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Photoacclimation of the seagrass *Halophila stipulacea* to the dim irradiance at its 48-meter depth limit

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Abstract

The seagrass *Halophila stipulacea* grows in the northern Red Sea from the intertidal to depths of ~ 50 m. Along that gradient, there is a > 1 order of magnitude difference in irradiance and the spectrum narrows from that of full sunlight to dim blue-green light. Based on these differences, we set out to estimate the molar ratios and potential contributions of photosystem II (PSII) and photosystem I (PSI) to light absorption, and photosynthetic electron transport rates (ETR), in plants growing at 1-m and 48-m depths. The amount of PsaC (a proxy for PSI) was three times higher in the deep-growing plants. On the other hand, the amount of PsbA (a proxy for PSII) did not differ significantly between the two depths. Thus, the PSII : (PSII + PSI) ratio (FII) was 0.62 in the shallow- and 0.41 in the deep-growing plants. Similar results were obtained by 77K emission fluorescence. Because ETR is linearly dependent on FII, it follows that the ETR vs. irradiance curves differed significantly if calculated based on the commonly used FII value of 0.5 or the FII values we found. As a result, the photosynthetic parameters ETR_{max} and α also differed when using the different FII values. Correct(ed) FII values should, therefore, be used in the calculation of photosynthetic ETRs. The ability of *H. stipulacea* to alter its amount of PSI relative to PSII according to the ambient irradiance and spectrum may be one reason why this organism can grow down to its exceptional depth limit in clear tropical waters.

In aquatic systems, irradiance is attenuated with depth, and the light spectrum in clear waters shifts from that of full sunlight in the intertidal to a narrower band of bluish light at depth. Accordingly, plants growing along a depth gradient have to acclimate to these differences in the optical properties of the water that surrounds them. In addition to changes in plant morphology (Schwarz and Hellblom 2002) and meadow architecture (Dalla Via et al. 1998; Peralta et al. 2002), marine angiosperms, or seagrasses, may also enhance chlorophyll (Chl) concentrations and photosynthetic efficiencies at low irradiances (reviewed in Ralph et al. 2007). Also spectral changes can affect light absorption by altering the composition and ratio of the two photosystems (PSs) and, thus, photosynthetic rates and plant growth (Fujita 1997; Falkowski and Raven 2007). Among marine macrophytes, such spectral changes were shown to influence the functionality of the two PSs as found for the green macroalgae *Ulva pertusa* and *Bryopsis maxima* (Yamazaki et al. 2005). Regarding seagrasses, only two studies have reported on changes in the PS composition: one in shallow-growing plants during various seasons (Major and Dunton 2000) and another along a narrow depth gradient down to 1.6 m (Major and Dunton 2002). To our knowledge there are no studies on photosystems' acclimatory mechanism in seagrasses growing at greater depths where both the irradiance and light spectrum change drastically.

In the Gulf of Aqaba (northern Red Sea), the seagrass *Halophila stipulacea* grows from the intertidal to 30–50 m depending on season. Along such depth gradients, the light environment changes from high levels of photosynthetically active radiation (PAR) and ultraviolet radiation (UVR) to a narrow blue-green spectrum containing virtually no red light nor UVR. In the summer, the midday PAR decreases from ~ 2100 μmol photons m^{-2} s^{-1} at the water surface to ~ 100 μmol photons m^{-2} s^{-1} at 50 m, which is the depth limit for this plant. In previous studies we investigated how *H. stipulacea* copes with high PAR and high UVR. It was found that midday PAR irradiances of > 400 μmol photons m^{-2} s^{-1}, corresponding to depth of < 10 m, caused chloroplasts to clump together as an apparent mechanism of photo-protection (Sharon and Beer 2008). This clumping also lowered the amount of incident PAR absorbed by the leaves, thus altering their calculated photosynthetic electron transport rates (ETR). In a subsequent study, we found that UVR was responsible for chloroplast movements in this organism (Y. Sharon and S. Beer unpubl.).

Although we have frequently observed the presence of *H. stipulacea* at depths of > 30 m, diving limitations in bottom time at such depths prevented in situ photosynthetic measurements there. However, recent improvements in diving techniques have now enabled the application of in situ pulse amplitude modulated (PAM)–fluorometric measurements in order to quantify photosynthetic rates down to 50 m. In order to understand *Halophila stipulacea*'s photo-acclimation strategy to the low irradiances and the blue-green–shifted spectrum at its depth limit, several aspects of the photosynthetic units were studied: in situ PAM-fluorometric measurements were, thus, combined with determinations of Chl *a* and *b* concentrations, measurements of the abundance of core subunits of PSI (PsaC), PSII (PsbA, D1 protein), and the large subunit

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(RbcL) of ribulose-1,5-bisphosphate carboxylase and oxygenase (Rubisco), as well as energy transfer by the two PSs.

Methods

Study site—The *Halophila stipulacea* meadows studied (during Jul and Aug 2009) are located ~ 200 m south and 300 m north of the Inter-University Institute (IUI) in Eilat, the Gulf of Aqaba, northern Red Sea (29°30′N, 34°55′E). These meadows extend down to ~ 50 m during the summer.

Spectral and irradiance measurements—PAR irradiance and spectral composition at various depths were measured for seven cloudless middays during the study period with a profile reflectance radiometer (PRR-800; BioSpherical Instruments) equipped with both spectral (300–875 nm, μW cm^{-2} nm^{-1}) and PAR (400–700 nm, μmol photons m^{-2} s^{-1}) sensors. This instrument was lowered down from a boat through the water column above the seagrass beds using a free-fall technique (Kirk 1994).

Chlorophyll fluorescence measurements—Chl fluorescence was measured in situ on cloudless days at 1-m and 48-m depth by PAM fluorometry using a Diving-PAM (Walz). This instrument was programmed to perform so-called rapid light curves (RLC) by irradiating a leaf with eight increasing irradiances (up to 1450 μmol photons m^{-2} s^{-1} and 1827 μmol photons m^{-2} s^{-1} for the deep- and shallow-growing plants, respectively) for 10 s each, where in the end of each irradiation the effective quantum yield ($\Delta F : Fm'$) was measured. Each sequence of measurements was preceded by minimal exposure to darkness (< 1 s) just after the opened dark-leaf clip (Walz) attached to the leaf was connected to the instrument's optical fiber, following the protocol of Beer et al. (2001). The photosynthetic ETR at each given irradiance was calculated as $\Delta F : Fm' \times$ PAR $\times$ absorption factor (AF) $\times$ FII, where PAR is the incident irradiance given by the Diving-PAM's actinic light source (as premeasured with the Diving-PAM's quantum sensor, calibrated against a quantum-sensor–equipped Li-250A light meter, LiCor), AF is the fraction of light absorbed by the leaf as determined under water (Sharon and Beer 2008), and FII (or the FII value) is the assumed fraction of photons absorbed by PSII (normalized to (PSI + PSII)). FII is often assumed to be 0.5, but varied here with depth (*see* Results). The ETR values of the RLCs were depicted against absorbed PAR (PAR_a; i.e., PAR $\times$ AF) as recommended by Saroussi and Beer (2007) and as performed previously for this plant (Sharon and Beer 2008).

Pigment quantification and protein extraction—Plants were collected for Chl and protein analysis. After the leaves were separated from the shoots, some were used for Chl extraction while others were frozen in liquid N_2 for further protein analyses.

Chl contents of the leaves were determined after extraction in N,N-dimethyl formamide for 24 h in darkness. Absorbance at 663, 647, and 625 was then measured spectrophotometrically (using an Ultraspec 2100 Pro; Amersham Biosciences), and Chl *a* and *b* concentrations were calculated on a leaf-area basis according to Moran (1982).

Middle parts of leaves with a known surface area were suspended in 250 μL cold 1$\times$ denaturing extraction buffer containing 140 mmol L^{-1} tris(hydroxymethyl)aminomethane (Tris) base, 105 m mol L^{-1} Tris–HCl, 0.5 m mol L^{-1} Ethylenediaminetetraacetic acid, 2% lithium dodecyl sulfate, 10% glycerol, and 0.1 mg : mL PefaBloc SC 4-(2-Aminoethyl) benzenesulfony fluoride hydrochloride protease inhibitor (Roche). These samples were then sonicated using a Fisher Scientific Model 100 Sonic dismembrator with a microtip attachment at a setting of 30%. To avoid overheating and improve extraction efficiency, the samples were then refrozen immediately in liquid N_2. Two such cycles of freezing followed by thawing were performed in order to maximize protein extraction with minimal degradation of representative membranes and soluble proteins (Brown et al. 2008; Levitan et al. 2010*a*). Following the disruption of the leaves, the samples were centrifuged for 3 min at 10,000 $\times$ *g* to remove insoluble material and unbroken cells. The total protein concentration was measured with a modified Lowry assay (Bio-Rad DC) using bovine gamma globulin as a comparative protein standard.

Target protein quantification—Key photosynthetic proteins subunits of PSI (PsaC), PSII (PsbA), and Rubisco (RbcL) were quantified using specific standards (AgriSera, Sweden) following the procedure described in Brown et al. (2008) and Levitan et al. (2010*a*). Primary antibodies (AgriSera, Sweden) were used at a dilution of 1 : 40,000 in 2% enzymatic chemiluminescence (ECL) advanced blocking reagent in Tris buffered saline (TBS-T) for PsbA, PsaC, and RbcL. The blots were incubated for 1 h with horseradish-peroxidase–conjugated rabbit anti-chicken secondary antibody (Abcam) for RbcL primary antibodies and with horseradish-peroxidase–conjugated chicken anti-rabbit secondary antibody (Abcam) for the PsbA and PsaC primary antibodies (diluted to 1 : 40,000 in 2% ECL). The blots were developed with ECL Advance detection reagent (Amersham Biosciences, GE Healthcare) using a charged coupled devise imager (DNR; M-ChemiBIS). Protein levels of the immunoblots were quantified using the Quantity One software (Bio-Rad) as calculated from standard curves for each blot (Brown et al. 2008).

Assuming that PsaC and PsbA (pmol μg protein^{-1}) are proxies for PSI and PSII, respectively, the FII factor was calculated as the amount of PSII relative to (PSI + PSII) according to PsbA : (PsaC + PsbA).

Energy transfer by photosystems—The distribution of excitation energy between PSI and PSII can be estimated by measuring the Chl fluorescence emission spectra at 77K (Butler 1978). The functionality of the two photosystems was estimated according to Schubert et al. (1995). A frozen glass cuvette containing a single leaf was scanned in the low-temperature holder of a spectrofluorometer (Cary Eclipse Fluorometer; Varian) with the excitation wavelength set to 435 nm. Emission of the leaf was measured at

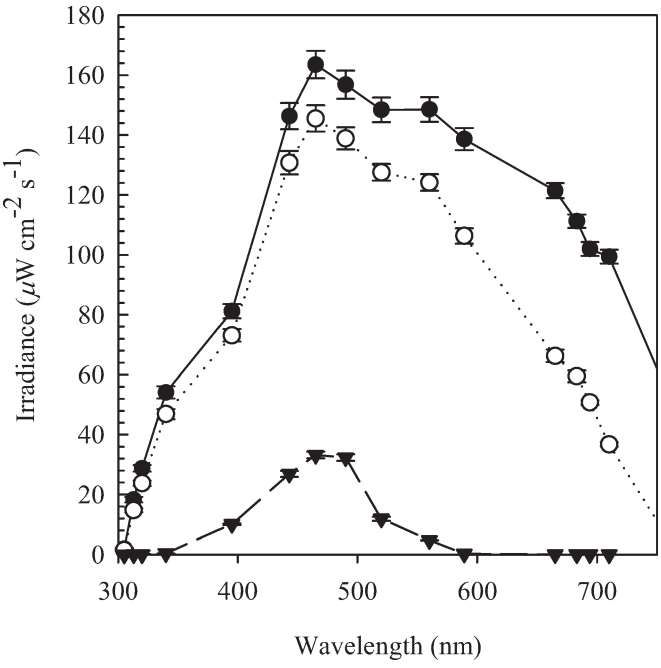

Fig. 1. In situ transmitted midday down-welling irradiance at the surface (closed circles) and at 1-m (open circles), and 48-m (closed triangles) depths offshore the IUI, Eilat, Israel. Data points are means ± SD of 20 measurements during seven different days in July and August 2009.

1-nm intervals from 650 nm to 750 nm. Ratios of PSI and PSII emission were determined from the peak heights at 717 nm and 686 nm, respectively. Assuming that these peak heights are proxies for the excitation energy of PSI and PSII, respectively, the FII factor was here calculated as the emission value of PSII relative to (PSI + PSII) according to $\lambda_{686} : (\lambda_{717} + \lambda_{686})$.

Statistical analyses—For the protein data ($n = 4$) we used a *t*-test for analyzing equality of means between groups ($p < 0.05$). For the 77K fluorescence data, Chl content, and RLC analysis ($n = 7$–8), *t*-tests were used to assess their significance ($p < 0.05$).

Results

The midday spectra (300–800 nm) from the surface and 1-m and 48-m depths are presented in Fig. 1. These spectra reveal high UVR (< 400 nm) and red (650–700 nm) wavelengths values at the surface and at 1 m, which were hardly detected at 48 m, where > 90% of the surface irradiance narrowed to blue-green wavelengths. Within this depth gradient, the PAR decreased > 1 order of magnitude, from ~ 1500 μmol photons m^{-2} s^{-1} at 1 m to ~ 100 μmol photons m^{-2} s^{-1} at 48 m, during midday (Table 1).

The contents of Chl *a* and *b* per leaf area were significantly different between plants growing at 1 m and 48 m (Fig. 2). The deep-growing plants contained almost three times more of both Chl *a* and *b* than the shallow-growing ones. Chl *a* : *b* ratio did not differ between the shallow- and deep-growing plants because both Chls increased similarly with depth. Although there was no significant difference in the amount of PsbA between the shallow- and deep-growing plants, the PsaC concentration was three times higher in the deep-growing ones (Fig. 3). Thus, the PsaC : PsbA ratio increased ~ three-fold in the deep-growing plants. Assuming that the amount of PsaC and PsbA subunits are quantitative proxies for the presence of PSI and PSII, respectively (Brown et al. 2008), the PSI : PSII ratio (as reflected by the PsaC : PsbA ratio) increased similarly in deep-growing plants. In the latter, the quantity of the large subunit of the RbcL was reduced to 60% compared with that contained in the shallow-growing plants. The RbcL : PsbA ratio was twice as high in the shallow-growing plants as in the deep-growing ones (2.23 vs. 1.12).

The 77K Chl fluorescence emission spectra are depicted in Fig. 4. The spectrum of the 48-m plants showed no differences between peak heights at 686 nm (for PSII) and 717 nm (for PSI). On the other hand, the fluorescence emission spectrum of the shallow-growing plants' Chl revealed a significant difference between those peak heights.

The FII factor (i.e., the probability of photons to be absorbed by PSII), was found to be 0.62 and 0.41 for the 1-m and 48-m growing plants, respectively, according to the protein data. This parameter influences the calculated ETR linearly (*see* Methods). Thus, when using these FII factors instead of the commonly used factor of 0.5, the difference between the ETR vs. irradiance curve of plants from the two depths is enhanced (Fig. 5). Accordingly, the photosynthetic parameters maximal electron transport rate (ETR_{max}), the initial slope of the ETR vs. irradiance curve (α), and the onset of light-saturation point (I_k) based on the curves differ too. Extrapolating the values of those parameters by using the curve-fitting equation of Platt et al. (1980) reveals that: (1) The ETR_{max} value of deep-growing plants is 60% vs. 30% that of the shallow-growing plants when using 0.5 or the correct(ed) FII values (0.62

Table 1. Midday irradiance, photosynthesis maximum (Pmax), the initial slope (α), and onset of light-saturation point (I_k) value derived from RLC performed in situ on 1-m– and 48-m–depth growing leaves of *Halophila stipulacea* when using either 0.5 or the correctly acquired PSII : (PSII + PSI) ratio (FII) values at each depth. Data are means ± SD of eight replicates. ns = not significant.

Depth (m)	FII	Midday irradiance	Pmax	*p*	α	*p*	I_k	*p*
1	0.5	1498±53	68.66±10.91	<0.05	0.36±0.03	<0.0001	189.52±41.12	<0.001
48	0.5	107±8	38.79±4.34	<0.05	0.61±0.016	<0.0001	63.17±7.12	<0.001
1	0.62	1498±53	103.68±13.52	<0.0001	0.45±0.039	ns	189±41.1	<0.001
48	0.41	107±8	32.74±3.49	<0.0001	0.50±0.018	ns	65±7.3	<0.001

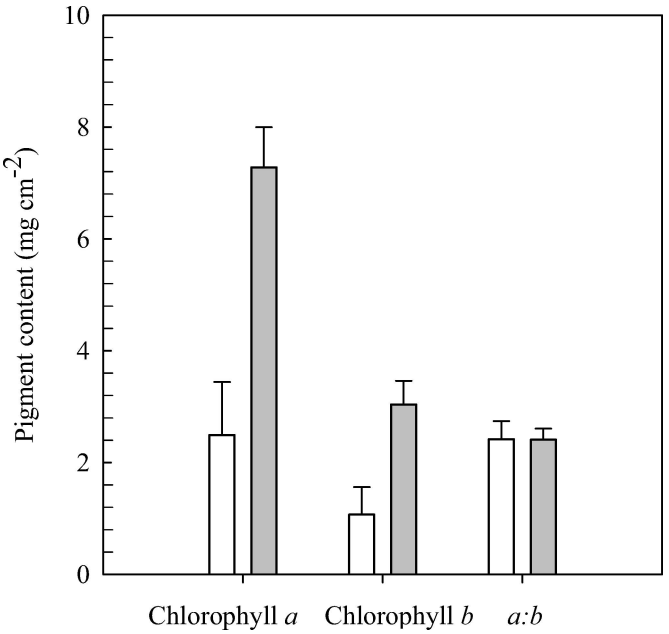

Fig. 2. Chlorophyll *a*, *b*, and *a*:*b* ratio concentrations of 1-m- (open bars) and 48-m-deep– (gray bars) growing *Halophila stipulacea* plants. Data points are means ± SD of eight replicates. Different letters represent statistically significant differences between each pair in a group.

and 0.41), respectively; (2) α differs significantly between plants from the two depths when using 0.5 as the FII value but no such difference is apparent when using the correct(ed) FII values for each depth; and (3) the difference in I_k is similar when using either 0.5 or the correct FII values (Table 1). Similar differences in these photosynthetic parameters were found when using the 77K fluorescence spectra peak heights to calculate the FII values.

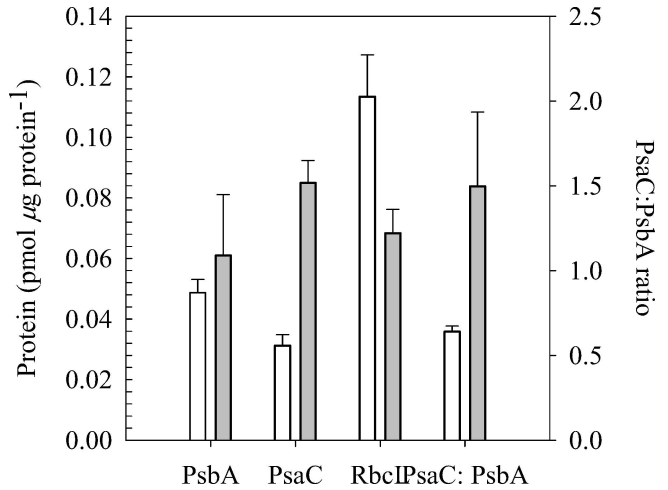

Fig. 3. Amounts of the photosynthesis-related proteins PsbA, PsaC, and RbcL, and PsaC:PsbA ratios of 1-m- (open bars) and 48-m-deep– (gray bars) growing *Halophila stipulacea* plants. Data points are means ± SD of four replicates. Different letters represent statistically significant differences between each pair in a group.

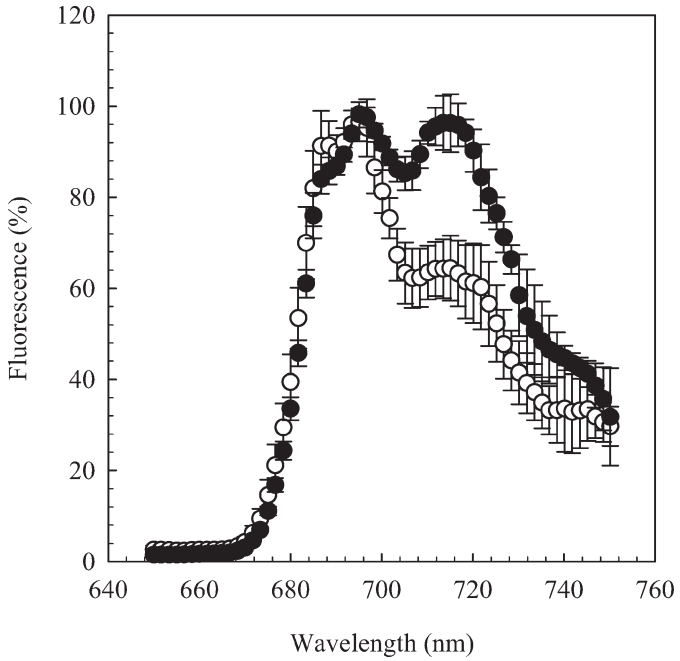

Fig. 4. The 77K fluorescence emission spectra of 1-m- (open circles) and 48-m-deep– (closed circles) growing *Halophila stipulacea* plants. Data points are means ± SD of eight replicates. Different letters represent statistically significant differences in peak heights between plants from the two depths.

Discussion

In this study, we investigated deep-growing plants of the seagrass *H. stipulacea*, which, at their depth limit in the summer (~ 50 m), received a down-welling PAR of ~ 5% of that at the surface. This low light requirement in comparison with the average one for seagrasses (e.g., 11% as reported by Duarte [1991]) could not be explained by a high photosynthetic:nonphotosynthetic biomass ratio because this plant has the same above- (leaves and stems) to below-ground (roots and rhizomes) biomass ratio (~ 1 on a dry-weight basis, data not shown) as do many other seagrasses (Duarte and Chiscano 1999). Thus, the ability to modify its photosynthetic system(s; e.g., photosystem ratio and Chl content), may contribute to its abundant growth under very dim irradiances.

Changes in Chl concentration are a known response of plants in their acclimation to variations in the light environment. We found that in deep-growing *H. stipulacea* leaves both Chl *a* and *b* were increased, as was also previously reported for this species (Sharon and Beer 2008; Sharon et al. 2009), as well as for other seagrasses (Wiginton and McMillan 1979; Dennison 1990; Abal et al. 1994). In terrestrial plants that grow within the shade of a leaf canopy, Chl *b* concentrations increase more than those of Chl *a* because Chl *b* utilizes a slightly narrower wavelength span in accordance with the spectrum found there (Martin and Churchill 1982; Marschall and Proctor 2004). In clear marine waters there is not only a narrowing of the spectrum with depth, but also a shift toward bluish light because of the selective absorption of water.

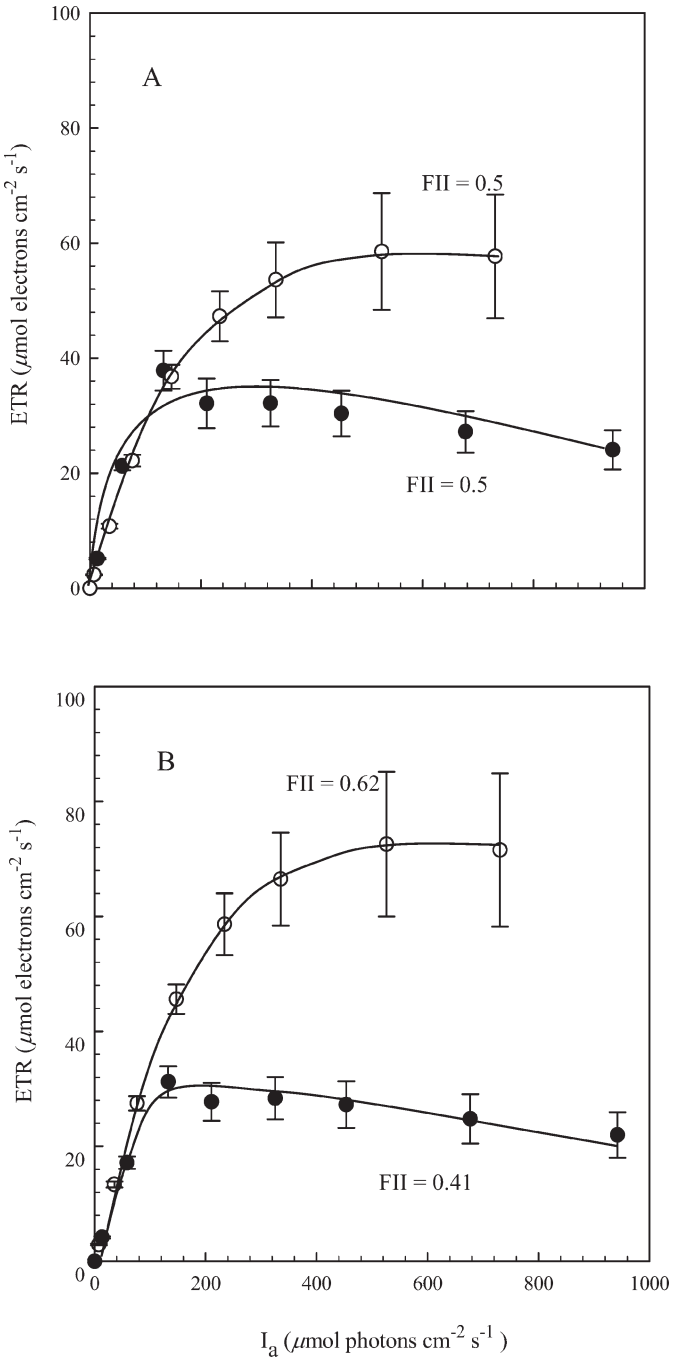

Fig. 5. Electron transport rates (ETR) derived from PAM fluorometry of 1-m- (open circles) and 48-m-deep– (closed circles) growing *Halophila stipulacea* plants as calculated (A) with an FII of 0.5, and (B) with the correct(ed) FII values as derived from the protein analysis.

Therefore, it may be that *H. stipulacea* does not feature the typical terrestrial-plant response to shading. Also the virtual absence of red light at the depth limit of this plant would not provide Chl *b* with any advantage in that spectral region, while the blue light reaching that depth would sustain the absorption of both Chl *a* and *b* in the blue-light region rather equally.

The almost three times higher PSI : PSII ratio in the deep-growing *H. stipulacea* plants was a result of changes mainly in the PSI content, similar to what was found for a cyanobacterium (Levitan et al. 2010*b*), some terrestrial plants and freshwater algae (Fujita 1997) and the macroalgae *Ulva pertusa* and *Bryopsis maxima* (Yamazaki et al. 2005). The latter authors suggested that the higher ratios of PSI : PSII may be derived from the need for a higher adenosine triphosphate and nicotinamide adenine dinucleotide phosphate (ATP : NADPH) ratio, which can be obtained by cyclic electron flow around PSI. In addition, those authors also found a higher level of excitation energy from light to PSI than to PSII as measured by 77K fluorescence. The high PSI : PSII ratio found here for *H. stipulacea* growing at its depth limit, together with the higher functionality of PSI as indicated by the 77K fluorescence emission, thus suggest a similar advantage to this plant under the extremely limiting light conditions found at 48-m depth. It is known that low-light grown chlorophytes usually change the number of the photosynthetic units (i.e., photosystems and Chl) rather than their size as a light-shade adaptation strategy (Falkowski and Owens 1980). Yet, we still do not know the reason why in this plant ATP vs. NADPH production at depth should or would be favored.

The increase in Rubisco, as reflected by its large subunit protein pool, correlates with the higher photosynthetic ETR under high-light conditions (in shallow water). The RbcL : PsbA ratio can, thus, represent Ek, or light saturation index (Brown et al. 2008). At light saturation, the electron transport rate from water to CO_2 is limited by the concentration of Rubisco per electron transport chain (Sukenik et al. 1987), here measured as RbcL : PsbA. For *H. stipulacea*, the RbcL : PsbA ratio was twice as high in the shallow-growing plants, probably reflecting higher carboxylation rates at the higher irradiance. This is similar to the findings of Schofield et al. (2003) for an algal symbiont during a season featuring high irradiances.

Another aspect of our study is to emphasize the need of using correct FII values when calculating ETRs, especially for plants featuring a wide depth-distribution pattern such as exemplified for *H. stipulacea*. When using FII values based on our protein quantification, the ETR_{max} values were ~ 2.5 times higher in the shallow-growing plants as compared to only ~ 1.5 times higher when using the commonly accepted value of 0.5. Interestingly, the initial slope of the RLC (α) did not change between shallow- and deep-growing plants when using correct(ed) FII values, suggesting the same photosynthetic efficiency at low irradiances for both plant types. We conclude that one should be cautious when using 0.5 as the FII value based on our present findings that the amount, functionality, and light absorption probability for each photosystem is clearly not equal in plants growing at extreme irradiances.

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