S×TS	1	0.58	11.10**	3.9
	T×TS	3	0.17	3.23*	1.1
	S×T×TS	3	0.18	3.37*	1.2
	Error	32	0.05		0.3
Respiration	S	1	9.40	40.8***	42.7
	T	3	1.58	6.86***	7.2
	TS	1	0.82	3.54^{ns}	3.7
	S×T	3	3.75	16.2***	17.1
	S×TS	1	3.88	16.8***	17.6
	T×TS	3	1.36	5.91**	6.2
	S×T×TS	3	0.97	4.20*	4.4
	Error	31	0.23		1.0

ns: non-significant.
* ($P<0.05$).
** ($P<0.005$).
*** ($P<0.001$).

excepting in July (Fig. 1). Seasonal and daily fluctuations in dissolved oxygen were similar to those recorded for the pH. The lowest concentrations (4.7–7.5 mg O_2 l^{-1}, in July) are largely explained by a decrease in the gas solubility as temperature raised (21–33 °C). A daily increase in oxygen was observed especially during morning rising tides (e.g. from 1.4 to 22.0 mg O_2 l^{-1}, in February). Alkalinity and DIC varied closely with maxima in November (3.57 mM alk, 3.21 mM DIC) and minima in July (1.91 mM DIC). Regardless of the season, levels were higher at LT than at HT (Table 1, Fig. 1). CO_2 concentration was highly variable on seasonal and daily basis. Like alkalinity and DIC, CO_2 levels peaked in November (49.41 μM, LT) and were minima in July (3.10 μM, HT).

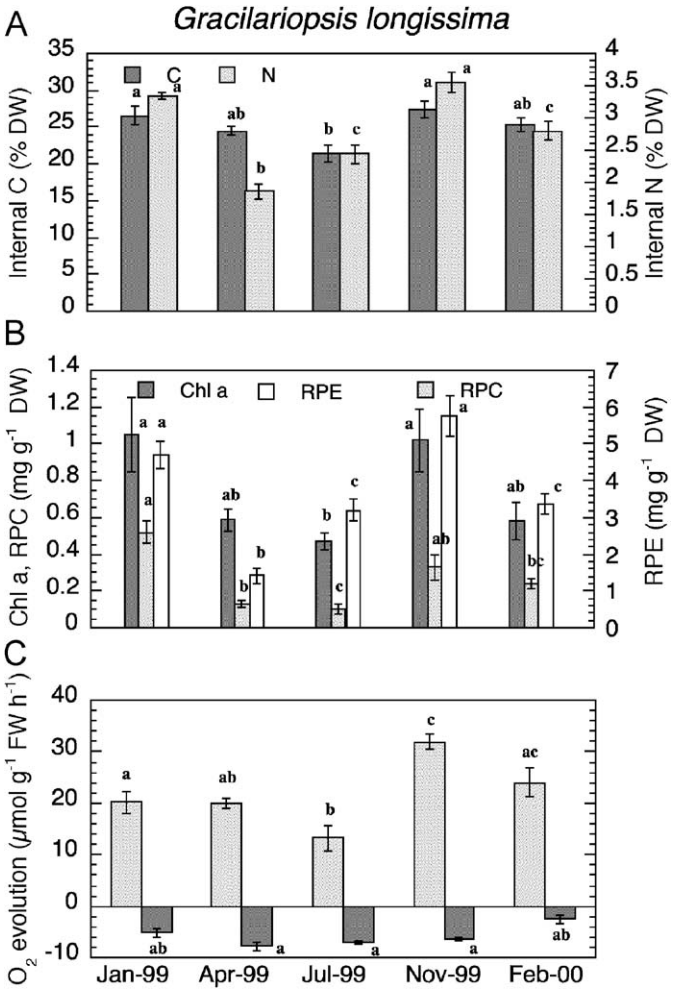

Fig. 2. *G. longissima*. Seasonal variations of (A) elemental composition (C, N); (B) photosynthetic pigments (chl *a*, RPE, RPC); (C) net photosynthesis (dotted bars) and dark respiration (full bars). Data are presented as means±S.E. (n=3). Different letters on columns indicate significant differences (P<0.05) among means.

In general, and irrespective of the tidal timing, CO_2 levels were high early in the morning decreasing along the day (e.g. Fig 1). The highest nutrient concentrations were also observed in November, especially during HT.

Species and seasonality explained a high percentage of the variance observed for tissue nutrients (C and N), pigments and oxygen evolution rates (Tables 3 and 4). Since the percentage of variance explained by the tidal state was not significant, data were pooled (Figs. 2 and 3). *G. longissima* showed higher C and N contents and lower chlorophyll *a* concentration than *E. intestinalis*. Overall, maxima were recorded in November (27.4% C, 3.56% N, 1.04 mg chl *a* g^{-1} DW in *G. longissima*; 19.3 % C, 2.56% N, 6.18 mg chl *a* g^{-1}

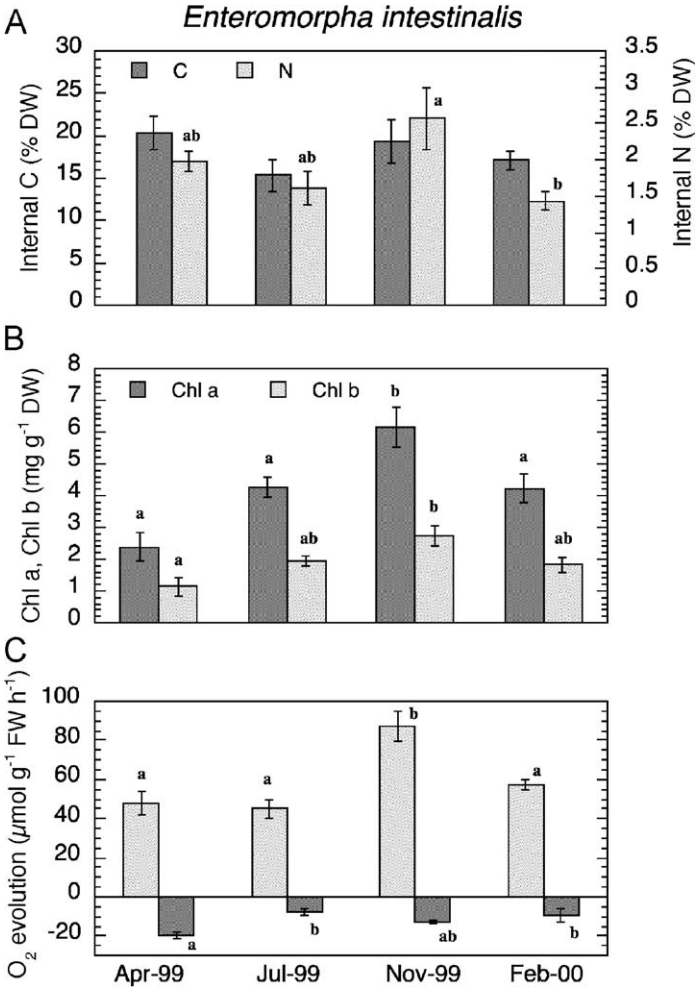

Fig. 3. *E. intestinalis*. Seasonal variations of (A) elemental composition (C, N); (B) photosynthetic pigments (chl *a*, chl *b)*; (C) net photosynthesis (dotted bars) and dark respiration (full bars). Data are presented as means±S.E. (*n*=3). Letters on columns as in Fig. 2.

Table 4
Two-way ANOVA on the effect of time (T), and tidal state (TS, high–low tide) on photosynthetic pigments (chlb in *E. intestinalis*; RPE and RPC in *G. longissima*), and on the effect of species (S) and time (T) on growth rate in *G. longissima* and *E. intestinalis* from Los Toruños salt marsh

Variable	Source of variation	*df*	MS	*F*	%Explained
Chl *b*	T	3	2.61	11.69***	55.4
	TS	1	0.79	3.56^{ns}	16.8
	T×TS	3	1.09	4.88*	23.1
	Error	16	0.22		4.7
RPE	T	4	17.13	21.59***	89.0
	TS	1	0.54	0.68^{ns}	2.8
	T×TS	4	0.78	0.99^{ns}	4.1
	Error	20	0.79		4.1
RPC	T	4	0.17	38.24***	53.1
	TS	1	0.11	24.36***	34.4
	T×TS	4	0.03	7.40***	9.4
	Error	20	0.01		3.1
Growth rate	S	1	0.004	1.84^{ns}	6.1
	T	11	0.03	10.70***	45.5
	S×T	11	0.03	13.91***	45.5
	Error	117	0.002		3.0

ns: non-significant.
*($P<0.05$).
***($P<0.001$).

DW in *E. intestinalis*) and minima in April (21.5% C, 1.87% N, 0.47 mg chl *a* g^{-1} DW in *G. longissima*) or July (15.3% C, 1.62% N, 2.40 mg chl *a* g^{-1} DW in *E. intestinalis*). Seasonal fluctuations in RPE and RPC (*G. longissima*) and chl *b* (*E. intestinalis*) were very close to that of the chl *a* (Figs. 2 and 3). Photosynthesis and respiration rates were always higher in *E. intestinalis* than in *G. longissima* (Figs. 2 and 3). Both species achieved the highest photosynthetic rates also in November (31.92 μmol O_2 g^{-1} FW h^{-1} in *G. longissima* and 87.12 μmol O_2 g^{-1} FW h^{-1} in *E. intestinalis*), while minimum values

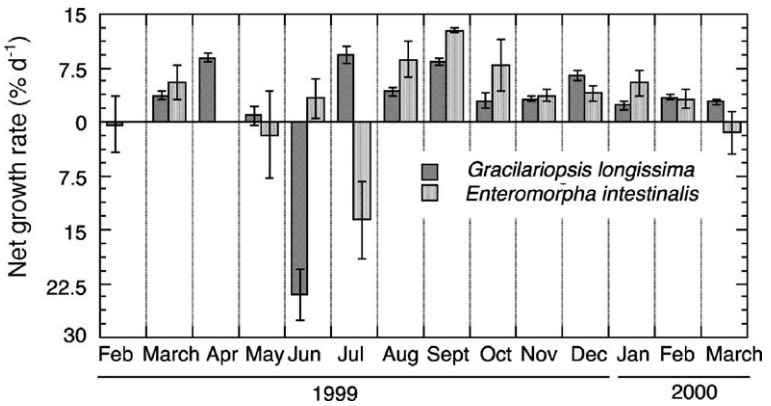

Fig. 4. Seasonal variations of growth rates in *G. longissima* and *E. intestinalis* measured in situ in a tidal creek of Los Toruños salt marsh. Data are presented as means±S.E. (n=5–10).

were recorded in summer. Seasonal differences but not species-specific, were observed in growth rate (Table 4, Fig. 4). However, punctual differences were observed in summer. Thus, *G. longissima* had large losses in June (-24% day^{-1}) and *E. intestinalis* in July (-13.6% day^{-1}). In general, positive growth was reached from late summer onwards,

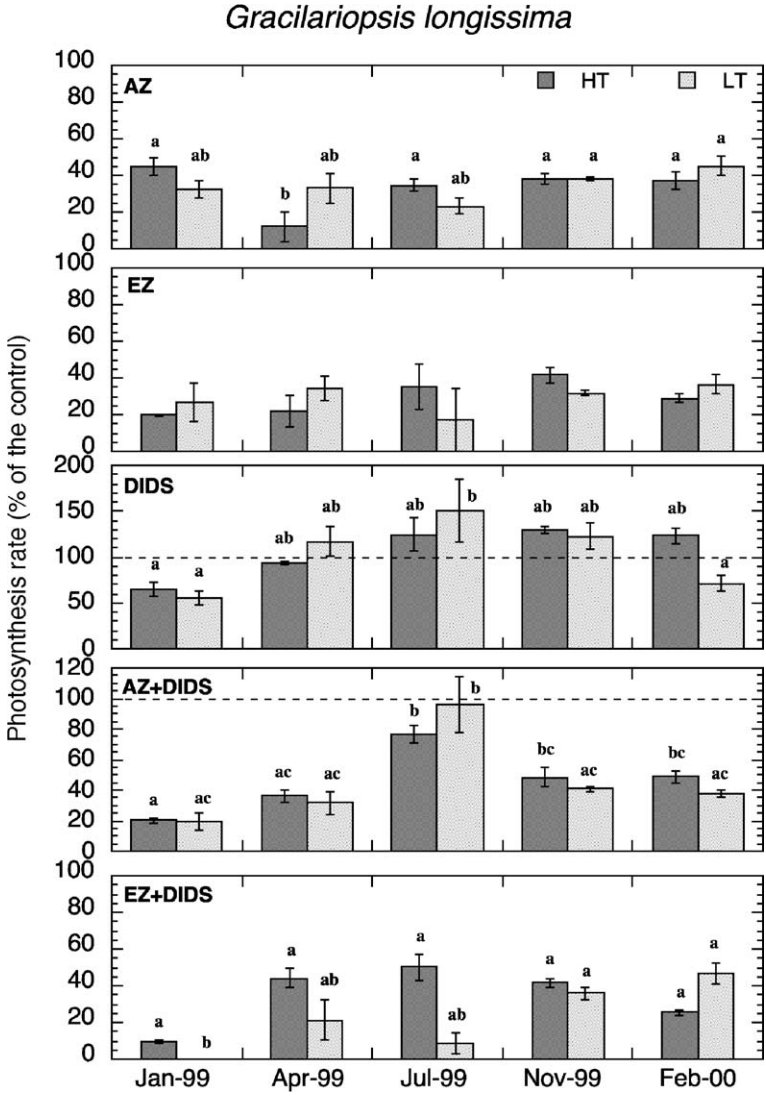

Fig. 5. *G. longissima*. Seasonal variation of the photosynthetic rates with inhibitors of inorganic carbon acquisition mechanisms (acetazolamide, AZ; 6-ethoxyzolamide, EZ; 4, 4′-diisothiocyanatostilbene-2,2′-disulfonate, DIDS) added during in situ incubations at high tide (HT) and low tide (LT). Data are presented as means±S.E. (n=3). Letters on columns as in Fig. 2.

peaking slightly in September (8% day^{-1} for *G. longissima* and 12.7% day^{-1} for *E. intestinalis*).

The addition of different inhibitors of carbon acquisition mechanisms on photosynthesis caused a rather species-dependent effect. Photosynthesis in *G. longissima* was partially inhibited by AZ (Fig. 5). The percentage of inhibition was nearly constant (50–55%)

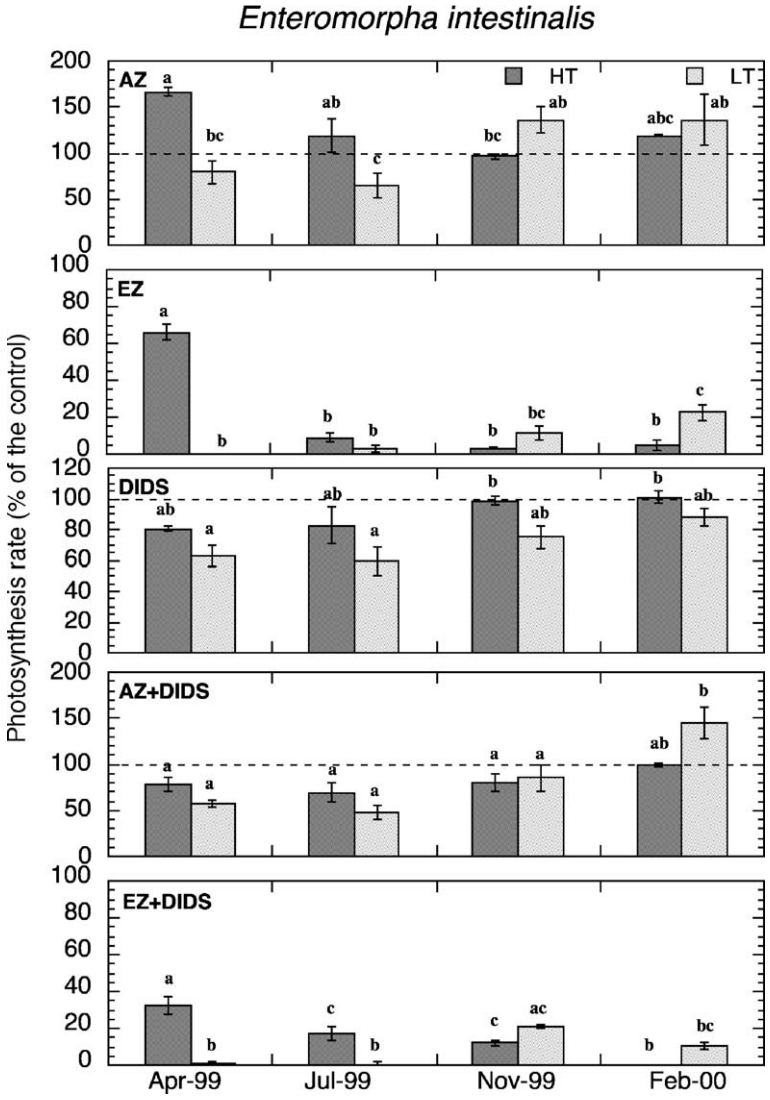

Fig. 6. *E. intestinalis*. Seasonal variation of the photosynthetic rates with inhibitors of inorganic carbon acquisition mechanisms (acetazolamide, AZ; 6-ethoxyzolamide, EZ; 4, 4′-diisothiocyanatostilbene-2,2′-disulfonate, DIDS) added during in situ incubations at high tide (HT) and low tide (LT). Data are presented as means±S.E. (n=3). Letters on columns as in Fig. 2.

throughout the study period suggesting a constitutive character for the external AC activity in the DIC acquisition for this species. A higher inhibition (60–65%) but a similar temporal pattern was observed after EZ addition. The effect of DIDS on *G. longissima* was seasonal, with inhibition in January (35–40%) while stimulation occurred from April (up to 150%) onwards. The responses to AZ+DIDS and EZ+DIDS were similar to that observed for DIDS, but with an enhanced inhibition. In general, the tidal state did not affect the response of *G. longissima* to the inhibitors.

In contrast, AZ showed little inhibition of, or even stimulated, photosynthesis in *E. intestinalis*. Thus, in April-99 and July-99, inhibition (ca. 20%) occurred only during LT, whereas stimulation (up to 165%) was observed during HT (Fig. 6). EZ caused a stronger effect than AZ, and percentages of inhibition for *E. intestinalis* (ca. 90%, with the exception of the data of April-99 at HT) were higher than for *G. longissima*. The effect of EZ (and EZ+DIDS) was dependent upon the tidal state. As observed in April-99 for AZ, the percentage of inhibition was much higher at LT (100%) than that at HT (35%). In contrast to *G. longissima*, the addition of DIDS did not stimulate photosynthesis in *E. intestinalis* and no significant seasonal variations were observed. Although percentages of inhibition were always higher at LT (25% mean seasonal value), they were not statistically different from those at HT (10%). The response to AZ+DIDS followed a seasonal variation close to the observed for AZ, but with inhibition values similar to those obtained for DIDS.

4. Discussion

As expected, seasonal variations and daily changes in inorganic carbon and related physico-chemical variables were recorded at the tidal creek of Los Toruños salt marsh where *G. longissima* and *E. intestinalis* proliferate. In general, and regardless of the season, tidal estate (LT or HT) or tidal timing, high levels of CO_2 (above the equilibrium values) and low O_2 concentrations and pH values were recorded early in the morning, likely as a consequence of the community respiration at night. Similar results were obtained in a Mediterranean lagoon were three macroalgal species (*Cladophora linum*, *Gracilaria verrucosa* and *Ulva* sp.) coexist (Menéndez et al., 2001).

In summer, during the afternoon low tide, the highest values of pH (8.95), salinity (42) and temperature (33 °C) were recorded. Such a fact, in conjunction with the stimulation of the photosynthetic activity (macro and microphytobenthos and phytoplankton) driven by elevated PFD levels, resulted in very low DIC (1.6 mM) and CO_2 levels (0.79 μM). Since the K_m (CO_2) of Rubisco for marine macroalge is usually high (30–90 μM, Beer, 1994; Badger et al., 1998) and CO_2 diffusion in seawater is 10^4 times slower than in air, photosynthesis and growth of species occurring in this habitat (mostly *G. longissima* and *E. intestinalis*) are expected to be Ci-limited. Previous laboratory experiments pointed out that *G. longissima* and *E. intestinalis* are both capable of using HCO_3^- as an exogenous source of Ci to overcome CO_2 limitations (Andría et al., 2001). Such mechanisms include an extracellular CA-mediated mechanism (more active in *G. longissima*) and a direct HCO_3^- uptake (via a DIDS-sensitive mechanism) (more relevant in *E. intestinalis*). However, acclimation experiments to fluctuating DIC levels showed that both mecha-

nisms are inducible and their relative contributions to C acquisition depend on DIC availability (Andría et al., 2001). The capacity to switch between HCO_3^- acquisition systems has been reported earlier for green macroalgae under "carbon stress" conditions (e.g. high pH or high PFD) (Axelsson et al., 1995; Larsson et al., 1997; Larsson and Axelsson, 1999).

The occurrence of (inducible) species-dependent strategies for DIC acquisition in intertidal seaweed has been considered as an adaptive mechanism to optimise the photosynthetic capacity in these periodically changing habitats (Axelsson et al., 1989a,b; Peckol et al., 1994; Rivers and Peckol, 1995). Thus, the potential over-excitation of the photosynthetic apparatus under low CO_2 supply at high PFD (usual when plants are photosynthesising at high rates) could be reduced by investing the excess of energy in HCO_3^- utilization processes (Kaplan and Reinhold, 1999), alleviating a likely Ci limitation under unfavourable growth conditions. The diminution in growth rate (even negative) observed in summer, along with the reduction of photosynthesis and tissue C content in *G. longissima* and *E. intestinalis* could be partially explained by the shortage in DIC levels, as measured previously under laboratory conditions in these species (Andría et al., 2001) and in *Chaetomorpha linum*, *G. verrucosa* and *Ulva* sp. from a Mediterranean coastal lagoon (Menéndez et al., 2001). However, besides the low DIC availability, the N cell quota was found to be below the optimum value of 2% DW (Hanisak, 1983) in July (*E. intestinalis*) and April (*G. longissima*) suggesting also a likely N limitation for growth. The summer drop in N content was more pronounced in *E. intestinalis* (1% DW) denoting the lower uptake and storage capacities of this specie compared to *G. longissima* when N ambient concentrations are low (Fujita, 1985; Anderson et al., 1996). In fact, *Gracilariopsis lemaneiformis* can store N in the phycobilisomes and reallocate it to sustain growth rate under conditions of N-limitation (Vergara et al., 1995). The observed drop of RPE and RPC in April (and July) besides the decrease caused by high PDF would indicate N-limitation (Vergara and Niell, 1993).

The involvement of either an extracellular CA or a HCO_3^- transporter has been widely assessed in macroalgae using specific inhibitors (e.g. AZ, EZ and DIDS) (Björk et al., 1992; Drechsler et al., 1993; Beer, 1994; Axelsson et al., 1995; Larsson et al., 1997; Andría et al., 1999, 2000; Larsson and Axelsson, 1999; Mercado et al., 1997, 1998; Mercado and Niell, 1999). All the cited works, excepting Larsson et al. (1997), have been carried out in the laboratory. The novelty of the present work is the in situ use of inhibitors in order to detect and identify potential seasonal or tidal patterns in both species studied. In our study, photosynthesis measurements were conducted in the field, at saturating PFD and DIC levels (2.2 mM) in unbuffered seawater at relatively high biomass density for a relatively long period of time (30 min), which may affect the interpretation of the results. In fact, DIC was added at saturating levels, even in excess when compared to natural DIC concentrations in some seasons (e.g. summer), and the pH increased during the incubations as a consequence of photosynthesis in unbuffered seawater. The high percentage of inhibition in *E. intestinalis* after EZ addition regardless of the season and tidal state confirms the previous observation that photosynthesis depends mainly on internal CA activity at saturated DIC levels in this species (Andría et al., 2001). However, in April and July, and mostly at low tide, both external CA activity and DIDS-sensitive HCO_3^- uptake contributed significantly to Ci acquisition.

In the case of *G. longissima*, the inhibition by AZ suggests a constitutive character for CA. In summer, DIDS did not inhibit, but stimulated photosynthesis, while the addition of DIDS+AZ relieves AZ inhibition. The addition of DIDS in unbuffered seawater would block OH^- extrusion and therefore photosynthetic pH alkalinization, favouring the existence of higher CO_2 levels compared to the control. Also, unlike chlorophytes such as *Ulva* sp., *G. longissima* is sensitive to Tris buffer (Andría, 2001), with inhibition of photosynthesis similar to that described by Axelsson et al. (2000) in *Laminaria saccharina*. The Tris buffer effect is presumed to be based on the abolition of the H^+ extrusion mechanism that favours CA-mediated bicarbonate transformation to CO_2 (Beer et al., 2002). Therefore, it can be argued that in summer, when there is a shortage of CO_2 availability, an active CO_2 transport would be overexpressed, and addition of DIDS could avoid local pH alkalinization, leading consequently to higher periplasmic CO_2 levels as a result of an active (H^+ pumping) CO_2 transport. The lack of AZ inhibition with DIDS reinforces the suggestion of an enhanced, active CO_2 transport under C limitation in summer.

In conclusion, populations of *Gracilariopsis logissima* and *Enteromorpha intestinalis* from Los Toruños salt marsh are subjected to large seasonal variations and abrupt daily changes in inorganic carbon levels and related physico-chemical variables. In contrast, photosynthesis, pigments, internal composition (N) and growth varied seasonally but not on tidal basis, as macrophytes integrated this short-term environmental variability. In both species, the lowest values were recorded in summer, as a result of extreme values of environmental variables. The use of carbon acquisition mechanisms inhibitors in situ showed a constitutive character of external CA in *G. longissima*, while *E. intestinalis*, relies on internal CA activity. With respect to the DIDS-sensitive bicarbonate transport in *G. longissima*, it was effective in winter but not in summer, suggesting the stimulation of a H^+ extrusion mediated CO_2 transport in periods of low CO_2.

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