Photophysiological Adaptations and Acclimations of the Seagrass

*Halophila stipulacea* to Extreme Light Environments

Thesis submitted for the degree

"Doctor of Philosophy"

By Yoni Sharon

Submitted to the Senate of Tel Aviv University
This work was supervised by Prof. Sven Beer
In memory of my father [29.6.1936-4.11.2008]
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Summary

This PhD thesis started with field observations, leading my curiosity to investigate a marine angiosperm (or seagrass) that grows from the intertidal down to the great depths of >30 m. Investigating the photophysiology of this plant enabled me to learn the special characteristics in its acclimation and adaptation to extreme light environments. One can also use the scientific findings of this current work in order to investigate other marine plants and their eco-physiological responses to extreme (both high and low) irradiances.

In the marine environment, light quantity and quality change greatly along depth gradients. Photosynthetic organisms inhabiting those gradients thus need to acclimate or adapt in different ways in order to utilize low light (which is composed of mostly blue wavelengths) in deep waters and to avoid chronic damage in shallow waters where visible irradiances and UV radiation are high. *Halophila stipulacea*, a tropical and sub-tropical seagrass, is an important ecosystem-engineering macrophyte in the Gulf of Aqaba, northern Red Sea. Meadows of this plant serve as nurseries for many fish and invertebrates species, stabilize the sediment in their habitat and take a significant role in the oxygen, nitrogen and carbon cycles. This seagrass inhabits a wide depth gradient (0 - ~50 m), featuring more than an order of magnitude differences in irradiance, in the clear waters of the Gulf of Aqaba. In this work, its photosynthetic light utilization mechanisms were investigated using fluorometric, optic, microscopic, spectrophotometric and biochemical methods, in order to understand how this plant can grow under very dim light at depth and survive under high light and UVR stress in the intertidal. Thus, the main goal was to explain the photophysiological strategies that allow this plant to acclimate and adapt to conditions along a depth gradient where differences in irradiance are extreme.
Our first findings quantified and found a rationale for the observation of diurnal chloroplast movements in high-light growing *Halophila stipulacea* plants. Along the morning hours till midday the chloroplasts clumped together and then dispersed evenly toward the late afternoon. These chloroplast clumping and dispersal were accompanied with changes in the absorption factor (AF) and, thus, affected the calculated electron transport rates (ETR) since the latter depends linearly on the amount of light absorbed by the photosynthetic tissues. This finding thus showed the importance of taking into account changing AF values along the day when calculating ETRs of *Halophila stipulacea*, and perhaps other seagrasses too if featuring diurnally changing AFs under high-light conditions (Ch. 1).

In continuation with our first findings, we investigated the spectrum responsible for chloroplasts clumping in high-light growing *Halophila stipulacea* plants. Since potentially harmful UVR is present in the shallow waters of the Gulf of Aqaba where part of its population thrives, we examined the effect of UVR on the clumping phenomenon. It was found that no chloroplast movements occurred when the plants were protected from 80% of the <400 nm wavelengths. In addition, UVR also affect the effective quantum and the ETRs. We conclude that the potentially harmful effects of UVR on the photosynthetic apparatus of *Halophila stipulacea* are mitigated by its activation of chloroplast clumping, which functions as a means of protecting most chloroplasts and perhaps the nucleus from high irradiances, including UVR (Ch. 4).

*Halophila stipulacea*'s successful growth under a broad irradiance gradient shows either a high plasticity or is caused by longer-term adaptations to the various depths, possibly resulting in the formation of ecotypes. In order to answer this question, we transplanted shoots of this seagrass between the extreme depths of its
distribution at the study time (8 and 33 m) in order to evaluate its acclimation potential to various irradiances. Our findings showed fast changes in photosynthetic responses and light absorption characteristics in response to changing light environments and suggested that *Halophila stipulacea* is a highly plastic seagrass with regard to irradiance, which may partly explain its abundance across a wide range of irradiances along the depth gradient that it occupies (Ch. 3). In addition, we used a novel PAM fluorometer to study the physiology of light-responses of native populations of *Halophila stipulacea*. We suggest that this seagrass acclimates to the widely varying irradiances across this depth gradient by regularly modulating down-regulation based non-photochemical quenching processes (Ch. 2).

Based on the extreme irradiance differences in *Halophila's stipulacea* light-environment along its depth gradient, we set out to estimate the potential contributions of PSII and PSI to light absorption, and photosynthetic ETRs, in plants growing at 1 and 48 m depth. Our results showed significant differences in the content of PSI in the two depth borders of *Halophila stipulacea*'s meadow. The latter difference also affects the FII value (the PSII:(PSII+PSI) ratio) which effects linearly the photosynthetic ETR calculations. We concluded that the ability of *Halophila stipulacea* to alter its amount of PSI relative to PSII according to the ambient irradiance and spectrum may be a basis for this organism's successful growth down to its exceptional depth limit in clear tropical waters (Ch. 5).
Index

Summary..........................................................................................................................1

1. General Introduction

1.1 Overview of relevant research fields

1.1.1 Seagrasses.................................................................6
1.1.2 Light in the marine environment.........................9
1.1.3 Light absorption and utilization in marine plants.....10
1.1.4 Chloroplasts movement and clumping in plants......12
1.1.5 Light absorption under UVR and high light environments...14
1.1.6 Acclimation processes of seagrasses.......................16
1.1.7 The photosystems' apparatus and stoichiometry........18

1.2 Research objectives.........................................................19

2. Research Chapters

2.1 Diurnal movements of chloroplasts in Halophila stipulacea and their effect on PAM fluorometric measurements of photosynthetic rates......21
2.2 Photosynthetic responses of Halophila stipulacea to a light gradient. I. In situ partitioning of non-photochemical quenching..........................25
2.3 Photosynthetic responses of Halophila stipulacea to a light gradient. II. Acclimation following transplantations.................................35
2.4 Chloroplast clumping in the seagrass Halophila stipulacea is triggered by UV radiation.................................................................40
2.5 Photoacclimation of the seagrass Halophila stipulacea to the dim irradiance at its 50 m depth limit.........................................................61
2.6 Measuring seagrass photosynthesis: methods and applications........67
1. General Introduction

1.1 Overview of relevant research fields

1.1.1 Seagrasses

Seagrasses (Angioperms: Monocotyledons) are a unique group of angiosperm that have adapted to the submerged marine environment. There, profoundly they influence the physical, chemical, and biological environments in coastal waters, performing as ecosystem engineers (Wright and Jones 2006) and providing several important ecological services to their environment (Costanza et al. 1997). Seagrasses modify water flow, nutrient cycling and food web structure (Hemminga and Duarte 2000). They are a vital food source for mega-herbivores such as green sea turtles, dugongs and manatees, and provide shelter and home for many animals, including commercially and recreationally important fishery species (Beck et al. 2001). In addition, seagrasses, through their root systems, stabilize sediments and generate large quantities of organic carbon through photosynthesis and carbon fixation.

Seagrasses are distributed in marine environments across the globe, from arctic to tropical regions, but dissimilarly to other taxonomic groups with global distribution, they display a low taxonomic richness (approximately 60 species worldwide, compared with approximately 250,000 terrestrial angiosperms). The three seagrass families (Hydrocharitaceae, Cymodoceaceae and Zosteraceae) evolved from a single lineage of monocotyledonous sometimes between 70-100 million yeas ago (Les et al. 1997). These plants are distinguished from other plant groups that have colonized the marine environment, such as salt marsh plants, mangroves, and marine algae, which are descended from multiple and diverse evolutionary lineages. Regardless of their low species richness, seagrasses have successfully colonized all but the most polar seas. While seagrass meadows are widely distributed, other major
coastal marine habitats are geographically restricted to much smaller latitudinal ranges (mangroves and coral reefs in tropical regions, kelp beds and salt marshes in temperate regions).

Seagrasses have developed through their evolution unique ecological, physiological and morphological adaptations to live in the submerged marine environment. These adaptations include internal gas transport from the shoots to the roots in order to oxygenate the latter, epidermal chloroplasts so as to optimize light capture, submarine pollination and marine dispersal (den Hartog 1970, Les et al. 1997). To supply photosynthate to their roots and rhizomes, seagrasses need some of the highest light levels of any plant group worldwide (about 11% of the surface irradiance on average; Duarte 1991, but nearly 25% of incident radiation in some seagrass species, compared with 1% or less for other angiosperm species; Dennison et al. 1993). These extremely high light requirements mean that seagrasses at their deeper distribution level are intensely sensitive environmental changes such as water clarity.

Seagrass meadows have important ecological roles in marine coastal ecosystems and provide high-value ecosystem services compared with other marine and terrestrial habitats (Costanza et al. 1997). For example, primary production from seagrasses with their associated macro- and microepiphytes exceeds that of many cultivated terrestrial ecosystems (Duarte and Chiscano 1999). In their environment, seagrasses also provide a major source of carbon to the detrital pool, some of which is exported to the deep sea. There it provides an important supply of organic matter in an extremely food-limited environment (Suchanek et al. 1985). Much of the excess organic carbon produced is buried within seagrass sediments, which are hotspots for carbon sinks in the biosphere (Duarte et al. 2005). In addition, seagrass organs; leaves,
rhizomes and roots, modify the hydrodynamics around them, trapping and storing both sediments and nutrients, and effectively filter nutrient inputs back to the coastal marine environment (Hemminga and Duarte 2000).

Biodiversity and richness of associated fauna and flora in seagrass meadows is greater than in nearby non-vegetated areas, and faunal densities are orders of magnitude higher inside the meadows (Hemminga and Duarte 2000). Seagrasses also serve as a nursery land, often to juvenile stages of species of finfish and shellfish, although the species vary by region and climate (Beck et al. 2001, Heck et al. 2003). The proximity of seagrass beds to other habitats, such as salt marshes (in temperate regions) or mangroves and coral reefs (in tropical regions), facilitates trophic transfers and exchange of habitat utilization by many species of fishes and invertebrates (Beck et al. 2001). It was suggested that this may be essential in maintaining the abundance of some coral reef fish species (Valentine and Heck 2005).

Seagrasses are often referred to being biological sentinels, or "coastal canaries". Changes in seagrass distribution and meadow density, such as an increase in water turbidity, change in the depth limit of the meadow (Abal and Dennison 1996) or widespread seagrass loss (Cambridge and McComb 1984), indicate important losses of ecosystem services that seagrasses provide. Seagrasses are sessile, and therefore are sensitive to water quality attributes such as chlorophyll-containing phytoplankton and turbidity, which affect the light reaching their leaves. In addition, seagrasses also live in shallow, protected coastal waters, directly in the path of watershed nutrient and sediment inputs. Thus they are highly susceptible to these inputs, unlike mangrove forests (which are largely unaffected by water quality) or coral reefs (which occur farther away from the inputs) (Orth et al. 2006). Another feature that makes seagrasses a precious biological indicator is that they integrate
environmental impacts over measurable and definable timescales (Longstaff and Dennison 1999, Carruthers et al. 2002). For example, increased coastal development leading to nutrient inputs in Cockburn Sound, Australia, led to large-scale losses of seagrasses into the 1990s, and seagrasses remain at low levels in the area till this day (Walker et al. 2006). The loss of seagrasses led to sediment re-suspension, which decreased fish populations.

1.1.2 Light in the marine environment

Light environments of the euphotic zone in coastal systems are highly extreme. In tropical mid-oceanic waters, irradiances can vary from 2300 µmol photons m$^{-2}$ s$^{-1}$ of photosynthetically active radiation (PAR) and high levels of UV radiation in surface waters to the 1% of surface irradiances necessary to maintain positive net photosynthesis of many algae at ca. 200 m depth (Lalli and Parsons 2002), while in coastal waters this 1% limit occurs at shallower depths. On a global average, the first 100 m are lighted with more than 1% of surface irradiance. This sunlit layer of the ocean, where 45% of the entire amount of photosynthesis in the world takes place (Falkowski and Raven 2007, ch.1), is called the euphotic zone.

A certain amount of incoming light is reflected away when it reaches the water surface, depending upon the state of the water itself. In calm, flat seas, less light will be reflected while in turbulent, wavy waters, more light will be reflected. Once the light penetrates the water, the irradiance decreases exponentially as light is absorbed and scattered along a depth gradient. The quality of light is thus shifted towards blue wavelengths at depth as photons with low energies (e.g. red) are absorbed by the water molecules in clear waters lacking particles. In turbid coastal waters, especially blue photons are scattered by particles (while the red photons are absorbed), such that
green light reaches deepest down. In Chapters 2.4 and 2.5, in situ measurements of coastal water spectrum will demonstrate these traits.

Light drives photosynthesis, leading to the production of oxygen and carbohydrates that are essential for plants to grow. For photosynthesis to occur in marine plants and in seagrass meadows specifically, the photons must penetrate the water column, enter the canopy of leaf blades, pass through a layer of possible epiphytes on the surface of the leaf and finally enter the leaf epidermis to reach the photosynthetic apparatus (Dalla Via et al., 1998). Light is attenuated at each of these steps. The level of light attenuation at the depth of the seagrass meadow is affected by external factors such as: (1) physical properties of the water (depth, dissolved organic matter content, sediment accumulation and re-suspension), (2) human activities (increased sediment run-off, increased nutrient load leading to eutrophication and algal blooms, over-fishing, aquaculture and dredging) as well as (3) regional weather patterns (e.g. clouds, extreme storms and altered rainfall patterns) (Ralph et al. 2007).

1.1.3 Light absorption and utilization in marine plants

Leaf absorption is regulated by factors such as pigment content, morphology, chloroplast movements and dispersal and physical properties of the area (Ralph et al. 2007, Sharon and Beer 2008). Light reduction can cause an increase in chloroplast density, increase in chlorophyll content (Czerny and Dunton 1995, Longstaff et al. 1999, Sharon and Beer 2008, Sharon and beer 2009), decrease in the chlorophyll a to b ratio and a decrease in UVR blocking pigments (Abal et al., 1994). Yet, interestingly, large variations in chlorophyll content of seagrass leaves result in relatively small variations in leaf absorbance (Enríquez et al. 1992, 1994, Cummings and Zimmerman 2003, Enríquez 2005, Sharon and Beer 2008). This phenomenon has
been ascribed to the package effect, whereby the relationship between light harvesting efficiency and chlorophyll content is non-linear due to pigment self-shading among thylakoid membranes, chloroplasts and cell layers (Kirk, 1994).

The strong package effect observed in seagrasses is because chloroplasts are restricted to the leaf epidermis in these plants. Seagrass leaves do not have specialized tubular palisade cells that facilitate penetration of light in the leaf (Vogelmann and Martin 1993), nor do they have a spongy mesophyll that increases the optical path length due to scattering at the interfaces between the air spaces and cells such as in terrestrial leaves (Terashima and Saeki 1983). In the face of these constraints, in most cases, seagrass leaves exhibit efficient and relatively uniform light harvesting capabilities across varying depths and water qualities (Cummings and Zimmerman 2003, Olesen et al. 2002) and significant differences in some others (Olesen et al. 2002, Enríquez 2005, Runcie et al. 2009). Meadow (i.e. height, complexity and density; Enríquez and Reyes 2005, Ralph et al. 2007) and leaf morphology (i.e. leaf thickness and leaf surface) may also effect the regulation of light absorption efficiency through changing the optical light path (Enríquez 2005).

Light absorption can be measured with an integrating sphere (Cummings and Zimmerman 2003, Drake et al. 2003, Runcie and Durako 2004, Thorhaug et al. 2006, Zimmerman 2006), using Shibata's (1959) spectroscopic determination of leaf absorbance (Enríquez et al. 1992, 1994, Enríquez and Sand-Jensen 2003, Enríquez 2005) or using the simplified method according to Beer (2001). Correct measurements and determinations of leaf absorbance should account for light that is transmitted, reflected and scattered as well as for light absorption by non-photosynthetic tissues. As for the photosynthetic tissue, although pigments have a crucial effect on the plant photosynthesis processes, it seems that they don't affect the optical absorption of the
leaf significantly. Enríquez et al. (1994) measured light absorption properties of 13 seagrasses and reported that although the species examined ranged widely in chlorophyll a density and light absorption properties, they absorbed, on average, 59% of the incident PAR. These findings are comparable with an overall mean of 57±6% PAR absorption reported for 8 seagrasses from the east and west coasts of Australia (Durako 2007). Thus, despite structural restrictions and a strong package effect, seagrass leaves are relatively uniform and efficient light-capturing organs.

One special study case is the light absorption by the species *Halophila stipulacea*. This tropical seagrass features diurnal chloroplast movements which affect significantly its optical properties (Drew 1979, Sharon and Beer 2008) and its photosynthetic rates. These chloroplast movements will be discussed in the coming section.

1.1.4 Chloroplasts movement and clumping in plants

Chloroplasts contain the primary photosynthetic apparatus of plants, and their intracellular distribution depends on environmental factors, especially the availability and quality of light. Chloroplast photo-relocation movements can be classified and divided into two categories; 1) avoidance responses, where chloroplast move away from light, and 2) accumulation responses, where chloroplast move toward light (Wada et al. 2003). Chloroplast accumulation responses are thought to maximize photosynthesis, whereas avoidance responses minimize photodamage to chloroplasts (Kasahara et al. 2002).

For seagrasses, in addition to the general trend in changing pigment contents according to the incident irradiance, at least one species features the ability to clump its chloroplasts and, thus, reduce the light absorption of the leaves under high
irradiances (observed in the seagrass *Halophila stipulacea*, Drew 1979). Movements of chloroplasts within plant cells have been described for a few algae and angiosperms (Marchant 1976, Furukawa et al. 1998, Kasahara et al. 2002, Kondo et al. 2004), including, as mentioned above, the seagrass *Halophila stipulacea* (Drew 1979). In the latter work, the clumping of chloroplasts was illustrated by drawings for intertidal plants under high-irradiance conditions, and this was suggested to be a mechanism for protecting the photosynthetic apparatus from excess light. In addition to protecting some of the chloroplasts, it is possible that the clumping phenomenon has a role in protecting also the cell nucleus from excess light, as it was found that the chloroplasts tend, in some cases, to clump around it (Kondo et al. 2004). Similar photoprotective movements of chloroplast were shown to be mediated by phototropin in mosses and algae (Suetsugu and Wada 2007) and by abscisic acid in a succulents plant (Kondo et al. 2004). It should be pointed out that most of the literature published so far is dealing mainly with molecular aspects of the signaling pathway that leads to chloroplast movements (Uenaka and Kadota 2007, Suetsugu and Wada 2007, Cho et al. 2007, Suetsugu et al. 2006, Luesse et al. 2006) or its biochemical-cellular mechanism (Shiihira-Ishikawa et al. 2007, Krzeszowiec et al. 2007, Paves and Truve 2007, Gabrys 2004), and not the eco-physiological role of this phenomenon. Therefore, there is a need to explore the ecological role of the phenomenon.

Since Drew's initial observation, movements of chloroplasts in seagrasses have not been studied further. The clumping of chloroplasts has an apparent effect on leaf transparency as reflected in the visually much paler leaves of *Halophila stipulacea* plants growing in shallow waters (<1 m) during daytime characterized by high irradiances (Yoni Sharon and Sven Beer, personal observations). The possible effect
of such chloroplast movements on light absorption by the leaves was only indicated once (Schwarz and Hellblom 2002), but was never measured.

1.1.5 **Light absorption under UVR and high-light environments**

In addition to high photosynthetically active radiation (PAR, 400-700 nm), coastal waters, especially clear tropical waters, also feature high levels of UV radiation (UVR, 280-400 nm) in their upper layers (Dunne and Brown 1996; Agusti and Liabres 2007; this study). In general, adaptations to UVR and high light assume the existence of mechanisms that protect the organism or reduce their harmful effects. Four basic mechanisms allow an organism to cope with stressful UVR and excess high light exposure; 1) avoidance, 2) reducing the stress by a physiological behavioral mechanism, e.g. through the synthesis of UV absorbing compounds or other protecting pigments, 3) repairing the damage produced and 4) acclimating to the stress (Helbling and Zagarese 2003). More specifically, under extreme light conditions plants evolve mechanisms enabling them to reduce the light absorption and, thus, high-light damage in their photosynthetic tissues. These adaptive processes include reduce leaf area and increase leaf thickness (Dawson and Dennison 1996), the production of mycosporine-like amino acids (MAA; Whitehead and Vernet 2000; Gao et al. 2007) and other UV-absorbing compounds (flavones and flavone-glycosides in seagrasses; Durako et al. 2003, Kunzelman et al. 2005, Meng et al. 2008). The accumulation of these compounds occurs mainly in the epidermis, creating a UVR screen for the underlying tissues. Epidermis tissue enriched with UV-absorbing compound may reduce up to 99% of the UV radiation (Robberecht and Caldwell 1978). Flavonoids and related pigments are considered to account for a large percentage of this attenuation (Dawson and Dennison 1996).
Also changing morphologies (Monro and Poore 2004), changing pigment contents (Dawson and Dennison 1996; Gao et al. 2007), down-regulating photosystem II (PSII; Figueroa et al. 2002) or shading by carbonate layers (Beach et al. 2006) can reduce the UVR levels and damage in the leaf. The latter may change also the light absorption characteristics of the photosynthetic tissue. Generally, reflectance of light by CaCO₃ has been largely ignored, or has been assumed to be minimal (Luning and Dring 1985, Henely 1993, Markager 1993). However, a few studies have shown that CaCO₃ can increase the reflection and, thus, reduce light absorption by photosynthetic tissues (Littler and Littler 1980, Beach et al. 2006).

Under anthropogenic stress, leading to increased nitrogen levels in the water, epiphytes that thrive under such conditions may decrease the UVR (as well as PAR) levels reaching the leaves (Trocine et al. 1981; Brandt and Koch 2003). It was reported that periphyton (defined as the complex matrix of living and dead organisms as well as mucus and sediment particles) developing on seagrasses can reduce the light levels and screen off UVB radiation, thus protecting their leaves from this radiation in shallow waters (Brandt and Koch 2003).

Despite the fact that seagrasses are referred to be, in general, shade-adapted plants, e.g. *Halophila stipulacea* is growing under high levels of PAR and UVR in the intertidal of the Red Sea. While it was shown that this high level of PAR and UVR inhibits seed germination in shallow waters (Malm 2006), and different morphology of the leaves was found in the deeper waters (Schwarz and Hellblom 2002), there is not much more information available about the photophysiological adaptation of this plant neither in shallow nor in deeper waters. While some seagrasses are known to be sensitive to high levels of PAR while others are sensitive to high UVR levels...
(Dawson and Dennison 1996), the cause which triggers adaptive mechanisms for high light and/or high UVR levels in *Halophila stipulacea* is still unknown.

Since many seagrasses thrive in tropical shallow waters, it may be that the midday chloroplast clumping found in at least one of them (*Halophila stipulacea*) has a protective role against UVR (in addition to high PAR). In light of this, the possible role of UVR, in addition to PAR, in mediating chloroplast movements in this seagrass, and the possible effects of UVR on some of its photosynthetic parameters will be discussed further in this work.

1.1.6 *Acclimation processes of seagrasses (from the community to the individual)*

While certain photosynthetic organisms have adapted to specific light regimes (e.g. low irradiances at depth or very high irradiances in the tropical intertidal), others show a high plasticity as evidenced in their ability to inhabit areas with either low or high irradiances. The ability of such organisms to develop mechanisms to utilize and/or cope with different irradiances may be crucial for their functioning at spatially different and temporally dynamic surroundings (Sultan 2000, Stewart and Carpenter 2003).

Terrestrial plants use various strategies in their adaptation to extreme light environments. Diurnal morphological changes allow flowers or leaves to face the sun or to avoid it. Trees of an entire forest can be flat-topped in response to light and moisture requirements (Raven et al. 1992). Plants growing under extremely high light carry sometimes salt-secreting hairs and bladders on their leaves in order to reflect some of the light, thus reducing the photon flux reaching the leaves (Raven et al. 1992). Shade-growing plants use physiological mechanisms to enhance pigment (chlorophylls) content and, thus, photosynthetic efficiency at low irradiances (Johnson
et al. 2000, Ralph et al. 2007). In contrast, high-light plants which are exposed also to high UV radiation can respond in a plastic way by producing UVB absorbing pigments, the so-called mycosporine-like amino acids (MAA) and flavonoids in greater amounts than low-light plants (Rozema et al. 2002). In addition, a higher stimulation of DNA repair genes is present in those high-light plants (Ries et al. 2000).

In the marine environment, many subtidal macroalgae have the capacity to detect variations in the amount of available light and its spectral proportions, and to respond to this variation in a plastic way. This is done both through changing morphologies (e.g. thallus shape and thickness) and physiology (e.g. pigment content, including those of the photoprotective xanthophyll cycle) in response to limiting or excess irradiances (Ralph et al. 2002, Monro and Poore 2004, Ralph et al. 2007).

Also seagrasses, are well-known for their plastic responses to different environments (Reusch 2001, French and Moore 2003, Peralta et al. 2005, Ralph et al. 2007, Sharon et al. 2009). They are distributed around the globe, from the arctic to tropical regions, where they inhabit areas with very differing irradiance regimes. In order to maximize light utilization in very dim environments, seagrasses use several mechanisms. On the community level, epiphytes may shade and protect seagrass leaves from over-exposure to damaging sun light (Trocine et al. 1981; Brandt and Koch 2003). On the population level, one mechanism includes a canopy architecture that reduces self shading where light is absent. On the organism level, chloroplast and thylakoid membrane dispersal (Dawson and Dennison 1996, White and Critchley 1999) and a change in pigment content may enhance or decrease light absorption according to the plants' demand in its light environment. One relevant example of the adaptations to irradiance is the longer and wider leaves of Halophila stipulacea
growing in deeper waters (Lipkin 1979, Schwarz and Hellblom 2002). In this present work the plasticity in the acclimation to light in *Halophila stipulacea* is discussed (see Chapter 2). Yet, though light-stress avoidance adaptations are known for seagrasses, the physiological adaptations/responses of deep-growing populations that photosynthesis under extreme low light conditions are still not clear. Here, I questioned the role of photosystem composition in the adaptation to low light at depth (see Chapter 5).

1.1.7 The photosystems' apparatus and its stoichiometry

In photosynthesis, the energy of absorbed photons is used to modify the electronic structure of pigment molecules to the extent that, in the reaction centers, an electron can be physically transferred from a donor to an acceptor. Thus, the light reactions in photosynthesis are photochemically catalyzed oxidation-reduction reactions. The main "players" of the light absorption in the photochemical electron transport are photosystem I (PSI with chlorophyll P700 at its reaction center) and photosystem II (PSII with chlorophyll P680 at its reaction center). The efficiency of photosynthetic electron transport depends on the coordinated interactions of photosystem II (PSII) and photosystem I (PSI) in the electron transport chain. Each photosystem contains different pigment-protein complexes that harvest light from slightly different regions of the visible spectrum. The light energy is utilized to excite an electron which leads to an electron-transport sequence in each photosystem. Some evidence has shown a large variability in the PSII/PSI stoichiometry from its generally assumed ratio of 1:1 in plants grown under different environmental irradiance conditions in terrestrial habitats (reviewed in Fujita 1997).
Relatively to terrestrial plants, little is known on macro-marine plants' photosystem composition or stoichiometry. Among marine macrophytes, spectral changes were shown to influence the functionality of the two PSs as reported for the green macroalgae *Ulva pertusa* and *Bryopsis maxima* (Yamazaki et al. 2005). Regarding seagrasses, only two studies have reported on changes in the Photosystems composition of shallow-growing plants during various seasons (Major and Dunton 2000) and along a depth gradient down to 1.6 m (Major and Dunton 2002). For seagrasses, it is still not known what are the stoichiometry and functionality of deep growing plants photosystems. In the current work (see chapter 5), protein analysis and 77K measurements are presented in order to investigate the mechanism of depth acclimation in the tropical seagrass, *Halophila stipulacea*. To our knowledge there are no studies on PS relations in seagrasses growing at such great depths where both the irradiance and light spectrum change drastically.

1.2 Research Objectives

*Halophila stipulacea* grows from the intertidal down to >50 m depth along a large light gradient. However, the mechanisms and characteristics of this plant to adapt and acclimate to this light gradient are not known. Accordingly, the specific aims of this work were to:

1. Quantify chloroplast movements and elucidate there importance on photosynthetic rates.
2. Investigate the acclimation of *Halophila stipulacea* to different light environments.
3. Investigate *Halophila stipulacea*'s excitation energy allocation to photochemistry along its light gradient.
4. Elucidate the role of UVR in chloroplast movements and photosynthetic rates in *Halophila stipulacea*.

5. Characterize the photosystems' stoichiometry and functioning of shallow and deep growing *Halophila stipulacea* plants.
Diurnal movements of chloroplasts in Halophila stipulacea and their effect on PAM fluorometric measurements of photosynthetic rates

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Abstract

Since diurnal chloroplast movements in Halophila stipulacea were described by Drew in 1979, this phenomenon has not been studied further for seagrasses. In addition to an apparent photoprotective role, such movements may affect the measurements of photosynthetic rates based on pulse amplitude modulated (PAM) fluorometry. This is because calculations of electron transport rates (ETR) are directly affected by the light absorption of the leaves (or the so-called absorption factor, AF), the latter of which changes with the movements of the chloroplasts. In this work, we therefore determined chloroplast clumping and dispersal, and measured AFs, chlorophyll contents and PAM fluorescence diurnally for H. stipulacea grown under two irradiance regimes. Diurnal chloroplast clumping occurred in high-light grown (HL) plants (450 mmol photons m\textsuperscript{-2} s\textsuperscript{-1} during midday), which was accompanied by a decrease in AF values (from 0.56 in the early morning to 0.34 at midday) but not in the chlorophyll content. Also, non-photochemical quenching (measured as NPQ) increased during the day in these plants. No such chloroplast movements and, thus, no diurnal changes in AF values (0.60 ± 0.04 throughout the day), and no changes in NPQ, were found in low-light grown (LL) plants (150 mmol photons m\textsuperscript{-2} s\textsuperscript{-1} during midday). As a consequence of the chloroplast clumping in HL plants, and its effect on AF values, maximal ETRs did not differ significantly between HL and LL plants. This finding thus shows the importance of taking into account changing AF values along the day when calculating ETRs of H. stipulacea, and other seagrasses potentially featuring diurnally changing AFs, under high-irradiance conditions.

Keywords: Chloroplast movement; Chloroplast clumping; Halophila stipulacea; PAM fluorometry; Photosynthesis

1. Introduction

Movements of chloroplasts within plant cells have been described for a few algae and angiosperms (Marchant, 1976; Furukawa et al., 1998; Kasahara et al., 2002; Kondo et al., 2004), including the seagrass Halophila stipulacea (Drew, 1979). In the latter work, the clumping of chloroplasts was illustrated by drawings for intertidal plants under high-irradiance conditions, and this was suggested to be a mechanism for protecting the photosynthetic apparatus from excess light. Since this initial observation, movements of chloroplasts in seagrasses have not been studied further. The clumping of chloroplasts has an apparent effect on leaf transparency as reflected in the visually much paler leaves of H. stipulacea plants growing in shallow waters (<1 m) characterised by high irradiances (Yoni Sharon and Sven Beer, personal observations). The possible effect of such chloroplast movements on light absorption by the leaves was only indicated once (Schwarz and Hellblom, 2002), but was never measured.

Pulse amplitude modulated (PAM) fluorometry has been used increasingly during the past decade for photosynthetic measurements in seagrasses (cf. Beer et al., 2001). In order to measure photosynthetic electron transport rates (ETR) by this method quantitatively, it is necessary to take into account the absorption of light by the photosynthetic pigments, or at least the so-called absorption factor (AF), of the leaves (Beer et al., 1998). If indeed chloroplast movement affects the leaf transparency (i.e. AF value), and if this occurs on a diurnal basis, it follows that such continuously changing AFs need to be taken into account when assessing photosynthetic rates on a diurnal basis using PAM fluorometry.

Given the potential importance of chloroplast movement on AF values and, thus, photosynthetic ETRs, we here set out to (a)
confirm microscopically chloroplast clumping and dispersal throughout the day in low- and high-light grown H. stipulacea leaves, (b) measure AFs diurnally and (c) evaluate the diurnal photosynthetic performance of H. stipulacea taking into account the potential fluctuations in the AF values. In doing so, we confirm the importance of using diurnally changing, chloroplast movement induced, AF values for assessing ETRs correctly.

2. Materials and methods

Whole plants, including roots and rhizomes, of H. stipulacea from a monospecific bed were collected together with their sediment at 30 m depth 200 m south of the Inter-University Institute (IUI) in Eilat, the Gulf of Aqaba, Northern Red Sea (29°30′.01N, 34°54′.57E). The seagrasses of this bed grow between 7 and >40 m depth. The midday irradiance at 30 m was ~60 mmol photons m⁻² s⁻¹ and the temperature was 21°C during the time of this study (February–March, 2007). The plants were transported to an outdoor water table (a table with a 20 cm margin, forming a shallow container) located at the IUI, where they were acclimated for 30 days in running seawater under shading nets providing either ~450 (‘‘high-light’’ grown, HL) or ~150 (‘‘low-light’’ grown, LL) mmol photons m⁻² s⁻¹ during midday, respectively. After this acclimation, the vigour of the plants was verified by night-time Fv/Fm measurements.

Chloroplast clumping was investigated using both light- and confocal microscopy (Nikon Eclipse TE2000-E, Japan). At each of the observation times along the day, leaves were quickly taken to the laboratory (within 10 min), placed on microscope glass slides, examined and photographed.

Chlorophyll contents of leaves were determined after extraction in dimethyl formamide (DMF) for 24 h in darkness. Absorbance was measured spectrophotometrically according to Moran (1982), and chlorophyll a and b contents were calculated on a leaf area basis.

Chlorophyll fluorescence was measured diurnally for individual leaves throughout cloudless days using the Diving PAM (Walz, Germany). An intact leaf growing on the water table was fixed at a distance of 10 mm from the instrument’s optical fibre using a so-called leaf distance clip (Walz, Germany) such that it received the sunlight remaining under the shading nets. The PAM fluorometer was programmed to take a yield (Y) measurement, together with irradiance, every 30 min using the “rep clock” mode. ETR was calculated as Y x PAR x 0.5 x AF. Non-photochemical quenching (NPQ) was calculated individually for each leaf as (Fm – FFm)/FFm; Fm was measured early in the morning before sunrise.

In order to determine AF diurnally, 10 leaves were removed at 5 different times during a cloudless day and absorption was measured under a lamp using the Diving PAM’s light sensor calibrated against a LiCor LI-189 (USA) quantum sensor. These AF measurements were done in an aquarium such that the reflection of the leaf was minimised in the water medium (cf. Beer and Björk, 2000). It is important to note that the absorption of the thin leaves is mainly due to the photosynthetic pigments. This was ascertained by measuring AF values of leaves from which all pigments had been extracted several times by DMF until they were close to transparent; these leaves showed AF values <0.04.

Fig. 1. Micrographs of Halophila stipulacea epidermal leaf cells photographed at various times during the day. The plants were grown on a sunlit water table at ~150 (panel a) and ~450 (panel b) mmol photons m⁻² s⁻¹ during midday. Black bars represent 10 mm.
3. Results

Night-time Fv/Fm values of the experimental plants were found to be 0.73 ± 0.02 (n = 8) and 0.71 ± 0.03 (n = 8) for LL and HL plants, respectively. In comparison, in situ measured night-time Fv/Fm values of plants growing at 30 m were 0.79 ± 0.02 (n = 8). These results were taken to confirm that the plants were not suffering photosynthetically from the 30 days of growth on the water table.

The epidermal cells of LL H. stipulacea leaves did not show any clumping of chloroplasts during the day (Fig. 1a). In contrast, HL plant leaf cells did feature diurnal clumping and subsequent dispersal of chloroplasts as illustrated in Fig. 1b. These results were confirmed using both light- and confocal microscopy, but only light-microscopic pictures are presented here. At 06:00, before sunrise, the chloroplasts were rather evenly dispersed in the cytoplasm of the cells. Migration started with sunrise, and the beginning of clumping can be seen at 08:00. Full clumping was observed at midday, and continued throughout the afternoon. In the late afternoon the chloroplasts dispersed throughout the cytoplasm, and full dispersal was observed a few hours after sunset (not shown).

Chlorophyll a + b contents showed no significant variations throughout the day (Fig. 2a). This was true for both LL and HL plants. However, the average chlorophyll a + b contents pooled for plants from the two irradiances showed a significantly higher (one way ANOVA, p < 0.00001) chlorophyll content in LL (14.86 ± 3.51 mg cm⁻²) than in HL (8.32 ± 3.37 mg cm⁻²) plants. The absorption factor, on the other hand, did vary throughout the day (Fig. 2b). While the trend showed decreased values during midday, the diurnal variation was significant (one way ANOVA, p < 0.01) for the HL plants only. In the lack of significant daily variations in chlorophyll content, we suggest that the daily oscillation in AF was due to the clumping and subsequent dispersal of chloroplasts.

ETRs of LL plants, calculated using corrected AF values for the various times of the day, followed the irradiance throughout the day (Fig. 3a). The highest rate was 29.2 mmol electrons m⁻² s⁻¹ at noon, which coincided with a peak in the irradiance reaching the water table. During this time, non-photochemical quenching values were below 0.3. This, and the fact that photosynthesis paralleled irradiance, confirms a virtual absence of photoinhibition. Under HL conditions, ETRs followed the irradiance only during the morning hours (Fig. 3b).

After 9:30, at irradiances above ~350 mmol photons m⁻² s⁻¹, ETRs decreased while the irradiance remained stable (until 12:30). Thus, ETR and irradiance did not parallel one another from 9:30 until 15:30. NPQ increased during the day, reaching maximum values of around 3 at 12:00–14:30 (400–200 mmol photons m⁻² s⁻¹). NPQ values were >2 during the time that the ETRs did not parallel irradiances, indicating photoinhibition by heat dissipation, e.g. via the xanthophyll cycle. After 15:30, ETR decreased in parallel with irradiance (below ~75 mmol photons m⁻² s⁻¹). The form of photoinhibition between 9:30 and 15:30 is dynamic as evidenced by a full recovery of the effective quantum yield in the late afternoon to the same values as during the morning hours at similar irradiances.
One striking consequence of taking the diurnally fluctuating AF values of HL plants into consideration when calculating ETRs can be exemplified in determining daily maximal ETRs. If early morning AF values of LL and HL plants are used, then significantly different (one way ANOVA, p < 0.001) maximal ETRs are derived for the two plant types (34.7 ± 2.43 and 47.0 ± 1.08 mmol electrons m⁻² s⁻¹ for LL and HL plants, respectively). If, however, the AF values at the time of measurements are used, then no significant (one way ANOVA, p > 0.05) differences in maximal daily ETRs between plants grown under the two irradiances are apparent (30.8 ± 3.01 and 35.6 ± 1.93 mmol electrons m⁻² s⁻¹ for LL and HL plants, respectively).

4. Discussion

One of H. stipulacea’s outstanding features is its wide depth distribution. In the Gulf of Aqaba, Northern Red Sea, it grows from the intertidal down to depths of 70 m (Lipkin, 1979). At depth, the thin leaves of this plant may be a characteristic of low-light adaptations. In addition, the leaves at depth are larger than in shallow waters (Lipkin, 1979; Schwarz and Hellblom, 2002) and the above- to below-ground biomass ratio is higher (Sharon, unpublished data). In shallow waters, the ability to withstand high irradiances may be facilitated by the ability of chloroplasts to move into clumps during the day and the apparently efficient mechanism for non-photochemical quenching.

While chloroplast movement was previously described for H. stipulacea in drawings (Drew, 1979), the present work shows for the first time photographs of this diurnally oscillating phenomenon in high-light grown plants. In some algae (e.g. the diatom Pleurosira, Furukawa et al., 1998) as well as both submerged (Vallisneria, Sakurai et al., 2005) and terrestrial (Arabidopsis, Kasahara et al., 2002) plants, similar movements of chloroplasts were induced by relatively low levels of blue light, and in the succulent CAM plant Zygocactus this movement was mediated by abscisic acid (Kondo et al., 2004). Similar mechanisms may be triggers for chloroplast movements in H. stipulacea, but we observed that clumping of chloroplasts occurred only in plants growing at midday irradiances above ~200 mmol photons m⁻² s⁻¹. Therefore, it seems that high irradiances are a prerequisite for chloroplast clumping irrespective of the mechanism involved.

In the Red Sea area, which may experience the highest irradiances in the world (e.g. Winters et al., 2003), there may be a clear need for photoprotection of marine organisms growing in shallow waters. H. stipulacea, like the genus Halophila in general, is characterised by very thin leaves of a few cell layers only. It is thus likely that the chloroplast clumping ability, causing self shading of the chloroplasts, is an important photoprotective feature in this plant. In addition to chloroplast clumping, an efficient non-photochemical quenching mechanism, probably via the xanthophyll cycle, is evidenced by the diurnal development of NPQ in high-light grown plants. In contrast, no such NPQ was observed for low-light grown plants. This, together with the absence of chloroplast clumping in these plants, indicated that there is no need for photoprotection in deeper growing plants.

The diurnal changes in AF caused by chloroplast movements in HL leaves of H. stipulacea have obvious consequences when determining photosynthetic rates by PAM fluorometry. For the latter, it is important to use correct AF values since ETRs strongly depend on them in a linear fashion (e.g. Beer et al., 1998). Thus, when leaves change their absorption through the day, it is important to take such changes in AF into account when calculating photosynthetic ETRs. This corrective practice can even change conclusions regarding maximum photosynthetic rates in low- and high-light plants: In this study, using, e.g. early morning AF values for the HL plants renders them significantly different maximal ETRs from LL plants. This difference is, however, not apparent when using the corrected AF values measured at the time of day when maximal ETRs were recorded.

References


Contribution to the Theme Section ‘Primary production in seagrasses and macroalgae’

Photosynthetic responses of *Halophila stipulacea* to a light gradient. I. *In situ* energy partitioning of non-photochemical quenching

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ABSTRACT: The quantum yield of photosystem II (φII, also termed ΔF/Fm’ or Fv/Fm in light- or dark-acclimated plants, respectively) of the tropical seagrass *Halophila stipulacea* was measured *in situ* using modulated fluorescence techniques over diel periods at a range of depths. Photosynthetic electron transport rates (ETRs), as derived from φII values at specific ambient photosynthetically available radiation (PAR) irradiances, increased in direct proportion to increasing irradiance in the morning and, at shallow sites (7 to 10 m), reached saturating rates and then declined in the afternoon with lower PAR-specific ETRs. On the other hand, plants at 32 to 33 m showed no saturation even at midday, and the percentage reduction in PAR-specific afternoon ETRs was less than that of the shallower plants. The use of an automated shutter in the measuring device enabled non-photochemical quenching due to down-regulation and basal intrinsic non-radiative decay to be distinguished. While midday values of down-regulation were lower in deeper water, basal intrinsic non-radioactive decay remained fairly constant at 30 to 40% at all depths, with more variation in shallow waters. The maximal φII (i.e. Fv/Fm) reached similar values at midnight regardless of depth. *H. stipulacea* acclimates to the widely varying irradiances across this depth gradient by regularly modulating down-regulation-based non-photochemical quenching processes, while dissipating a large proportion of light energy through intrinsic decay regardless of depth.

KEY WORDS: Depth gradient · *Halophila stipulacea* · Irradiance · Nonphotochemical quenching · PE curves · Photosynthesis · Pigments

INTRODUCTION

Seagrass meadows dominate many shallow estuaries and marine embayments, yet at a global scale seagrasses are in decline (Orth et al. 2006). While this decline has been largely attributed to human activity through increased light attenuation caused largely by higher sediment loads and algal densities, other factors such as eutrophication, heavy metals and petrochemical pollution have a significant impact on seagrass survival (Orth et al. 2006, Ralph et al. 2007). The capacity for a seagrass species to conduct photosynthesis such that the net carbon balance is positive is vital for the survival and resilience of a meadow. Thus considerable research effort over the last half-century has maintained a focus on seagrass growth and photosynthesis (e.g. Larkum 1976, Lee et al. 2007). The seagrass *Halophila stipulacea* is an integral component of the Gulf of Aqaba coral reef ecosystem in the Red Sea (Edwards & Head 1987), where it can be found in extensive monospecific beds. *H. stipulacea* has a generally wide ecological range, growing from the intertidal to depths > 50 to 70 m (Hulings 1979, Lipkin 1979, Beer & Waisel 1982). This is in con-
trast with the seagrass *Halodule uninervis* that coexists in shallow waters in the northern Red Sea, Gulf of Aqaba, but does not extend as deeply (Beer & Waisel 1982). Mechanisms that enable the survival of *H. stipulacea* in shallow and deep waters may include an insensitivity to pressure due to its lack of substantial gas lacunae (Beer & Waisel 1982) and its capacity for chloroplast clumping (Schwarz & Hellblom 2002, Sharon & Beer 2008).

Since the advent of submersible modulated fluorometers in the 1990s, chlorophyll fluorescence techniques have been widely used in the examination of *in situ* seagrass photophysiology (e.g. Beer et al. 1998, 2000), and the effects of varying irradiance with increasing turbidity or depth have been examined in some detail (Lee et al. 2007). However, most *in situ* fluorescence studies have been constrained by the necessity for divers to make the measurements, limiting studies to a combination of maximum depth, frequency and duration of measurements at these depths. Fewer studies have taken advantage of logging functions available in commercially available instruments (e.g. Winters et al. 2003, Schwarz et al. 2003); other studies have developed custom devices for continuous measurements over time of multiple replicate samples (Runcie & Riddle 2004, 2006, Runcie & Durako 2004).

The quantum yield (\(\phi\)) of a light-dependent process is defined as the ratio of the rate of that process to the rate of photon absorption. In unstressed green plants and algae, the light-dependent process of photochemical energy conversion uses approximately 83% of light absorbed, thus the quantum yield of photosynthesis is 0.83. Modulated fluorescence techniques are particularly useful for measuring the quantum yield of photochemical energy conversion in photosystem II (PSII), \(\phi_{\text{PSII}}\), and by difference, the quantum yield of non-photochemical energy conversion in PSII, \(\phi_{\text{NPQ}}\).

Diel changes in \(\phi_{\text{II}}\) describe how a phototroph responds to irradiance and can be used with other measurements to estimate net carbon gain (Beer et al. 2000, Longstaff et al. 2002). However, irradiance energy absorbed by PSII that cannot be directed to photochemistry is (by definition) directed to several classes of non-photochemical quenching (NPQ) processes. Of these, the generation of singlet oxygen can cause intensive damage to internal structures if the absorbed energy is not effectively dissipated through other NPQ processes (Müller et al. 2001).

Kornyeyev & Holaday (2008) separated NPQ into 4 components: (1) fluorescence is generally a very small proportion of NPQ (a few percent at most) and is generally disregarded; (2) regulated, dark-reversible dissipation is generally activated in response to irradiance stress; (3) constitutive thermal dissipation can be regarded as the (assumed) invariant ~27% of light directed to non-photochemical processes when absorbed by non-stressed phototrophs; and (4) induced thermal dissipation is associated with PSII photo-inactivation and is sometimes termed photo-inhibition. Kramer et al. (2004) pooled the 2 thermal dissipation components together and termed them basal intrinsic non-radiative decay. For simplicity we adopt this latter term to describe thermal dissipation.

As part of the recent Group for Aquatic Primary Productivity (GAP) workshop in Eilat (April 2008, see www.gap-aquatic.org/), we studied various photo-physiological aspects of the seagrass *Halophila stipulacea*, *in situ*. In the present study, we determined how irradiance energy absorbed by PSII is allocated to photochemistry versus non-photochemical processes in plants exposed to very different irradiances along a wide depth gradient. By determining diel changes in electron transport rates at various depths (8 to 33 m), we aimed to distinguish differential responses in terms of photochemical and non-photochemical use of the contrasting ambient photosynthetically available radiation (PAR) irradiances at each depth. The specific objectives of the present study were to determine how excitation energy is allocated within *H. stipulacea* to photochemistry, down-regulation and basal intrinsic non-radiative decay processes when exposed to different irradiances (at various depths), and how the allocation to these processes varies during the onset of irradiance stress and recovery. A parallel study was also conducted to examine acclimatory responses of *H. stipulacea* transplanted across the 3 depths (Sharon et al. 2009, this Theme Section).

**MATERIALS AND METHODS**

**Study sites and seagrass species.** *Halophila stipulacea* (Forsskål) Ascherson was examined *in situ* at 8, 18 and 33 m depth just south of the Inter-University Institute (IUI) in Eilat, the Gulf of Aqaba, northern Red Sea (29° 30'N, 34° 55'E) during April 2008. Automated chlorophyll fluorescence measurements were set up at each depth using SCUBA. Individual leaves separated from each other by ≥10 cm were examined. Selected samples were taken to the laboratory for leaf absorbance determinations.

In *in situ* irradiance and \(K_d\) measurements. Irradiance was measured *in situ* during fluorometer deployments using cosine-corrected irradiance sensors incorporated in the custom fluorometers, and is termed incident irradiance or \(I_o\). Data was compared with measurements in W m\(^{-2}\) obtained at the nearby research station and converted to μmol photons m\(^{-2}\) s\(^{-1}\) by multiplying by 4.6 (McCree 1981). Occasional in-air measurements and depth profiles using a LiCor 190SA quantum sensor
were also taken from a boat in the vicinity of the study site; those in-air irradiance values were similar to values measured at the research station. All light sensors were calibrated against the LiCor 190SA quantum sensor. The light attenuation coefficient $K_d$ was calculated from depth profile data.

**Absorbance measurements.** The absorption factor (AF) of 12 different leaves was assessed and averaged at each depth (8, 18 and 33 m) throughout the day (06:00, 10:00, 12:30, 16:00 and 18:00 h) by determining the reduction of ambient irradiance (or artificial irradiance at dawn and dusk) caused by a single leaf placed over the light sensor of a Diving-PAM fluorometer (Walz, Effeltrich). This reduction in irradiance was converted to a proportion. For example, a 50 % reduction in irradiance caused by placing a leaf over the light sensor corresponded to an AF value for that leaf of 0.5 (Sharon & Beer 2008). It was assumed that absorbance by photosynthetic pigments contributed 96 % of the overall absorbance in *Halophila stipulacea* (as found earlier, Sharon & Beer 2008); the absorbance of other compounds was therefore ignored. The measured AF values were regressed against time separately for morning and afternoon measurements and thus 2 linear relationships were determined for each depth. The trajectory of these regression lines indicated the direction and rate of change in absorbance throughout the day (a proxy for chloroplast clumping, Sharon & Beer 2008), and enabled the estimation of AF by interpolation at each depth for each fluorescence measurement.

Twelve absorbance values were obtained at each depth at (approximate) each time; differences between mean AF values at each depth at dawn and dusk were compared using ANOVA after testing for homogeneity of variances.

**In situ modulated fluorescence measurements.** Two designs of custom submersible fluorometer (Aquation, Australia) were used that excite chlorophyll *a* fluorescence with a modulated blue light (470 nm light-emitting diode, LED) before and during a 0.8 s blue-enriched white saturating pulse provided by a 10 W LED (Luxeon, Lumileds Lighting). A far-red LED (735 nm) incorporated into the fluorometers was activated for 5 s prior to saturating pulse measurements to facilitate the opening of PSII centres and hence determine $F_o'$ (i.e. minimal fluorescence intensity with all PSII reaction centers open in a light-acclimated state). The sensor head of one fluorometer design closed over the sample when taking a measurement, thus placing the sample in darkness (Fig. 1). The sensor head could then be left in place for a predetermined interval, thereby dark-acclimating the sample. Fluorescence measurements made immediately prior to closing of the automated shutter provided background fluorescence values which could then be subtracted from subsequent values obtained from the seagrass samples when the sensor head was in a closed position. The other fluorometer design simply positioned the sensing part of the fluorometer close to the sample which was held in a clear acrylic sample holder; samples were exposed to ambient irradiance throughout the course of measurements. Each fluorometer was connected by cable to an independent submersible data-logger and power supply; previous experiments (authors’ unpubl. data) demonstrated that the system can operate for at least 2 d on a single charge. Several fluorometers of both designs were deployed at each site and a single seagrass blade was held in the sample holder of the fluorometer with an upwards orientation; the irradiance sensor incorporated into each fluorometer likewise faced upwards. Care was taken during the deployments to minimise any disturbance of the seagrasses. The fluorometers were left in position for at least 24 h. While the second design of fluorometer was programmed simply to measure $\Delta F/F_m$ every 15 min, the dark-acclimating shutter fluorometers were additionally programmed to remain closed for a 15 min interval once every 2 h, thereby also allowing 12 measurements of $F_o/F_m$ during a 24 h interval. Examination of the $\varphi_{II}$ data directly after the $F_o/F_m$ measurements suggested no apparent effect of the 15 min dark treatment on subsequent $\varphi_{II}$ measurements, which would have manifested as an increase in $\varphi_{II}$ values directly after each $F_o/F_m$ measurement.

**Diel photosynthesis–irradiance curves.** ETRs were calculated according to Genty et al. (1989) as the product of ambient irradiance, $\varphi_{II}$, AF at the time of measurement and 0.5 to account for electron sharing between photosystem I (PSI) and PSII. Diel photosynthesis–irradiance ($P$-$I$) curves (Longstaff et al. 2002), (where ‘$I$’ represents non-wavelength-specific irradi-
Absorbance values of *Halophila stipulacea* at 8 m declined significantly from ~0.60 to a midday minimum of 0.34, rising again in the afternoon to values similar to, but slightly lower than, predawn values (Fig. 2). We ascribe this difference in AF to the chloroplast clumping phenomenon as described by Sharon & Beer (2008). In contrast, AF values of the deeper growing plants only declined slightly during the day, with marginally greater predawn and dusk values compared to the 8 m plants.
At each depth, the absolute value of the rate of decline in AF during the morning was similar to that during the afternoon (Fig. 3). The magnitude was greatest for shallow seagrasses and least for the deepest seagrasses, indicative of a more marked response in shallow water. Data describing AF acquired at dawn satisfied the assumptions of homogeneity, and AF at 8 m (0.558 ± 0.03; all reported values are mean ± SD) was significantly less than values at the deeper sites which did not differ significantly (33 m: 0.604 ± 0.05; 18 m: 0.632 ± 0.03; \( F = 14.2, p > 0.001 \)). AF measured at dusk was significantly different at the 3 sites (8 m: 0.509 ± 0.03; 18 m: 0.642 ± 0.04; 33 m: 0.598 ± 0.06; \( F = 26.6, p > 0.000 \)).

**Morning and afternoon fluctuation in \( \Phi_{II} \), \( \frac{F_v}{F_m} \) and ETR**

Minimum midday values of \( \Phi_{II} \) at 8, 18 and 33 m dropped to less than 0.1, 0.2 and 0.3, respectively, reflecting the decline in midday irradiance with depth (Figs. 4–6). After sunset, \( \Phi_{II} \) of plants at all depths increased rapidly to values around 0.6, followed by a slow gradual increase throughout the night. \( \frac{F_v}{F_m} \), as measured after in situ dark-acclimation for 15 min, declined during the day with declining \( \Phi_{II} \) and recovered rapidly at dusk assuming the same values as \( \Phi_{II} \) during the night. \( \frac{F_v}{F_m} \) continued to rise slightly after dawn while \( \Phi_{II} \) commenced its decline.

ETRs tended to increase rapidly during the morning and decline more slowly in the afternoon. This can be seen more clearly in Fig. 7 where morning values of ETRs for shallow plants are far greater than afternoon values for the same absorbed irradiance. The reduced differences between morning and afternoon ETRs of deeper plants is largely a consequence of the reduced irradiance, as can be seen when comparing ETRs where \( I_a \) is ~20 to 160 µmol photons m\(^{-2}\) s\(^{-1}\) for experiments conducted at the 3 depths (Fig. 7). Clearly, ETR\(_{max}\) calculated from pooled morning and afternoon

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**Fig. 4. Halophila stipulacea.** Diel variation in electron transport rate (ETR), quantum yield of PSII (\( \Phi_{II} \)) and incident irradiance (\( I_i \)) of a single representative leaf at 8 m depth.

**Fig. 5. Halophila stipulacea.** Diel variation in electron transport rate (ETR), quantum yield of PSII (\( \Phi_{II} \)) and incident irradiance (\( I_i \)) of a single representative leaf at 18 m depth.
Fig. 6. Halophila stipulacea. Diel variation in electron transport rate (ETR), quantum yield of PSII ($\Phi_{II}$) and incident irradiance ($I_i$) of a single representative leaf at 33 m depth.

Table 1. Halophila stipulacea. Observed maximum electron transport rate (ETR$_{max}$, µmol electrons m$^{-2}$ s$^{-1}$) and calculated photosynthetic parameters ETR$_{max}$, initial slope of the P-I curve ($\alpha$; electrons photon$^{-1}$), saturation irradiance $I_k$ (derived from calculated and observed ETR$_{max}$ values) and incident irradiance $I_i$ (µmol photons m$^{-2}$ s$^{-1}$) measured in situ at 8, 18 and 33 m. ETRs were derived from multiple modulated chlorophyll fluorescence measurements and estimates of absorbed irradiance ($I_a$) obtained over intervals ≥ 24 h. Each value represents parameter estimates (± SD) derived from a single experiment conducted over a 24 h interval on an individual leaf. calc.: calculated; obs.: observed.

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<th>Depth (m)</th>
<th>ETR$_{max}$ (calc.)</th>
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<th>$I_k$ (observed)</th>
<th>$I_i$ (max)</th>
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<td></td>
<td>20.69 ± 2.8</td>
<td>9.22</td>
<td>0.58 ± 0.0</td>
<td>15.9</td>
</tr>
</tbody>
</table>

Fig. 7. Halophila stipulacea. Representative photosynthesis–irradiance curves measured over ≥24 h at 8, 18 and 33 m depth (see also Table 1). Absorbed irradiance ($I_a \times AF \times 0.5$) was used to calculate electron transport rate and was used for the abscissa. Closed and open circles represent morning and afternoon measurements, respectively.

data underestimated observed ETR$_{max}$ when noonday irradiance was high (Table 1, Fig. 7), while calculated values for the deeper plants more closely approximated observed values. Observed values of ETR$_{max}$ ranged from 7 to 21 µmol electrons m$^{-2}$ s$^{-1}$, with lower values observed at all depths and higher values only observed at shallow and intermediate depths (Table 1).
The daily mean initial slope of the P-I curves, \( \alpha \), ranged from 0.41 to 0.80, with greater values at the shallow site. While calculated values of saturation irradiance (\( I_s \)) were higher for deeper plants, values derived from observed ETR\(_{\text{max}}\) values showed no obvious differences between depths and ranged between 12 and 33 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \).

**Non-photochemical quantum yields**

At 8 m depth, \( \Phi_{\text{NPQ}} \) declined from midday values of around 0.6 to near zero at night (Fig. 8). At 18 m depth, \( \Phi_{\text{NPQ}} \) similarly dropped to near zero at night, but was generally around 0.4 at midday, except for an (presumably erroneous) anomaly where \( \Phi_{\text{NPQ}} \) reached its maximal value of 1 at midday (Fig. 9) and \( \Phi_{\text{NO}} \) was underestimated at <0. At 33 m depth, \( \Phi_{\text{NPQ}} \) only rose to about 0.3 at midday, also dropping to zero at night. \( \Phi_{\text{NO}} \) was steady at about 0.4 in plants at all depths during the night. At 8 m depth, this value declined with light onset, and then increased at midday to about 0.5 (Fig. 8). A similar pattern could be seen at 18 m where a strong midday decline was evident (Fig. 9). The decline was also apparent, but far less marked, for plants at 33 m depth (Fig. 10). The range of \( \Phi_{\text{NO}} \) values declined with reduced irradiance from about 20% for the shallow plants to about 10% for the deep plants.
DISCUSSION

The rate of decline in absorbance of *Halophila stipulacea* was greatest in shallow waters, supporting previous observations that high light exposure causes a physiological response in this species (Sharon & Beer 2008). This response was less marked for the deeper plants, with progressively decreasing rates of change over the day with increasing depth. These data suggest that, with increasing depth, there is a diminishing requirement for chloroplast translocation throughout the day. Consequently, resources used for chloroplast movement in shallow plants can be reallocated to other processes. This capacity to acclimate during the day to irradiance, electron transport estimates become invariant (Belshe et al. 2007). For example, diel changes in absorbance of *Halophila stipulacea* due to chloroplast clumping clearly influenced calculated ETRs (Sharon & Beer 2008), and Saroussi & Beer (2007) argued that $I_a$ rather than $I_i$ should be used in $P-I$ curves when calculating $a$ and $I_{\infty}$. Both studies demonstrated that apparent differences in ETR due to seasonal or depth effects may be non-significant when the $P-I$ curve is constructed using $I_a$ rather than $I_i$. The results of the present study show wide variation in maximum ETRs of $P-I$ curves measured across the depth range (Table 1). The differences in values of ETR$_{\text{max}}$ and $a$ derived from curves using pooled data versus observed values of ETR$_{\text{max}}$ demonstrate the additional complexity of analysing diel $P-I$ curves when representative parameter values are desired, and the use of observed data may be generally advised if representative parameters are required.

The wide variation in parameter estimates across the depth gradient in the present study may in part be due to variability in leaf condition and orientation, as leaves were fixed in a horizontal position to enable measurement. As the propagated errors associated with $I_k$ values were considerably greater than those associated with ETR$_{\text{max}}$ and $a$, $I_k$ should be treated with caution when used to compare treatments.

At 8 m depth, $\phi_{\text{NO}}$ declined below 0.1 (Fig. 4). When $\psi$ reaches this critical value (due to increasing irradiance), electron transport estimates become less suitable as descriptors of photosynthetic rates (Beer & Axelsson 2004) as more electrons are diverted from photochemistry. Thus, *Halophila stipulacea* at 8 m has more than enough light to conduct photosynthesis during the day, and accordingly diverts the excess energy absorbed into NPQ (Fig. 8). Also, the fact that ETRs were lower in the afternoon than before noon at comparable irradiances points towards a dynamic photo-inhibition taking place after several hours of high irradiance. A similar phenomenon of hysteretic diurnal ETRs was also described for shallow-growing corals in the Gulf of Aqaba (Winters et al. 2003) and the seagrass *Thalassia testudinum* (Belshe et al. 2007, 2008). At the 18 and 33 m sites, $\phi_{\text{NO}}$ does not exceed this threshold value (Figs. 5 & 6) and accordingly diverts less excess energy into NPQ. Even at 33 m there is substantial NPQ, with 30% of exciton energy diverted to this sink at noon (Fig. 10). However, this may not be surprising as *H. stipulacea* has been observed at depths far in excess of 33 m (Lipkin 1979).

The automated dark-acclimation function enables the measurement of dark-acclimated fluorescence in the field without user intervention, as well as the application of a far-red light. Until now this not been possible and, as pointed out by Maxwell & Johnson (2000) and others, this enables the significant limitations of dark-acclimating samples in the field to be overcome. Using this technique, our field measurements enable us to determine the extent that long-term NPQ ($q_l$, or photoinhibitory quenching; Müller et al. 2001) contributes to the decline in photon conversion efficiency relative to the other more rapidly relaxing NPQ components qE (energy-dependent quenching) and qT (state-transition quenching). As expected, all 3 forms of NPQ are active during the day, hence the difference between effective and maximal quantum yield values. However, at night, short-term relaxation of qE and qT has occurred and the remaining NPQ ($q_l$) is evident for both effective and maximal quantum yields. Here, $q_l$ can be seen as the difference between the predawn maximum quantum yield and nighttime quantum yield.

In addition to this ability to quantify these 3 different phases of NPQ according to relaxation kinetics, *in situ* dark-acclimation also enables a detailed deconvolution of *in situ*-derived NPQ components over a diel cycle and enables the direct estimation of NPQ parameters described in the literature (Kramer et al. 2004, Kornyeyev & Holaday 2008). Here, the relatively straightforward deconvolution described by Kramer et al. (2004) is employed, where the term for basal intrinsic non-radiative decay, $\psi_{\text{NO}}$, includes both constitutive thermal dissipation and thermal dissipation associated with PSII photo-inactivation (Kornyeyev & Holaday 2008).

$\psi_{\text{NO}}$ remained fairly constant at between 30 and 40% at all 3 depths, with slightly more variation in shallow
waters and slightly lower values in deep waters. Thus, *Halophila stipulacea* dissipates a large proportion of light energy through intrinsic decay apparently regardless of depth within the depth range examined. The water clarity in the Gulf of Aqaba is such that photosynthetic organisms at 33 m are unlikely to be light limited (Winters et al. 2003). As $\varphi_{NO}$ describes the dissipation of excess energy and is relatively constant throughout the diel period in the deepwater plants, it partly represents a constitutive property of *H. stipulacea* rather than a high-light-induced acclimatory mechanism. The reduced capacity for photosynthetic photon conversion during the day in deep water leaves occurs at a depth where one might consider irradiance becomes negligible at the depth limit of this species; such an approach might be used to suggest a species’ depth limit. The present study invites the question of the cost of maintaining a constitutive capacity to dissipate excursions versus the cost of maintaining a down-regulatory capacity for dissipation.

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Photosynthetic responses of *Halophila stipulacea* to a light gradient. II. Acclimations following transplantation

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ABSTRACT: *Halophila stipulacea* is the dominant seagrass in the Gulf of Aqaba (northern Red Sea), where it grows from the intertidal to depths exceeding 50 m. Its successful growth under such a broad irradiance gradient shows either a high plasticity or is caused by longer-term adaptations to the various depths, possibly resulting in the formation of ecotypes. In April 2008 we transplanted shoots of this seagrass between the extreme depths of its distribution at the study site (8 and 33 m) in order to evaluate its acclimation potential to various irradiances. We compared photosynthetic parameters derived from light response curves generated by PAM fluorometry (so-called rapid light curves, RLC) and measured chlorophyll *a* and *b* concentrations. RLCs from the shallow (~400 µmol photons m⁻² s⁻¹ at midday) and deep (~35 µmol photons m⁻² s⁻¹ at midday) sites were characteristic for high- and low-light growing plants, respectively, and the transplanted seagrasses acclimated to their new environments within 6 d, at which time their RLCs resembled those of the original plants growing at the depths to which they had been transplanted. Concentrations of both chlorophyll *a* and *b* decreased or increased when the plants were transferred to high- vs. low-light environments, respectively, but the chlorophyll *a*: *b* ratios remained constant. These fast changes in photosynthetic responses and light absorption characteristics in response to changing light environments points to *Halophila stipulacea* as being a highly plastic seagrass with regard to irradiance, which may partly explain its abundance across a wide range of irradiances along the depth gradient that it occupies.

KEY WORDS: Acclimation · Depth gradient · *Halophila stipulacea* · Irradiance · Photosynthesis · Pigments · Seagrass

INTRODUCTION

In addition to diurnal changes in insolation, the marine environment is characterised by strongly attenuated irradiances along a relatively short depth gradient. The ability of marine macrophytes to develop mechanisms to utilise and/or cope with different irradiances may therefore be crucial for their functioning at temporally dynamic as well as spatially different surroundings (Sultan 2000). Some marine macroalgae have indeed been shown to possess the capacity to cope with variation in irradiance as well as spectral composition in a plastic way (e.g. Monro & Poore 2005, Mata et al. 2006). Regarding submerged marine angiosperms (seagrasses), much information has been acquired regarding their photophysiological responses to irradiance (Olesen et al. 2002, Durako et al. 2003, Silva & Santos 2003, recently reviewed by Ralph et al. 2007), but whether these responses are the result of adaptations (long-term selection processes resulting in eco-
types) or acclimation processes (short-term plastic responses) are largely unknown.

Halophila stipulacea is the dominant seagrass in the clear waters (light attenuation coefficient \( K_d \) [400 to 700 nm] = 0.1) of the Gulf of Aqaba (northern Red Sea). There it grows abundantly in fine sediments from the intertidal down to depths >50 m (Lipkin 1979, Y. Sharon pers. obs.) where, unlike shallow-growing species, it is not affected by hydrostatic pressure (Beer & Waisel 1982). Its photosynthetic responses to irradiance at various depths are characterised by decreasing maximal photosynthetic rates (measured as relative electron transport rates, rETR) and decreasing onsets of saturating irradiances \( (I_k) \) at increasing depths from 7 to 30 m (Schwarz & Hellblom 2002). For plants grown at higher irradiances, midday chloroplast clumping was quantified by Sharon & Beer (2008), and it was suggested that this is an important photoprotective mechanism for shallow-growing plants. Thus, while acclimation to daily temporal changes in irradiance was shown for high-light plants of Halophila stipulacea, it is not known whether the lower rETRs of this seagrass at low irradiances such as demonstrated by Schwarz & Hellblom (2002) are also due to acclimation processes or can only be explained by longer-term adaptations.

As part of the recent Group for Aquatic Primary Productivity (GAP, see www.gap-aquatic.org/) workshop in Eilat (April 2008), we studied in situ various photo-physiological aspects of the seagrass Halophila stipulacea. In the present study, we tested the photosynthetic plasticity of this plant as a response to in situ irradiance. This was done by comparing pigment contents and photosynthetic parameters derived from pulse-amplitude modulated (PAM) fluorometry following reciprocal transplantations from the 2 edges of an H. stipulacea meadow.

**MATERIALS AND METHODS**

The Halophila stipulacea seagrass bed studied was located ca. 200 m south of the InterUniversity Institute (IUI) in Eilat, the Gulf of Aqaba, northern Red Sea (29° 30' N, 34° 54' E). The vertical extension of the bed varies between seasons, and was between 8 and 33 m at the time of the present study (April 2008). Midday irradiances at the 2 experimental depths at this time were ~400 and ~35 \( \mu \text{mol} \, \text{photons} \, \text{m}^{-2} \, \text{s}^{-1} \) at 8 and 33 m, respectively; most days were cloudless during the 2 wk experimental period. The water temperature was 23°C. All plants were accessed by SCUBA diving using NITROX.

In order to measure the plasticity of the photosynthetic responses of Halophila stipulacea to irradiance, we transplanted plants reciprocally from one site to another. Some 30 to 50 shoots, including rizhomes (possibly belonging to the same clone) and roots, from the 2 depths were removed with their sediments (ca. 3 l) into mesh bags, and 3 such bags were placed into dug-out depressions at each of the sites (both vertically and horizontally for same-depth controls). Photosynthesis and pigment measurements were performed just prior to transplantation and following 6 and 14 d of exposure to the various sites.

Chlorophyll fluorescence was measured on cloudless days at 09:30 to 10:30 h at the 2 sampling depths using an underwater fluorometer (Divine-PAM). Individual leaves (from different shoots, \( n = 5 \)) were placed into a leaf-clip and rapid light curves (RLCs) were initiated immediately. Each leaf was irradiated with increasing pre-set photosynthetically active radiation (PAR) steps \( (0, 37, 56, 104, 160, 227, 339, 512 \) and 818 \( \mu \text{mol} \, \text{photons} \, \text{m}^{-2} \, \text{s}^{-1} \) for 5 s, and the effective quantum yield of Photosystem II (PSII) was recorded after each step. It was found that 5, 10 and 30 s of exposure to each level of irradiance yielded the same results, so 5 s was chosen in order to minimise the diving time. Electron transport rates (ETR) were calculated as \( \text{ETR} = \frac{Y \times I}{AF \times 0.5} \) where \( Y \) is the effective quantum yield of PSII, \( I \) is the incident irradiance, \( AF \) is the absorption factor and 0.5 is the assumed proportion of light absorbed between PSII and PSI. AFs were estimated in situ by measuring the light absorbed by the leaves as described elsewhere (Beer & Björk 2000, except that ambient light was used instead of a lamp), and assuming that all light was absorbed by the photosynthetic pigments of the leaves (Sharon & Beer 2008). ETR vs. PAR curves were generated after correcting incident PAR with AF so as to depict absorbed PAR on the x-axes (according to Saroussi & Beer 2007; this is logically called for since the ETRs of the y-axis also include AF values). The maximal photosynthetic rate \( \left( P_{\text{max}} \right) \) and onset of light saturation \( \left( I_k \right) \) were calculated after fitting the ETR data to equations by Platt et al. (1980). The maximal effective quantum yield \( \left( Y_0 \right) \) was taken as the first yield measurement of the RLC (according to Saroussi & Beer 2007; this value is equal to \( a \) as commonly calculated from the initial slope of the RLC). Initially, in order to find out if the plants from the deep and shallow sites had the same potential photosynthetic capacity, PSII maximum quantum yield of photo-chemistry in PSII \( (F_{\alpha} / F_{\text{m}}) \) of dark-adapted plants were recorded for those plants at dawn (06:00 h).

Chlorophylls \( a \) and \( b \) (chl \( a + b \)) were extracted in \( N'N'\text{-dimethylformamide} \) from cut-out leaf sections within 20 min of collection (\( n = 5 \)). The absorbance was measured in a spectrophotometer at 664, 647 and 625 nm and concentrations were calculated according to Moran (1982).

Statistical analyses were performed using Sigma Stat 3.00. Differences were considered significant at \( p < 0.05 \).
RESULTS

*In situ* \( F_v / F_m \) values (mean ± SD) of native plants were found to be 0.76 ± 0.02 (n = 10) and 0.76 ± 0.01 (n = 10) for shallow (8 m) and deep (33 m) plants, respectively. Thus, no significant difference was found in the maximum photosynthetic efficiency of PSII in *Halophila stipulacea* at the different bathymetric sites during the time of these measurements.

The average RLCs (n = 5) of *Halophila stipulacea* from the 2 different sites are depicted in Fig. 1. The \( P_{\text{max}} \), \( I_k \) and \( Y_0 \) (equalling \( a \)) values derived from these curves were 39.7 ± 3.8 \( \mu \text{mol electrons m}^{-2} \text{s}^{-1} \), 65.0 ± 7.1 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) and 0.61 ± 0.03 mol electrons mol photons\(^{-1} \), respectively, for the shallow plants and 20.6 ± 1.4 \( \mu \text{mol electrons m}^{-2} \text{s}^{-1} \), 41.1 ± 4.4 \( \mu \text{mol photons m}^{-2} \text{s}^{-1} \) and 0.50 ± 0.04 mol electrons mol photons\(^{-1} \), respectively, for the deep plants. All parameters were significantly different between the shallow and deep plants (Mann-Whitney \( U \)-test, \( p < 0.001 \)).

\( P_{\text{max}} \) of the shallow plants decreased significantly (Mann-Whitney \( U \)-test, \( p < 0.001 \)) 6 d after transplantation to the deep site, and kept decreasing slightly after 14 d of exposure to the dimmer environment (Fig. 2). Conversely, deep plants increased their \( P_{\text{max}} \) significantly (\( p < 0.001 \)) 6 d after transplantation, and did not change over the remaining 8 d. Thus, \( P_{\text{max}} \) values of shallow plants became quantitatively similar to those of deep plants after 6 d and vice versa. The control plants, which were dug up and replanted at the same depths in order to appraise whether mechanical uprooting had affected them, showed much less of a change in \( P_{\text{max}} \) over time.

\( I_k \) changed similarly to \( P_{\text{max}} \) at the different environments to which the plants were transferred (Fig. 3). Also, \( I_k \) of the transplanted seagrasses became quantitatively similar to that of the plants originally growing at the 2 depths. While the \( Y_0 \) val- ues of the transplanted plants also showed a pattern of change with time such that they came to resemble those of the plants originally growing at the 2 depths (Fig. 4), this change was slower than for the other parameters.

The chl \( a+b \) content of shallow plants increased between 6 and 14 d following transplantation to the deeper site (Fig. 5). Conversely, the deep plants showed a decreased chl \( a+b \) content after 6 d following transplantation to the shallow site. There was no significant difference in chl \( a:b \) ratios following the transplantations (Fig. 6).
DISCUSSION

Our results indicate that *Halophila stipulacea* is a highly plastic seagrass that can acclimate to various light environments quickly. This was shown before for plants grown under high-light conditions, where chloroplast movements were suggested as a means to both capture photons under diurnal low-light periods (when chloroplasts are dispersed) and apparently protect the leaves from photodamage during midday (by clumping the chloroplasts, Sharon & Beer 2008). In the present study, we showed that the photosynthetic apparatus also features plasticity and it can acclimate to various irradiances along a depth gradient according to classical adaptation strategies of high- and low-light plants. Further, this plasticity is in line with the thought that specimens of *H. stipulacea* growing at various depths in a meadow are not ecotypes, although the maximal irradiance during midday spans at least 1 order of magnitude.

The apparent lack of ecotype formation at various depths within a *Halophila stipulacea* meadow may stem from its largely vegetative growth pattern (through rhizome elongation) resulting in large areas belonging to the same clone. Further, we have observed that the vertical extension of the meadow differs between seasons. During the summer, the studied meadow extends from 5 m to a depth of 45 m (Y. Sharon pers. obs.), while during the winter the meadow’s lower limit only extends to ~30 m. This may be due to irradiance and/or temperature; the lowest irradiance in the Gulf of Aqaba is in January and the lowest temperature in March (Winters et al. 2006). The present study suggests that the plasticity in the response to seasonal irradiance levels would allow for the successful acclimation to the different depths as observed throughout the year. This would then explain the dynamic seasonal meadow extensions as based on clonal growth, although *H. stipulacea* meadows were found to be highly polymorphic between depths in the Mediterranean (Procaccini et al. 1999).

On the cellular level, the fast (within 6 d) adjustment of $P_{\text{max}}$ to irradiance points towards rapid changes in the carbon fixation reactions during the acclimation to high or low midday irradiances. These changes could involve key enzymes of the Calvin cycle, e.g. Rubisco, or other reactions following those of the primary photochemistry. The fact that $P_{\text{max}}$ values in the transplanted seagrasses became similar to those of the plants growing originally at the transplant depths shows that these acclimations are not only qualitative but also quantitative. The finding that $I_k$ values varied plastically in concord with $P_{\text{max}}$ is also in agreement with acclimation to high and low irradiances. While chl *a + b* content also showed plasticity, the response in the plants transplanted from the high- to the low-light environment was slower than in the low-
high-light transplants, indicating a longer time is required for the production of new chlorophyll than its loss. The lack of changes in chl a:b ratios in all treatments suggests that this parameter has no role in in situ acclimation processes, such as reported by Durako et al. (2003) for 2 other Halophila species. While terrestrial shade-adapted plants often increase chl b contents relative to chl a, it may be that the spectral changes with depth prevent such a change in H. stipulacea.

In addition to the differences in photosynthetic responses at different irradiances, Halophila stipulacea has been reported to feature various leaf morphologies with depth (e.g. longer and wider leaves in deeper waters, Lipkin 1979). Also, changes in Pₘₐₓ and Iₔ along a depth gradient have been reported (Schwarz & Hellblom 2002). However, in neither case was it verified whether those changes could be due to acclimation processes or were the outcome of longer-term adaptations possibly resulting in ecotype formations. Olesen et al. (2002) have suggested that some seagrasses (i.e. Cymodocea nodosa) can acclimate and respond differently to changing light conditions than others (i.e. Posidonia oceanica). While C. nodosa displayed reduced light requirements with depth, both species acclimated mainly at the population structure level by reducing self-shading in deeper waters.

This is the first work reporting that Halophila stipulacea’s wide depth distribution across a broad range of irradiances can be explained by its plastic photosynthetic abilities allowing for successful acclimation both spatially and seasonally. While most of the differences between shallow (i.e. high-light) and deeper (low-light) growing plants measured in the present study follow classical sun- vs. shade-acclimation patterns, others do not. For the latter, the higher Y₀ (i.e. a) in shallow-growing plants and the stable chl a:b ratios may be a result of spectral changes along the depth gradient in the water column, which still need to be elucidated.

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Chloroplast clumping in the seagrass *Halophila stipulacea* is triggered by UV radiation

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Running title: UVR triggers chloroplast clumping in *Halophila stipulacea*
ABSTRACT

It was shown previously that high irradiances caused chloroplasts to clump in the seagrass Halophila stipulacea during midday (Sharon and Beer 2008). Since potentially harmful UVR is present in the shallow waters of the Gulf of Aqaba where part of its population thrives, we examined the effect of UVR on the clumping phenomenon. This was done by acclimating plants to high midday irradiances under filters that did or did not transmit UVR. It was found that no chloroplast movements occurred when the plants were protected from 80% of the <400 nm wavelengths. Accordingly, UVR caused the clumping, which resulted in a 44% reduction in the absorption cross section of the leaves and, therefore, a concomitant lowering of midday electron transport rates. In addition, the effective quantum yield decreased by 26% during midday and the pre-dawn maximal quantum yield decreased by 10%. On the other hand, UVR had no effect on chlorophylls contents or on the characteristic absorption spectra of flavones and flavone-glycosides. We conclude that the potentially harmful effects of UVR on the photosynthetic apparatus of Halophila stipulacea are mitigated by its activation of chloroplast clumping, which functions as a means of protecting most chloroplasts and perhaps the nucleus from high irradiances, including UVR.

Key words: Chloroplast clumping; Halophila stipulacea; Photosynthesis; UV radiation; Seagrasses
INTRODUCTION

While the mechanisms underlying chloroplast movements in plants have been studied on the intracellular and molecular levels (Gabrys 2004; Sakuri et al. 2005; Luesse et al. 2006; Krzeszowiec et al. 2007; Suetsugu and Wada 2007; Yamashita et al. 2007), their ecophysiological role(s) is less understood. These movements are in many plants induced by blue light (Gabrys 2004; DeBlasio et al. 2005; Sakuri et al. 2005; Luesse et al. 2006; Krzeszowiec et al. 2007; Shiihira-Ishikawa et al. 2007). In a few studies, it was suggested that also UV, or "near to UV", wavelengths could trigger chloroplast movements (Yatsuhashi 1996; Rodrigues et al. 2007). Interestingly, Kondo et al. (2004) reported that in many cases the chloroplast clumps are formed near or around the nuclei.

The only seagrass so far for which chloroplast movements has been described is Halophila stipulacea (Drew 1979; Sharon and Beer 2008). In the latter work, it was found that chloroplast clumping occurred during midday only in plants growing under high irradiances (450 μmol photons m⁻² s⁻¹), while lower-irradiance grown plants maintained their chloroplasts evenly dispersed during the day. It was then suggested that the chloroplast clumping during midday may have a protective role against high irradiances.

In addition to high photosynthetically active radiation (PAR, 400-700 nm), coastal waters, especially clear tropical waters, also feature high levels of UV radiation (UVR, 280-400 nm) in their upper layers (Dunne and Brown 1996; Agusti and Liabres 2007; this study). Various marine plants respond to such high UVR levels by producing mycosporine-like amino acids (MAA; Whitehead and Vernet 2000; Gao et al. 2007) and other UV absorbing compounds (flavones and flavone-glycosides in seagrasses; Durako et al. 2003, Kunzelman et al. 2005, Meng et al. 2008), changing morphologies (Monro and Poore 2004), changing pigment contents (Dawson and Dennison 1996; Gao et al. 2007), down-regulating photosystem II (PSII; Figueroa et al. 2002) or shading by carbonate layers (Beach et al. 2006). Also epiphytes
may decrease the UV (as well as PAR) levels reaching the leaves (Trocine et al. 1981; Brandt and Koch 2003). Since many seagrasses thrive in tropical shallow waters, it may be that the midday chloroplast clumping found in at least one of them (Halophila stipulacea) has a protective role against UVR (in addition to high PAR as suggested by Sharon and Beer 2008). In light of this, we investigated the possible role of UVR in mediating chloroplast movements in this seagrass, and the possible effects of UVR on some of its photosynthetic parameters.

MATERIALS AND METHODS

Whole plants (including roots and rhizomes) of Halophila stipulacea, together with the surrounding sediments, were collected at 20 m depth from the densest part of a monospecific meadow (extending from 7 to 33 m at the time of this study; the tidal range is <40 cm) located ca. 200 m south of the Inter-University Institute (IUI) in Eilat, Gulf of Aqaba, northern Red Sea (29°30’N, 34°54’E) during March and April, 2007. These plants were grown in cups containing natural fine-sandy sediment on an outdoor water table (a table with a 20 cm margin, forming a shallow container) through which natural seawater at 21°C was flowing. Thus, the leaves were submerged 5-10 cm under the water surface. The water table was shaded by a neutral-density net allowing 50% of the natural sunlight to reach the plants. Typical midday irradiances under the net were ~850 µmol photons m⁻² s⁻¹ during midday. The light under the shading net was further screened through UV-filters (supplied by the GKSS Forschungszentrum, Germany) placed just above the water surface over some of the plants, while control-filters that transmitted UVR (from the same supplier) was used for the other plants. Both filters reduced PAR by ~20%, while the UV-filter reduced wavelengths <400 nm by >80% (see Table 1 and Fig. 2). The wavelength-dependent transmittance properties of the filters were verified using a miniature-fibre optical spectrometer (USB 2000, Ocean Optics, USA). All plant measurements were done after 14 days of acclimation to the water-table
conditions (except measurements of Fv/Fm, which were performed more often). This time was found to be enough for the plants to feature diurnal chloroplast clumping under the chosen conditions, and was later also found to be sufficient for the acclimation of several photosynthetic parameters to changing light regimes (Sharon et al. 2009).

The solar irradiance (PAR and UVR) reaching the water surface was measured by a CM11B (Kipp & Zonen, The Netherlands) sensor at the IUI pier (displayed also at [www.meteo-tech.co.il/eilat-yam/eilat_daily.asp](http://www.meteo-tech.co.il/eilat-yam/eilat_daily.asp)). Spectral radiation measurements were done using a profile reflectance radiometer (PRR-800, BioSpherical Instruments Inc., USA) equipped with both spectral channel (300-875nm, µW cm⁻² nm⁻¹) and PAR (400-700 nm, µmol photons m⁻² s⁻¹) sensors. Underwater spectral measurements of downwelling UVR, PAR and infrared radiation were also done using the PRR-800, which was lowered from a boat offshore the IUI using a free-fall system (cf. Kirk 1994) in order to avoid boat shading and to keep the instrument in a vertical posture.

Chloroplast clumping was verified through microscopic observations on live leaves within 2 min of collection (using a Nikon YS100, Japan). The absorption cross sections (or absorption factor, AF) of the leaves (i.e. the fraction of incident irradiance absorbed by the photosynthetic pigments of the leaves) were estimated along the day by measuring the PAR above and below a submerged leaf with the Diving-PAM’s quantum sensor (while subtracting the absorption of non-chlorophyllous pigments, see Sharon and Beer 2008). Chlorophyll contents of the leaves were determined after extraction in dimethyl formamide for 24 h in darkness. Absorbance was then measured spectrophotometrically according to Moran (1982), and chlorophyll a and b contents were calculated on a leaf area basis. Total carotenoids were measured by their absorption maxima at 480 and 510 nm according to Gradinaru et al. (2000). Absorption spectra (300-400 nm) with special attention to absorption at 340 and 350 nm of the UV absorbing pigments (flavones and flavone-glycosides; Meng et al. 2008) were
Chlorophyll fluorescence was measured by pulse-amplitude-modulated (PAM) fluorometry using a Diving-PAM (Walz, Germany). Effective quantum yield (ΔF/Fm’) measurements were carried out throughout the day using a leaf-distance clip (Walz, Germany) and photosynthetic electron transport rates (ETR) were calculated as ΔF/Fm’ × PAR × AF × 0.5; PAR was measured with the Diving-PAM’s quantum sensor calibrated against a quantum-sensor-equipped Li-250A light meter (LiCor, USA). Maximal quantum yields (Fv/Fm) were measured in darkness at pre-dawn hours every morning for the first week of acclimation and after 14 days.

RESULTS

The midday underwater spectra (300-800 nm) from two depths and the integrated total PAR (400-700 nm) and UVR (300-400 nm) at those depths, as well as on the water-table under the control- or UV-filters, are shown in Fig. 1 and Table 1, respectively. The spectra (Fig. 1) reveal high UVR values at 6 m depth that could hardly be detected at 30 m. Similarly, there were measurable amounts of red light (650-750 nm) available at 6 m but not at 30 m. In all, approximately 45% and 5% of surface PAR was present at 6 and 30 m depths, respectively, while the corresponding percentages for UVR were 50% and 3% (Table 1). The PAR measured under the filters on the water-table was 45% of that of full sunlight, while UVR was 41% and 7.4% under the control- and UV-filters, respectively (see Fig. 2 for the spectral transmission of these filters). Unlike for UVR, there was no reduction in red wavelengths by the UV-filter (Fig. 2).

The epidermal leaf cells of plants grown on the water-table under control-filtered conditions featured midday clumping of chloroplasts, followed by their dispersal during the
evening, while no such chloroplast movements could be detected in the UV-filtered plants (Fig. 3). While peaking at midday, chloroplast aggregation and dispersal was observed between 09:00 and 15:00 and was paralleled by decreasing and increasing AF values, respectively. Because the only difference between the treatments was the presence and virtual absence of UVR, it is concluded that the UVR dose present for the control-filtered plants caused the chloroplast movements that resulted in their midday clumping. More precisely, wavelengths between 280 and 380 nm caused the clumping (since these wavelength were the absolute cut-of points for the control- and UV-filters, respectively). In comparison, also leaves of plants growing at 6 m depth clumped their chloroplasts during midday while those at 30 m did not (Runcie et al. 2009 and personal observations). These chloroplast movements resulted in a significant (44%) change in the AF of the control-filter-grown plant leaves from early morning to midday while the UV-filtered plants did not show any significant differences in AF during the day (Fig. 4); the AF values of the plants growing under the control-filters were significantly lower than those of the UV-filtered plants between 10:00-14:00.

Chlorophyll a+b contents showed no significant differences between the UV- and control-filtered water-table plants after 14 days: Chlorophyll a contents were 2.3 ± 0.17 and 2.6 ± 0.27 mg cm\(^{-2}\) for the control- and UV-filtered plants, respectively, while chlorophyll b contents were 0.3 ± 0.1 mg cm\(^{-2}\) in both control- and UV-screened plants. Also carotenoid absorption spectra peaks (at 480 and 510 nm) showed no significant difference between the two types of water-table grown plants after 14 days of acclimation. Similarly, the absorption spectra in the region where flavones and flavone-glycosides absorb (340–350 nm), nor the peak maxima of anthocyanin (290 nm), showed significant differences between treatments with or without UVR.

Photosynthetic ETRs of the plants grown on the water-table with or without UV screening are presented in Fig. 5. Since these ETRs were calculated according to their respective AF
values during the various times of the day (see Fig. 4, cf. Sharon and Beer 2008), it follows that the differences in ETRs between control- and UV-filtered plants are partly due to the differences in AFs (here termed an "indirect" UVR-effect). The maximum ETR reached at around midday by the UVR-protected plants coincided with the highest PAR reaching the water-table while the ETRs for the control-plants levelled off after ca. 09:00 in the morning. This, and the fact that the midday decrease in ETR in the control-filtered plants was more than the 44% that could be accounted for by the decrease in AF, indicates an excessive decrease of effective photosynthetic quantum yields by UVR during the high-irradiance hours of the day. Indeed, $\Delta F/Fm'$ values were significantly lower in the control-filtered than the UV-filtered plants from around 11:30 and throughout the afternoon (Fig. 6), confirming a higher down-regulation of PSII in the former as caused by UVR (here termed a "direct" UVR-effect).

$Fv/Fm$ values of plants acclimated to control-filter conditions decreased slightly but significantly from 0.79 to 0.71 ($\pm$ 0.02) after 14 days of acclimation (and a significant decrease was observed already after 3 days) while there was no significant change in the $Fv/Fm$ values of the plants growing under the UV-filters (Fig. 7).

**DISCUSSION**

In a previous study, we suggested that the midday chloroplast clumping observed in *Halophila stipulacea* had a photoprotective role against high PAR during midday (Sharon and Beer 2008). Here, we found that it is UVR that triggers the diurnal chloroplast movements that cause their midday clumping under high-PAR conditions. This was realised at midday UVR values of ca. 2000 µW m$^{-2}$ on the water table (while UV-screening under those same high PAR values prevented clumping). In nature, at depth where clumping does not occur, reduced UVR is usually accompanied with low PAR, but the UVR/PAR ratio may also change (here it was reduced by 46% from 6 to 30 m). Thus, it is possible that also the
UVR/PAR ratio has an effect on the clumping phenomenon in shallow waters, but this was not quantified here. While the surface irradiance characteristics of the region where this study was conducted (northern Red Sea) are among the highest in the world (Winters et al. 2003), we were surprised to find high UVR doses similar to those on the shaded water table also in situ at 6 m depth. Indeed, midday chloroplast clumping was observed at that depth (Runcie et al. 2009). Thus, apparently, the successful adaptation of this plant to shallow waters (~6 m at the site studied) is partly based on the ability of its chloroplasts to shade one another in order to protect them from high irradiances, including UVR. In addition, the surrounding chloroplasts may, besides shading one another, protect the nucleus and its genetic material from excess UV to prevent UV damage (such as suggested by Kondo et al. 2004).

Another distinction from our earlier work is that while PAR correlated negatively with chlorophyll concentrations (Sharon and Beer 2008), manipulating UVR under constant PAR did not cause any changes in chlorophyll nor carotenoid contents in *Halophila stipulacea*. Supportively, also another *Halophila* species (*Halophila johnsonii*) did not show differences in absorption peak maxima for any photosynthetic and UV-protecting pigments when acclimated to various PAR and UV treatments (Kunzelman et al. 2005). Surprisingly, UVR apparently did not induce the formation of flavones and flavone-glycosides as indicated by similar UV absorption values in methanol extracts of UVR-exposed and UVR-protected plants. This is unlike other tropical shallow-growing seagrasses (Dawson and Dennison 1996; Meng et al. 2008), corals (Dunlap and Shick 1998), microalgae (Geo et al. 2007) and macroalgae (Karsten et al. 1998), many of which do protect their tissues from UVR by the formation of UV-absorbing pigments. Regarding seagrasses, one other *Halophila* species (*Halophila ovalis*) did, however, produce less UV-blocking pigments than other genera (Dawson and Dennison 1996).
While the reduction in midday ETRs was largely an indirect effect of UVR via the decreased AF values caused by the clumping of chloroplasts, UVR also affected photosynthesis directly by reducing quantum yields. The daily reduction in effective quantum yield (ΔF/Fm’) can be seen as a dynamic form of photoinhibition (or down-regulation of PSII) since values in the evening returned to morning values. One mechanism that can explain this midday reduction in effective quantum yields is the operation of the xanthophyll cycle, in which excess light energy is dissipated as heat (Demmig-Adams and Adams 2006). In addition, but to a lesser degree than the effective quantum yield, the maximal quantum yield (Fv/Fm) was also reduced by UVR. Since Fv/Fm is measured in pre-dawn darkness, its lower values reflect an incomplete recovery from high irradiances that might indicate a photo-acclimatory response to UV stress as resulting of a regulating reduction in the PSII repair mechanism. Thus, as part of the more general negative effects of UVR on phytoplankton (Agusti and Liabres 2007) and other marine photosynthetic organisms (Sinha et al. 1998), the chloroplast-based photosynthetic apparatus of some seagrasses is also influenced by UVR. Perhaps logically, this radiation also activates mechanisms such as chloroplast clumping and a dynamic down-regulation of photosystem II that protect at least Halophila stipulacea against it.
REFERENCES


Table 1: Midday values of PAR and UVR and percentage transmittances (%) of the in situ downwelling (at 6 and 30 m) and 50%-shaded water-table (under the control- and UV-filters) PAR (400-700 nm) and UVR (300-400 nm). The water-table values were measured on April 10, 2007, and the in situ values were measured on April 1, 2008 offshore the IUI, with the PRR-800 profiling radiometer.

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<tr>
<th></th>
<th>PAR</th>
<th>UVR</th>
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<tr>
<td></td>
<td>(µmol photons m(^{-2}) s(^{-1}))</td>
<td>(%)</td>
</tr>
<tr>
<td>In situ, 6 m</td>
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<td>Water-table + control-filter</td>
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<tr>
<td>Water-table + UV-filter</td>
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</table>
FIGURE LEGENDS

**Fig. 1**: In situ transmitted downwelling irradiance (Ed) at 6m (open circles) and 30m (closed circles) measured with the PRR-800 (BioSpherical Instruments) on April 1, 2008, offshore the IUI.

**Fig 2**: Transmission spectra of control- and UV-filter (lines marked in graph) as measured using the USB-2000 (Ocean Optics).

**Fig. 3**: Light-microscopic photographs of epidermal cells of *Halophila stipulacea* during midday (at 850 µmol photons m\(^{-2}\) s\(^{-1}\)) grown for 14 days under a control-filter (a) and under a UV-filter (b). Black bars represent 10 µm.

**Fig. 4**: Daily changes in the absorption cross section (or absorption factor, AF) of plants grown on the water-table for 14 days under control-filters (open circles) or UV-filters (closed circles), and PAR (400-700nm) measured above the plants on the water-table (full line) and UVR (300-400nm) under the control-filter (dotted line; there was no measurable UVR under the UV-filter). Data points of AF are means of 10 replicates ± SE.

**Fig 5**: Daily changes in electron transport rates (ETR) of plants grown on the water-table for 14 days under control-filters (open circles) or UV-filters (closed circles), and PAR (400-700nm) measured above the plants on the water-table (full line) and UVR (300-400nm) under the control-filter (dotted line; there was no measurable UVR under the UV-filter). Data points are means of 8 replicates ± SD.

**Fig. 6**: Daily changes in effective quantum yield (ΔF/Fm’) of plants grown on the water-table for 14 days under control-filters (open circles) or UV-filters (closed circles) and PAR (400-700nm) measured above the plants on the water-table (full line) and UVR (300-400nm) under the control-filter (dotted line; there was no measurable UVR under the UV-filter). Data points are means of 8 replicates ± SD.
**Fig. 7:** Maximal quantum yields (Fv/Fm) as a function of time for acclimated to water-table conditions (~850 μmol photons m$^{-2}$ s$^{-1}$ PAR during midday) under control-filters (open circles) and UV-filters (closed circles). Data points are means of 8 replicates ± SD.
Fig. 1: In situ transmitted downwelling irradiance (Ed)

Fig. 2: Transmission spectra of control- and UV-filter
Fig. 3: Light-microscopic photographs of epidermal cells of *Halophila stipulacea*

Fig. 4: Daily changes in the absorption cross section
Fig. 5: Daily changes in electron transport rates

Fig. 6: Daily changes in effective quantum yield
Fig. 7: Maximal quantum yields
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 ETRmax, and a 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 macroalgal *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 mmol photons m⁻² s⁻¹ at the water surface to ~100 mmol 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 mmol 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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(Rubisco) 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, mW cm⁻² nm⁻¹) and PAR (400–700 nm, mmol 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) for 10 s each, where in the end of each irradiation the effective quantum yield (DF: Fₙ/Fᵢ) 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 DF: Fₙ/Fᵢ × PAR 3 absorption factor (AF) 3 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 (PARₐ; i.e., PAR 3 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 N₂ 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 mL cold 1.3 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⁻¹ Pefabloc SC 4-(2-Aminoethoxy) benzenesulfonyl fluoride hydrochloride protease inhibitor (Roche). These samples were then sonicated using a Fisher Scientific Model 100 Sonic dismembrator with a micropip attachment at a setting of 30%. To avoid overheating and improve extraction efficiency, the samples were then refrozen immediately in liquid N₂. 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). Following the disruption of the leaves, the samples were centrifuged for 3 min at 10,000 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. (2010a). 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 mg 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
1-m and 48-m depths are presented in Fig. 1. These spectra reveal high UVR (,400 nm) and red (650–700 nm) radiation, and RLC analysis (n=20) were used to assess their significance (derived from RLC performed in situ on 1-m– and 48-m–depth growing leaves of Halophila stipulacea) 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

$$I_{686} : (I_{717} + I_{686}).$$

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 a and b 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 nmol photons m$^{-2}$ s$^{-1}$ at 1 m to ,100 nmol 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 PsbA concentration was three times higher in the deep-growing ones (Fig. 3). Thus, the PsbA ratio increased three-fold in the deep-growing plants. Assuming that the amount of PsbA 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 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 PSI), 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$_{\text{max}}$), the initial slope of the ETR vs. irradiance curve (a), and the onset of light-saturation point (I$_s$) 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$_{\text{max}}$ 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 vs. 0.41).

Table 1. Midday irradiance, photosynthesis maximum (Pmax), the initial slope (a), and onset of light-saturation point (I$_s$) 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.

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<th>Depth (m)</th>
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<th>Midday irradiance</th>
<th>FII</th>
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<td>48</td>
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<td>0.5</td>
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<td>63.62</td>
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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.
and 0.41), respectively; (2) a 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.

**Discussion**

In this study, we investigated deep-growing plants of the seagrass *H. stipulacea*, which, at their depth limit in the summer (\( \sim 50 \) m), received a down-welling PAR of \( \sim 5\% \) 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 (\( \sim 0.5 \) 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.
Halophila dim irradiance acclimation

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. 2010b), 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 CO₂ 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<sub>max</sub> 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 (a) 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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Measuring seagrass photosynthesis: methods and applications

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ABSTRACT: This review originates from a keynote lecture given at the recent 8th Group for Aquatic Productivity (GAP) workshop held in Eilat, Israel. Here we examine the most important methodologies for photosynthetic measurements in seagrasses and evaluate their applications, advantages and disadvantages, and also point out the most relevant results. The most commonly used methodologies are based on oxygen (O2) evolution and chlorophyll fluorescence measurements. O2-based methodologies allowed for the first approaches to evaluate seagrass productivity, whereas chlorophyll a fluorescence has more recently become the choice method for in situ experiments, particularly in evaluating photosynthetic responses to light and assessing stress responses. New methodologies have also emerged, such as O2 optodes, underwater CO2 flux measurements, geo-acoustic inversion and the eddy correlation technique. However, these new methods still need calibration and validation. Our analysis of the literature also reveals several significant gaps in relevant topics concerning seagrass photosynthesis, namely the complete absence of studies on deep-growing populations that photosynthesize under extreme low light conditions and the uncertainties about the true degree of seagrass carbon limitation, which limits our ability to predict responses to global changes.

KEY WORDS: Seagrass photosynthesis · O2 evolution · Chlorophyll a fluorescence · Carbon uptake · CO2 flux

INTRODUCTION

Early studies on seagrass photosynthesis (e.g. Drew 1978) usually began by explaining that seagrasses are relatively primitive monocotyledons, closely related to freshwater plants but able to live in the marine environment. Presently, many introductions start by highlighting the importance of seagrasses in overall marine productivity and their subsequent economical importance (e.g. Duarte & Cebrián 1996, Costanza et al. 1997, Duarte & Chiscano 1999). This shift of emphasis indicates the evolution of seagrass perception in global biological science and reflects the contribution of the increasing number of physiological and ecophysiological studies dealing with this plant group.

Seagrass photosynthesis was, until recently, most commonly measured in laboratory experiments, usually by incubating leaf segments in closed chambers and determining initial and end O2 values, or by following continuous O2 evolution in the chambers with Clark-type electrodes. These methods have provided most of the fundamental information on seagrass responses to factors such as light, temperature and nutrients (comprehensively reviewed by Lee et al. 2007) and on the mechanisms of carbon uptake and fixation by these plants (Beer et al. 2002). However, they are extremely intrusive, as they involve plant removal from the natural environment and a high degree of manipulation (Beer et al. 2001).

In situ measurements of photosynthetic activity in seagrasses were made possible after the development
of a submersible pulse-amplitude modulated (PAM) fluorometer (applied initially by e.g. Beer et al. 1998, Ralph et al. 1998, Björk et al. 1999, Beer & Björk 2000). Recently, an infrared gas analysis (IRGA) technique has been adapted for in situ continuous dissolved CO₂ flux measurements using incubation chambers connected to the analyser at the surface (Silva et al. 2008). While PAM fluorometry determines photosynthetic traits of individual plants, the IRGA method measures the rates of gas exchange at the community level. A combination of both methods may give real-time information on both the photosynthetic characteristics of seagrass plants and the community CO₂ exchange of undisturbed seagrass meadows.

In situ experiments are less intrusive and deal with the plants in their natural habitat, hence the photosynthetic measures incorporate the influences of all ambient parameters. However, the control of experimental conditions is limited, and insights on specific processes are difficult to obtain. On the other hand, laboratory experiments allow for the control and manipulation of most external parameters and are most suited to obtain very specific information, e.g. data on biochemical processes related to photosynthesis. The general downside of laboratory experiments is the difficulty in extrapolating results to the natural environment, e.g. time-related patterns of photosynthetic activity.

We review the fundamental aspects of the most important methodologies used for photosynthetic measurements in seagrasses, detailing their field of application and highlighting the most relevant results achieved. Some new and promising techniques are presented and a critical analysis of the major advantages and disadvantages of each method is given. Significant gaps in this area of research are discussed and presented as potential pathways for future work.

**MEASURING SEAGRASS PHOTOSYNTHESIS**

**O₂ measurements**

Measuring the O₂ evolved during photosynthesis is one of the oldest and simplest ways of quantifying the photosynthetic activity of plants. In the aquatic environment, O₂ concentration can be determined: (1) chemically by Winkler titration, (2) polarographically using O₂ electrodes, or (3) optically using O₂ optodes.

The Winkler method

When applied to seagrasses, the Winkler method (see Strickland & Parsons 1972, adapted to field use by Drew & Robertson 1974, Grasshoff et al. 1983) usually involves enclosing the plants in sealed containers (or ‘bottles’) and leaving them to incubate for a period of time. The increase or decrease of the O₂ concentration during the incubation period provides a measure of either net photosynthesis (in the illuminated transparent bottles) or respiration (in darkened bottles), respectively. Gross photosynthesis can be calculated by correcting the net photosynthetic rates obtained with the rate of dark (mitochondrial) respiration. The Winkler method remains one of the most accurate ways of measuring dissolved O₂ concentrations, arguably better than most commercial field-going O₂ probes. Nevertheless, there are a number of downsides to this methodology, namely: (1) the need to detach and cut down plant samples (since whole plants usually do not fit into the bottles), compromising integrity and inducing stress (except when this method is used to measure O₂ before and after whole-community incubations); (2) the difficulty in maintaining adequate homogenisation of the medium during incubations, leading to increased boundary layers and resulting in underestimations of photosynthetic rates (Koch 1994); (3) the likelihood of O₂ saturation and/or inorganic carbon depletion during the incubation period, both resulting in photosynthetic inhibition due to, for example, photorespiration (Beer 1989) and carbon limitation, respectively (neither verifiable in real-time as only initial and final O₂ concentrations are determined); and (4) the difficulty in ensuring a proper homogeneous or in situ-like illumination of the plant sample. This method thus allowed for the very first approaches to in situ and laboratory experiments of seagrass productivity: Drew (1978, 1979) investigated the seasonal variation in the photosynthetic activity of Cymodocea nodosa and Posidonia oceanica, generated photosynthesis-irradiance (P-E) curves and measured dark respiration in C. nodosa, Halophila stipulacea, Phyllospadix torreyi, Posidonia oceanica, Zostera angustifolia and Z. marina, determining the effects of temperature on photosynthesis and calculating compensation and saturation irradiances for those species. Over the years, the effects of light, temperature, salinity and pressure on seagrass photosynthesis were further investigated using this technique in Halodule uninervis, Halophila stipulacea and Halophila ovalis (Beer & Waisel 1982, Wahbeh 1983), Z. muelleri (Kerr & Strother 1985), C. nodosa (Pérez & Romero 1992, Zavodnik et al. 1998, Olesen et al. 2002) and Posidonia oceanica (Olesen et al. 2002). Recently, a few studies focusing on whole-community metabolism also used Winkler titrations to determine O₂ concentrations before and after incubations in benthic chambers (Barrón et al. 2004, Gazeau et al. 2005a,b). Nevertheless, the long incubation times used in these studies raise the concern that photosynthesis may be underestimated due to the decreased carboxylase activity of Rubisco in response to
the increase in O$_2$ and decrease in CO$_2$ concentrations within the incubation chambers.

O$_2$ electrodes

Electrochemical sensors for O$_2$ measurements are commonly known as Clark-type O$_2$ electrodes (after their inventor, Leyland C. Clark). These electrodes are composed of a platinum cathode and a silver anode separated by a salt bridge. An electron flow is set in motion when a control unit applies a small electric potential to the system. The current flow is proportional to the O$_2$ consumed at the cathode and can be either analogically plotted or converted into a digital signal to be recorded by a control unit (Walker 1987). Clark-type electrodes are available in a number of options such as handheld units, bench oximeters or incorporated in complete commercially available setups. Whereas handheld units, with low accuracy, are mainly useful for indicative measurements of dissolved O$_2$ (DO) concentrations, bench-top oximeters, with better performance, have been used in conjunction with customized incubation chambers, allowing for versatile and tailor-made apparatuses (Smith et al. 1984, Fourqurean & Ziemann 1991, Koch 1994, Masini et al. 1995, Masini & Manning 1997). Complete systems comprised of Clark-type electrodes attached to water-jacketed incubation chambers are also available on the market, namely those by Hansatech Instruments and Rank Brothers. These laboratory systems provide the highest available resolution and accuracy and have become standard in photosynthetic research. O$_2$ electrodes such as these are probably among the best tools to investigate photosynthetic mechanisms and determine the photosynthetic capacities of seagrasses. Photosynthetic capacity refers strictly to the photosynthetic rate obtained under ideal conditions, i.e. with non-limiting dissolved inorganic carbon (DIC) supplies, nutrients and light and at optimum temperature (Jones 1994). Such demanding conditions are relatively easy to reproduce and maintain in a small, highly controlled environment such as an O$_2$ electrode-coupled reaction vessel, but almost impossible to achieve in any other environment.

O$_2$ electrodes are mostly used in 2 general types of experimental setups: (1) those in which whole plants or plant sections are incubated for a selected period of time in a sealed container and O$_2$ concentrations are determined at the beginning and at the end of the incubations (endpoint incubations), and (2) those in which an O$_2$ electrode is coupled to the incubation chamber and O$_2$ evolution is continuously monitored. Using endpoint incubations, Evans et al. (1986) evaluated temperature acclimation effects on the photosynthetic rates of Zostera marina and Ruppia maritima leaf tips (from Chesapeake Bay) while Terrados & Ros (1995) investigated the seasonal variation of temperature effects on the photosynthesis and respiration of shallow Mediterranean Cymodocea nodosa. Koch & Dawes (1991) searched for differences in P-E curves between 2 ecotypes of R. maritima from Florida and North Carolina and Enríquez et al. (1995) determined a series of P-E relationships in plant sections in an attempt to identify a relationship between the photosynthetic activity of several Mediterranean macrophytes and their morphological characteristics. Invers et al. (1997) investigated the effects of pH on photosynthetic rates of Zostera noltii, C. nodosa and Posidonia oceanica; Ramirez-Garcia et al. (1998) incubated pre-desiccated leaves of Phyllospadix scouleri and Phyllospadix torreyi in glass bottles to assess the effects of different previous air-exposure periods on the photosynthetic activity; and Ruiz & Romero (2001) compared light response curves among Posidonia oceanica samples harvested from an in situ multilevel shading experiment. Finally, Alcoverro et al. (1998, 2001) examined the seasonal and age-dependence of Posidonia oceanica photosynthetic parameters and its annual carbon balance; Invers et al. (1999, 2001) evaluated the role of carbonic anhydrase in the inorganic carbon supply to Posidonia oceanica and C. nodosa; and Bintz & Nixon (2001) measured respiration and photosynthetic rates of whole-plant Z. marina seedlings at 3 different light levels. Hence this type of setup has been used to determine photosynthetic light response curves and to evaluate the effects of seasonality, morphological characteristics, leaf age dependence, shading, temperature, desiccation and pH on seagrass photosynthesis.

Systems based on continuous O$_2$ measurement have been used both in laboratory and field experiments. Most laboratory work has been done using the commercially available integrated systems. Dennison & Alberte (1982) investigated the effects of light availability on Zostera marina photosynthesis and growth; seasonal variations in photosynthetic light responses were evaluated in Z. noltii from the Netherlands (Vermaat & Verhagen 1996), in Z. marina and Phyllospadix scouleri from California (Cabello-Pasini & Alberte 1997) and in 2 Texan Thalassia testudinum populations (Herzka & Dunton 1997); Kaldy & Dunton (1999) determined P-E relationships in T. testudinum laboratory-grown seedlings; Cabello-Pasini et al. (2002) compared the maximum photosynthetic rates of an open-ocean Z. marina population with that from a coastal lagoon; Invers et al. (2001) measured continuous O$_2$ evolution in Z. marina and P. torreyi from the eastern Pacific; Peralta et al. (2000, 2005) compared photosynthetic light response curves between 2 Z. noltii morphotypes;
Silva & Santos (2004) correlated photosynthetic O₂ release with electron transport rates in the same species; and Cayabayab & Enríquez (2007) determined the photosynthetic light responses of *T. testudinum* plants submitted to 3 distinct light treatments. These integrated O₂ measuring systems have the advantage of measuring net gas exchange, which may be correlative to growth, but their often small size and the fact that the plants (or usually only leaves) must be enclosed, limits the reliability of the results for extrapolation to true, unobstructed, *in situ* conditions.

The above-mentioned systems have also been widely used in experiments addressing the mechanisms of carbon uptake, particularly those concerning the use of HCO₃⁻ by seagrasses. Using Clark-type O₂ electrode-based experimental setups, the use of HCO₃⁻ as a DIC source was demonstrated for *Zostera muelleri* from Australia (Millhouse & Strother 1986), *Thalassia testudinum* from Florida (Durako 1993), *Posidonia australis* from Australia (James & Larkum 1996) and *Z. marina* (Beer & Rehberg 1997). Beer & Koch (1996) used this kind of system to simulate primitive inorganic carbon conditions in the Cretaceous and compare the photosynthetic performance of *Z. marina* and *T. testudinum* under those conditions with that under present day ocean conditions. Zimmerman et al. (1997) evaluated the effects of CO₂ enrichment on *Z. marina* productivity and light requirements with a similar apparatus. Björk et al. (1997) investigated inorganic carbon use in 8 seagrass species from the Eastern African coast, by measuring the response of photosynthesis to increased inorganic carbon levels. In the same study, *Halophila ovalis*, *Cymodocea rotundata* and *Syringodium isoetifolium* also revealed their ability to use HCO₃⁻ as an inorganic carbon source. Hellblom et al. (2001) assessed the effects of commonly used buffers on the photosynthetic rates of *Z. marina* and were able to identify 2 mechanisms of HCO₃⁻ utilization, one of them firstly reported for seagrasses based on extruded protons-mediation of HCO₃⁻ uptake. Mercado et al. (2003) investigated the affinity for DIC and the presence of different mechanisms of HCO₃⁻ use in 2 morphotypes of intertidal *Z. nolitii* from southern Portugal. They found that this species had a low affinity for HCO₃⁻, particularly the lower intertidal morphotype. Further, photosynthetic pathways, mechanisms of inorganic carbon acquisition, effects of carbon enrichment and buffer additions and the molar relationships between O₂ release and electron transport have been elucidated using such O₂ electrode systems. On the other hand, extrapolation of the results obtained in these small-volume, laboratory-bound systems to *in situ* conditions may be rather incorrect.

A few authors have built custom chambers with coupled electrodes for specific experiments. Smith et al. (1984) used a custom-made 2-chambered apparatus connected to a Clark-type O₂ electrode to demonstrate and quantify the transport of O₂ from the shoots to the root-rhizome system of *Zostera marina*. In a very elegant experimental design, the authors incubated intact shoots, separating the leaves from the root-rhizome system in 2 adjacent compartments with separate media and illumination. By quantifying the O₂ taken up or released by the root-rhizome system in the absence or presence of shoot illumination, it was possible to elucidate the role of the light environment in the maintenance of root aerobiosis. This line of research was further pursued by Greve et al. (2003) and Binzer et al. (2005), using microelectrodes inserted directly into seagrass rhizomes. These studies significantly advanced our understanding of metabolic integration in seagrasses. They provided the first *in situ* measurements of the fraction of photosynthetically evolved O₂ that is channelled down internally to below-ground tissues and eventually leaked to ensure aerobicism in the rhizosphere. Fourquean & Zieman (1991) used a custom-made transparent acrylic chamber with a coupled Clark-type polarographic O₂ probe to determine the light-response curves of whole *Thalassia testudinum* leaves from south Florida. Koch (1994) examined the effects of hydrodynamics and boundary layer thickness on the photosynthetic rates of *T. testudinum* and *Cymodocea nodosa*. In that study, plant samples were incubated in a temperature-controlled microcosm with an externally recirculated incubation medium. O₂ evolution was continuously monitored by a Clark-type O₂ electrode installed at an intermediate point of the system. Masini et al. (1995) and Masini & Manning (1997) determined photosynthetic light responses in *Postonia sinuosa*, *P. australis*, *Amphibolis griffithii* and *A. antarctica* in a custom-built tubular incubation chamber with an external water recirculation circuit. Water flowing in the circuit was passed through a high-resolution O₂ sensor, recording O₂ values every 15 s.

Some researchers brought high precision O₂ electrodes to the field, connecting them to benthic incubation chambers to monitor the whole-community O₂ release and/or consumption. Dunton & Tomasko (1994) and Major & Dunton (2000) incubated, respectively, whole *Halodule wrightii* and *Syringodium filiforme* plants from south Texas, *in situ*, using transparent acrylic chambers placed on the seabed. Continuous O₂ evolution within the chambers was followed using a high-resolution dissolved O₂ probe installed on a boat at the surface. In both studies, the authors also conducted laboratory measurements of P-E curves in leaf segments using water-jacketed incubation chambers with coupled O₂ electrodes. Field and laboratory measurements were compared, and in both studies the photosynthetic efficiency (α), expressed as the initial
slope of P-E curves, was shown to be highly overestimated in laboratory measurements. Other significant differences, observed in the compensation intensity ($I_c$) and in respiration, highlighted the advantages of in situ measurements. Benthic chambers with continuous O$_2$ monitoring were also used by Plus et al. (2001) to determine primary production and respiration of Zostera noltii beds in the Thau lagoon in South France.

O$_2$ optodes

Optodes are sensors for optical detection of chemical species. The common measuring principle is the use of an indicator dye (immobilized on the tip of an optic fibre or on a planar surface), the colour of which changes as a function of variations in the concentration of the analyte. Optodes can be used to quantify a number of analytes, e.g. O$_2$ and CO$_2$. Several indicator dyes can be used to build O$_2$ optodes, providing that their luminescence intensity and lifetime are affected by O$_2$ concentration. A measurement is obtained when an excitation light is supplied via the fibre and the luminescence quenching of the dye is guided in the opposite direction and digitally imaged (Glud et al. 2001, Glud 2008). The quenching magnitude is then directly proportional to the O$_2$ concentration (Kühl & Polerecky 2008). Optodes, mostly in their planar configuration, were developed as a tool for 2-dimensional measurements of O$_2$ profiles in benthic communities. The 2-dimensional O$_2$ imaging allows a higher degree of spatial integration than single-point microsensor measurements, and thus planar O$_2$ optodes are seen as a powerful tool to describe spatial and temporal benthic O$_2$ distribution patterns and their dynamics (Glud et al. 2001, Glud 2008). In recent studies (Jensen et al. 2005, Frederiksen & Glud 2006), planar optodes were used for the first time to investigate O$_2$ leakage in the rhizosphere of the seagrass Zostera marina. In both cases, O$_2$ imaging revealed high spatial and temporal dynamics of the O$_2$ distribution in the rhizosphere and identified root tips as the zone where the oxic areas were more significant, and thus where most leakage occurred. One apparent drawback of planar optodes is that the measurements are conducted along an artificial wall, which represents a significant alteration of the natural sediment structure, with possible effects on O$_2$ spatial dynamics. Tapered O$_2$ micro-optodes provide 1-dimensional measurements and are seen as a possible alternative to micro-electrodes, with the advantage of not consuming O$_2$. Miller & Dunton (2007) essayed the use of micro-optodes to measure photosynthetic rates in incubated Laminaria hyperborea discs, and their results agreed with published ones obtained by established methods.

Both planar and tapered O$_2$ optodes appear as promising techniques for use in seagrass photosynthetic research. Their full potential is yet to be explored, and further studies are necessary, particularly to compare and validate O$_2$ optodes against other established methods.

Chlorophyll fluorescence

When photons within the photosynthetically active radiation (PAR) region strike the photosynthetic pigment molecules, these become excited. Their excitation energy is, during the following de-excitation, transferred towards the reaction centre’s special chlorophyll a molecules, whose electrons also become excited and are channelled to an electron acceptor molecule (Quinone A, QA). However, a significant portion of the energy released by de-excitation (or decay) back to ground-level does not enter the photochemical process and is, instead, released (or dissipated) as heat or fluorescence, i.e. the emission of photons of longer wavelength than the ones absorbed (Schreiber et al. 1998). It is the nature of an inverse relationship between chlorophyll a fluorescence and photochemistry that enables the use of the former to be used in the investigation of many aspects of the photosynthetic process.

Conventional fluorometers emit identical actinic (or photosynthesis-causing) and excitation lights. The high-wavelength fluorescent light is separated from the actinic light by filters before reaching the instrument’s detector. This allows for a strong fluorescence signal, but makes quenching analysis and quantum yield measurements difficult. In modulated fluorometers, light is emitted in a succession of alternate light–dark periods, which enables a more clear separation of the fluorescence signal from ambient light. PAM fluorometers, the more recent type, emit continuous short measuring-light pulses of red or blue light. As the fluorescence signal caused by this measuring light is captured during the very short pulse periods, external disturbances, background signals and transient artefacts are eliminated and do not mask the fluorescence signal (Schreiber et al. 1998).

In PAM fluorometers, the short pulses of measuring light induce the emission of a fluorescence signal termed $F_m$ or $F_l$ (depending on whether the plant was dark-adapted or not, respectively). When a saturating light pulse of some 0.8 s duration is applied to the plant sample, all reaction centres become reduced (or ‘closed’) and the fluorescence emission is maximal ($F_m$ or $F_m'$, again dependent on the previous dark-adaptation or not, respectively; complete notation of fluorescence terms can be found in van Kooten & Snel 1990). In the case of dark-adapted plants, the maximum pho-
tochemical efficiency of PSII is then given by $\Phi_{\text{PSII}} = F_m - F_o / F_m$ or simply $F_o / F_m$. This parameter, besides expressing the potential for PSII photochemistry, is commonly used to investigate the onset and recovery of stress situations due to its sensitivity to plant stress (Beer et al. 2001). In illuminated samples, the same measurement provides the effective quantum yield ($Y$) which is given by $Y = F_m' - F_o / F_m' = \Delta F / F_m' (Genty et al. 1989)$. When $Y$ is measured at steady-state at a known irradiance ($I$), the rate at which electrons are carried through the transport chain past Photosystem II (PSII) (electron transport rate, ETR) can be estimated, $\text{ETR} = Y \times I \times AF \times 0.5$, where AF is the absorption factor (Beer et al. 2001) and 0.5 expresses an equal distribution of the absorbed photons between PSII and PSI (Schreiber et al. 1988). Absolute ETRs are mandatory, for instance, when comparisons of ETR and gross photosynthetic O₂ evolution are made (Beer et al. 2001). Unlike terrestrial plants, where an AF value of 0.84 is often accepted as a valid value, seagrass leaves present high variability in this parameter (Durako 2007)

Chlorophyll fluorescence measurements have been applied to seagrass research from the mid-1990s. Two main types of fluorometers have been used to investigate a wide array of questions. A non-modulated or continuous excitation fluorometer (PEA, Hansatech Instruments) was used in laboratory experiments by Dawson & Dennison (1996) to assess the effects of increased ultraviolet radiation and elevated PAR on various morphological and physiological parameters of 5 Australian seagrass species. The same type of instrument was also used by Enríquez et al. (2002) to examine variations in photosynthetic activity along the leaves of *Thalassia testudinum* in the Caribbean Sea. Continuous excitation fluorometers allow for high temporal resolution measurements, an essential condition to investigate the kinetics of the Kautsky induction curve (Buschmann & Lichtenthaler 1988, Enríquez et al. 2002). Given that this kind of fluorometer does not allow the separation of fluorescence emission from ambient light, measurements can only be performed in dark-adapted samples, which restricts their usefulness to high quality $F_o / F_m$ determinations and the above-mentioned induction kinetics analysis.

Most research involving fluorescence determination of seagrasses has been conducted using PAM fluorometers, mainly those produced by Walz. This instrument allows measurements to be conducted in full sunlight, thanks to a special emitter-detector unit that separates the fluorescence signal from ambient light (Schreiber et al. 1988). Most PAM fluorometers are portable and one model, the Diving-PAM, is adapted for underwater operation. The coupling of these characteristics opened the way for autonomous measurements of effective quantum yields on plants exposed to natural conditions. Novel, automated, multi-channel chlorophyll fluorometers, first described by Runcie & Riddle (2004) and able to withstand prolonged deployments, were used to investigate light acclimation processes of *Halophila stipulacea* (Runcie et al. 2009, this Theme Section) during the recent 8th Group for Aquatic Productivity (GAP) workshop. Those instruments allow autonomous measurements to be conducted for longer periods (>24 h), opening new and exciting possibilities in seagrass photosynthetic research. Whether in the field or in laboratory experiments, PAM fluorescence has been used in the evaluation of time- or space-related variations in photosynthesis, with insights on the dynamic behaviour of the photosynthetic apparatus of several seagrass species (Silva & Santos 2003). *P-E* curves can be obtained by PAM fluorescence when $Y$ is determined along an irradiance gradient. If this gradient is imposed by the fluorometer’s own actinic light source, usually only short (up to minutes) light steps are allowed, due to instrument limitations. The resulting curves are named rapid light curves (RLCs) due to this feature, and must be interpreted differently from conventional light response curves, although they are graphically similar. The critical difference between RLCs and conventional *P-E* curves is that the ETRs used to draw the curves are not measured at steady-state conditions, given the short exposure at each light step (Ralph & Gademann 2005 and references therein). Still, RLCs provide important ecophysiological information, namely through the interpretation of curve defining parameters, similar to those of conventional light response curves (Beer et al. 1998, Ralph & Gademann 2005, Saroussi & Beer 2007).

Conventional light response curves can be determined in nature using chlorophyll fluorescence, as long as an external light source supplies more extended periods of illumination at each light step. This type of curve has been obtained mainly with the goal of comparing ETRs with gross O₂ production in the search for an expedient and field-going methodology to estimate photosynthetic rates. In these experiments, O₂ production and ETRs are simultaneously measured at several irradiances, always at steady state. Considering that 4 mol of electrons are carried through the transport chain for each mol of O₂ produced, there is a theoretical molar ratio of 0.25
between O₂ production and ETR (Walker 1987). In order for ETR measurements to be used as proxies for estimations of actual photosynthetic production, 2 basic conditions must be met: both a linear relationship between O₂ production and ETR, and a 0.25 O₂/ETR molar ratio must be observed, preferably along a comprehensive range of light levels (Silva & Santos 2004). Beer et al. (1998) published the first comparison between photosynthetic O₂ evolution and ETRs in sea-grasses. The authors examined the O₂/ETR relationship in Cymodocea nodosa, Halophila stipulacea and Zostera marina and found molar values of 0.3, 0.12 and 0.5, respectively, although only C. nodosa presented a clearly linear relationship between the evolved O₂ and the transported electrons. Beer & Björk (2000) observed a linear relationship and a ratio of 0.28 in Halophila ovalis, and a contrasting curvilinear relationship and a ratio of 0.57 in Halodule wrightii. Silva & Santos (2004) obtained a linear relationship and a ratio of 0.15 in Zostera noltii. Enriquez & Rodriguez-Román (2006) verified a linear relationship and a ratio of 0.25 for Thalassia testudinum, but only for irradiances <170 µmol m⁻²s⁻¹. Common to all these studies are: (1) the observation of quite disparate molar ratios, many of them far from the theoretical stoichiometry and ranging from 0.12 to 0.57, (2) the recording of different initial slopes of ETR and O₂ response curves, and (3) the fact that ETR tends to saturate at higher irradiances than O₂ evolution. Putative explanations for problems concerning ETR calculations are electron-cycling around PSI and non-photochemical quenching in PSI reaction centres at saturating light levels (Franklin & Badger 2001). Uncertainties in O₂ evolution may be related to photorespiration, which can be an important O₂ sink (e.g. Flexas & Medrano 2002 and references therein), or the Mehler reaction, in which O₂ is photo-reduced at PSI with the production of superoxide radicals (Asada 1999). The Mehler reaction alone can represent up to 10% of the ETR (Makino et al. 2002), corresponding to a similar deviation in the theoretical stoichiometry of the photosynthetic process. The conversion of net to gross photosynthesis is also likely to introduce additional errors (discussed in Silva & Santos 2004), depending on how dark respiration is estimated.

One of the most common applications of PAM fluorescence in seagrass research has been the study of temporal and spatial variation in photosynthesis patterns. In a set of laboratory experiments, Ralph (1996) examined the effects of daily irradiance patterns in the photosynthetic activity of Halophila ovalis, comparing laboratory-grown plants with wild ones, and observed very different patterns among the 2 groups in response to different light regimes. Since these experiments, and taking advantage of the portability and water resistance of the new Diving-PAM, most of the studies addressing similar questions have been conducted in situ, either in the intertidal area or underwater, using SCUBA. Daily variation in seagrass photosynthesis, as influenced by ambient irradiance, has been investigated in situ for Zostera noltii and Cymodocea nodosa in southern Europe (Silva & Santos 2003), Posidonia australis in Australia (Runcie & Durako 2004), Halophila stipulacea in the Red Sea (Sharon & Beer 2008) and Thalassia testudinum in Florida (Belshe et al. 2008). Spatial variation in photosynthetic activity has also been investigated, at scales ranging from within- to among-shoot variability up to landscape-level patterns. Ralph & Gademann (1999) measured Fv/Fm ratios along the leaves of Posidonia australis while assessing the effect of epiphytes on the plants’ photosynthesis. Durako & Kunzelman (2002) evaluated the variability of photosynthesis within the shoots of T. testudinum in Florida, and went further, examining differences between shoots from healthy and die-off patches and looking at temporal variation among ca. 300 sampling stations distributed among several basins. Turner & Schwarz (2006) conducted a similar study on Z. capricorni from New Zealand. Cayabyab & Enriquez (2007) evaluated the photoacclimatory responses of T. testudinum plants to 3 distinct light treatments, comparing the daily variation in photochemical efficiency between basal and apical leaf sections.

Light availability is often regarded as the most important single parameter controlling seagrass distribution. Responses to light have been widely explored by many authors and from a number of different perspectives. A few studies have particularly addressed the relationships between photosynthesis and both depth- and turbidity-related light attenuation. Using the Diving-PAM, Ralph et al. (1998) measured the in situ photosynthetic responses of Posidonia australis, P. sinuosa, Amphibolis antarctica, A. griffithii and Halophila ovalis from shallow Australian waters (0 to 6 m), comparing them with laboratory measurements of the deeper growing P. angustifolia (27 m) and Thalassodendron pachyrhizus (46 m). Despite the use of 2 contrasting experimental approaches, the authors found significantly different patterns of photosynthetic behavior between shallow and deeper growing seagrasses. Schwarz & Hellblom (2002) measured the photosynthetic light responses of Halophila stipulacea growing at different depths in the Red Sea. They found patterns of acclimation to distinct light environments, even though they only sampled a small depth range (7 to 30 m from a known 0 to 70 m total range). The same approach was used by Durako et al. (2003) to compare the photobiology of Halophila johnsonii and H. decipiens along a depth gradient in Florida, with the goal of explaining their relative depth distributions. Additionally, Collier et al. (2008) used Diving-PAM measurements to com-
plement their physiological characterization of Australian *Posidonia sinuosa* along a very small vertical gradient down to 9 m depth. Comparing a broader spectrum of irradiance controlling factors, Campbell et al. (2003, 2007) used PAM fluorescence to evaluate the combination of light with other environmental factors on the distribution of several tropical seagrass species, comparing 4 major Australian habitats (estuarine, coastal, reef and deepwater).

Chlorophyll fluorescence has provided detailed descriptions of variation in seagrass photosynthetic performance at a number of spatial and temporal scales, essentially through the analysis of the quantum efficiency fluctuations. Photosynthetic responses to light have also been elucidated across a wide range of natural conditions of light quantity and quality.

PAM fluorescence has also been used to investigate inorganic carbon limitations to seagrass photosynthesis. Schwarz et al. (2000) used a Diving-PAM connected to a small chamber to evaluate in situ the inorganic carbon limitation to *Halophila ovalis* and *Cymodocea serrulata* photosynthesis. The underwater operating chamber was used to hold and isolate still-attached leaves, while permitting the addition of inorganic carbon, buffers or inhibitors to the leaf-adjacent medium. PAM fluorescence was also used by Enríquez & Rodríguez-Román (2006) to analyze the photosynthetic response of *Thalassia testudinum* to carbon limitation, as influenced by water flow, in a set of laboratory experiments.

Chlorophyll fluorescence has proven to be a very useful tool in assessing the onset and recovery of numerous types of stress on seagrasses. As discussed above, the potential quantum yield of PSII, $F_v/F_m$, is a very useful indicator of stress conditions, and therefore is widely used in studies addressing a wide variety of stress situations. Desiccation is a major stressor in intertidal seagrasses. Hanelt et al. (1994) investigated low-tide stress in *Thalassia hemprichii* in South China and observed a midday depression in $F_v/F_m$, particularly when low tide occurred around solar noon, when desiccation effects were enhanced by high irradiances. The desiccation tolerance of several tropical intertidal seagrasses was evaluated by Björk et al. (1999) in Zanzibar. Interestingly, in that study, shallow intertidal species were overall more resistant to desiccation than deeper growing ones, leading the authors to conclude that desiccation tolerance is not the key factor in determining the vertical positioning of seagrass species in the intertidal zone, but rather their ability to withstand high irradiances. In a subsequent investigation in Zanzibar, Beer et al. (2006) compared the photosynthetic responses of *H. ovalis*, *Cymodocea rotundata* and *Thalassia hemprichii* in low-tide conditions, to find that the first species can only survive in monospecific tidal pools, given that the presence of the other 2 plants raises the pH above its compensation point.

The combined effects of desiccation and high temperatures were compared in Australian *Posidonia australis* and *Amphibolis antarctica* by Seddon & Cheshire (2001). *Thalassia hemprichii* and *Halodule uninervis* from Taiwan were also compared by Lan et al. (2005) regarding their relative capacity to deal with both high irradiances and air exposure. The stress responses to high irradiance regimes have been mostly investigated in laboratory experiments, under well-controlled conditions, namely for *Halophila ovalis* (Ralph & Burchett 1995, Ralph 1999a) and *Zostera marina* (Ralph et al. 2002). Figueroa et al. (2002) and Kunzelman et al. (2005) approached UV radiation stress by evaluating the responses of *Posidonia oceanica* and *Halophila johnsonii* to combinations of PAR with UV-A and/or UV-B, respectively. Other types of stress, whose effects have been evaluated through the PAM fluorescence technique, include thermal stress (*Halophila ovalis*, Ralph 1998; *H. ovalis*, Zostera capricornis, *Syringodium isoetifolium*, Campbell et al. 2006), osmotic stress (*H. ovalis*, Ralph 1998), herbicide toxicity (*H. ovalis*, *Z. capricornis*, *Cymodocea serrulata*, Haynes et al. 2000; *H. ovalis*, Ralph 2000), ammonium toxicity (Brun et al. 2008) or even a combination of factors such as temperature, light and salinity (Ralph 1999b).

Overall, PAM fluorometry has the advantage of being quick and non-intrusive, and since no enclosures are needed, it is particularly suited for *in situ* measurements. On the other hand, since respiration is ignored in the measurements (as only photosynthesis *per se* is measured), it is often impossible, or at least difficult, to compare such measurements with growth rates. Indeed, the most suitable questions to ask using PAM fluorometry are those concerning the photosynthetic light responses to the whole range of ambient parameters.

### Dissolved inorganic carbon uptake and CO$_2$ fluxes

Gas molecules, with the exception of those composed by 2 identical atoms, absorb radiation at extremely narrow bandwidths within the infrared region of the spectrum. CO$_2$ has its main absorption peak at $\lambda = 4.25$ µm, with 3 secondary peaks at 2.66, 2.77 and 14.99 µm (Long & Hallgren 1985). Infrared gas analysis (IRGA) has therefore long been used to measure, with high accuracy, the evolution of CO$_2$ exchanged in either the photosynthetic or respiratory process in terrestrial plants. A number of laboratory and portable gas analyzers are available, with varying configurations, from closed to semi-closed or even open systems, depending on the type of enclosure and air pathway.
(Long & Hallgren 1985). Compared to the previously discussed methodologies, direct assessments of DIC uptake or CO2 flux measurements have rarely been used to estimate seagrass productivity; 4 distinct types of approaches have been described.

(1) Direct measurement of CO2 uptake by individual leaves, which are enclosed in a mini-cuvette with temperature and humidity control coupled to a portable infrared gas analyzer. Given the considerable extensions of intertidal seagrass meadows worldwide, and the array of information likely to be obtained in situ by these gas-exchange instruments (to which PAM fluorometry can also be coupled), it is surprising that only a few studies have used this approach (Leuschner & Rees 1993, Leuschner et al. 1998, both with Zostera marina and Z. noltii). These highly controlled and accurate systems are, however, limited to intertidal habitats, as no adaptations are yet made to operate them underwater.

(2) DIC uptake at the community level has been mostly investigated using benthic incubations in closed chambers, where water is retained for a few hours. Initial and final DIC values are derived from variations in pH and alkalinity. As long incubation times are required for pH and alkalinity variations to occur, photosynthesis tends to saturate as Rubisco carboxylase activity decreases in response to the increasing O2 and decreasing CO2 concentrations within the chambers. No assessment has yet been made of how underestimated the carbon uptake measurements may be when using this technique. Using both transparent and dark chambers, the net community production and dark respiration are measured, providing an overall gross community production value. Studies based on this approach have been conducted on Zostera marina (Ibarra-Obando et al. 2004, Martin et al. 2005) and Posidonia oceanica communities (Barrón et al. 2006), complemented with O2 measurements.

(3) Silva et al. (2005, 2008) proposed a variation to the benthic incubation method by introducing water recirculation in the chambers and reducing the length of the incubations to minutes instead of hours. In this system, water from the chambers is recirculated by a peristaltic pump at the surface and flows through an equilibrator, which allows the partial pressure of CO2 (pCO2) to be continuously monitored in the gas phase by an infrared gas analyzer. It is important that pH and alkalinity are measured before and after the incubation period, so as to provide an estimate of the total DIC flux. This method may also underestimate seagrass community production as CO2 uptake is being regenerated through carbonate equilibrium dynamics. Further discussion on the technical and analytical aspects of this method can be found in Abril (2009) and Silva & Santos (2009).

(4) Air–sea CO2 fluxes have also been used to estimate the net community production of seagrass-dominated coastal systems (Gazeau et al. 2005b). The air–sea CO2 flux is computed from the air–sea gradient of pCO2, the gas transfer velocity and the solubility coefficient of CO2. These fluxes can be determined either by following pCO2 evolution inside a floating bell system (Frankignoulle & Distèche 1984) or by simultaneous measurements of both air and water pCO2 (Gazeau et al. 2005a, 2005b).

Other methods

Radioactively labelled carbon (14CO2 or H14CO3-) was one of the first (Beer et al. 2001) methods to determine CO2 uptake. The 14C technique has mostly been used to investigate the mechanisms of carbon uptake in seagrasses (Beer & Waisel 1979, Abel 1984) and its productivity (Williams & McRoy 1982), but nowadays it is only rarely used.

New methodological possibilities are emerging, particularly those addressing large-scale evaluations of seagrass community production. Hermand et al. (2001) and Hermand (2006) summarized the applicability of geo-acoustic inversion techniques to monitor photosynthetic O2 release in Posidonia oceanica meadows. The working principle, verified in this technique, was that the presence of non-dissolved gases in the leaves’ aerenchyma and the production of O2 microbubbles sticking to the blade surface interfere with the impulse response of broad-band acoustic transmissions and can be correlated with whole-meadow photosynthetic activity. This method may provide continuous measurements of oxygen produced by seagrasses, and may be useful in monitoring seagrass productivity in large coastal areas. However, more developments of this technique, including calibration with more established methods and extrapolation to different seagrass species, are necessary.

The eddy correlation technique, used in terrestrial ecosystems, was adapted to aquatic environments by Berg et al. (2003). This technique determines the sediment–water fluxes of dissolved O2. The general operating principle lies in the simultaneous measurement of vertical water column velocity and O2 concentration at a point a given distance from the sediment surface (Berg & Huettel 2008). This is a completely non-intrusive approach, capable of integrating considerable sediment areas (Berg et al. 2007). Although the technique’s full potential is yet to be explored, it appears very promising, particularly in heterogeneous environments where its integrating capacity is an advantage. If used above seagrass meadows, this method may provide whole-ecosystem metabolism
information. The first attempt to determine oxygen fluxes and ecosystem metabolism in seagrasses was made by Hume et al. (unpubl. data) in a Zostera marina community.

CONCLUSIONS

Table 1 summarizes the applications, advantages and disadvantages of the methods presented above to measure seagrass photosynthesis and production. In roughly 3 decades of research on seagrass photosynthesis, much information has been gained at different levels through the use of the various methodologies described here. O₂ measurements, either by Winkler titration or by electrodes, provide accurate rates of net photosynthetic gas exchange in the light and respiration in darkness. However, limitations include the need to use enclosures which, invariably, alter natural irradiances and water flow conditions. O₂ electrodes are also usually employed in the laboratory, with obvious limitations related to the removal of plants from their natural environments. Measurements of CO₂ employing IRGA are much more sensitive than O₂ measurements and thus require lower incubation times. While the IRGA technique is probably the best for measuring rates of intertidal seagrasses exposed to the air, its recent adaptation for measuring dissolved CO₂ fluxes of underwater communities may underestimate them. Currently, the most employed method for in situ photosynthetic measurements is PAM fluorometry. It is fast (a measure of ETR can be obtained within 1 s), and no enclosures are necessary. One major restriction is that respiration rates cannot be obtained by PAM fluorometry, and photosynthetic rates thus cannot be readily converted to production rates. However, this method is superb for investigating the stresses that impede photosynthesis (by measuring \( F_s/F_m \)) as well as for estimating photosynthetic responses to irradiance (including the use of RLCs). In the future, it is desirable that autonomous instruments that measure both chlorophyll fluorescence and CO₂ gas exchange become available, so that many of the relevant seagrass photosynthetic parameters can be measured simultaneously over extended time periods.

Being rooted plants, seagrasses present the most complex physical structure among marine autotrophs, as plants simultaneously occupy 2 distinct environments, the water column and the sediment. The integrated physiology of above- and below-ground tissues adds a considerable degree of complexity to metabolic studies, namely in the accurate determination of CO₂ fixation and O₂ production rates. In fact, although it may represent more than half of the whole-plant O₂ consumption (Fourqurean & Zieman 1991), root-rhizome respiration is rather difficult to measure in situ, and highly unrealistic if measured in laboratory conditions. On the other hand, a significant portion of the photosynthetically evolved O₂ is conveyed down from the leaves to the below-ground tissues in order to support respiration and also to maintain some degree of aerobiosis in the rhizosphere, which is often surrounded by anoxic sediment (Larkum et al. 1989). In this context, despite several laboratory studies (Smith et al. 1984, Connell et al. 1999), it was Greve et al. (2003) and Binzer et al. (2005) who firstly provided accurate in situ measurements of O₂ leakage by sea-grass roots and rhizomes, using O₂ micro-electrodes. O₂ optodes (Glud 2008) added a second dimension to these measurements. Whereas respiration measurements at the plant level are fairly simple in the laboratory, their extension to the community level has low value. On the other hand, community measurements still present a great number of uncertainty factors (outlined in Middelburg et al. 2005). One of the paths to explore further in seagrass photosynthetic production research is the assessment of the metabolic contribution of other autotrophic and heterotrophic components of seagrass communities. Process discrimination and separate metabolic evaluations are essential, in particular sediment respiration, to avoid considering the sediment as just a black box within the community. This will help obtain accurate metabolic budgets for seagrass communities, thus closing the gap between gross and net primary production.

This review of the published literature on seagrass photosynthesis, although oriented to methodological aspects, also provided an assessment of the status of seagrass photosynthesis research. We agree with a recent review of the impact of light limitation on seagrasses (Ralph et al. 2007): although a great deal is known on seagrass ecophysiology, much information is still missing, including many fundamental photo-biological data. Our analysis of the literature revealed, for example, that even though seagrasses have a wide depth distribution gradient, down to 50–70 m (den Hartog 1970), hardly any work has been done on deep populations. This is a major gap in knowledge, especially given the importance of understanding the biology of plants living in extreme environments and taking into account that seagrass declines worldwide are attributed largely to reductions in light availability (Ralph et al. 2007 and references therein). The availability of underwater instruments such as the Diving-PAM, and the fact that such deep populations are within the depth range for technical diving, provides an opportunity for narrowing these gaps in knowledge.

With the development of the new large-scale assessment techniques described above, research questions
Table 1. Applications, advantages and disadvantages of the most common methods used in seagrass photosynthesis and community metabolism studies

<table>
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<th>Method</th>
<th>Applications</th>
<th>Advantages</th>
<th>Disadvantages</th>
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| O₂ titration (Winkler)              | • Photosynthesis and dark respiration of whole plants or leaf cuts incubated in bottles (laboratory or in situ)  
• O₂ analysis of water samples from benthic chambers (field) | • High accuracy  
• Low price | • Intrusive (if plants are incubated in bottles)  
• Problems related to containment in closed chambers  
• Initial and final O₂ concentrations only  
• Cumbersome |
| O₂ electrodes coupled to small reaction chambers | • Photosynthesis and dark respiration of leaf cuts (laboratory) | • High resolution  
• High accuracy  
• Continuous O₂ measurements  
• Highly controlled conditions  
• Possibility to manipulate the incubation medium | • Intrusive  
• Highly artificial  
• Spectral quality of artificial light sources |
| O₂ microelectrodes                  | • O₂ consumption by below-ground tissues (in situ)  
• O₂ leakage into the rhizo-sphere (in situ)  
• O₂ production or consumption in custom-made chambers  
• Used in the eddy correlation technique (in situ) | • Not very intrusive (small diameter electrodes)  
• Fast response time  
• Positioning possibilities | • Small spatial resolution  
• Fragile in field conditions |
| O₂ optodes                          | • O₂ mapping of seagrass rhizosphere (in situ) | • Not very intrusive  
• 2-dimensional measurements  
• Very sensitive at low O₂ concentrations  
• Does not consume O₂  
• Long-term stability | • Slower response than microelectrodes  
• Technique still under development |
| PAM fluorescence                    | • In situ and laboratory measurements of photosynthetic efficiency at the plant level | • Non-intrusive  
• Portability  
• Autonomous underwater equipment  
• Possibility of continuous measurements | • Measures light reactions only  
• Does not allow respiration measurements or thus production estimates |
| CO₂ evolution                       | • In situ measurements of community uptake and release of CO₂, in incubation chambers | • Non-intrusive  
• Integration of whole-community metabolism  
• Highly reliable in air-exposed conditions | • Possibility of underestimating CO₂ uptake in underwater conditions  
• Problems related to containment in closed chambers |
| Geo-acoustics                       | • In situ large-scale estimation of community O₂ production | • Large-scale application, suitable for ecosystem level studies  
• Continuous measurements | • Underdeveloped technique |
| Eddy correlation                    | • In situ sediment–water fluxes of dissolved O₂  
• Community level O₂ fluxes metabolic studies | • Non-intrusive  
• Autonomous underwater equipment  
• Good surface integrating capacity  
• Continuous measurements | • Underdeveloped technique |
pertaining to seagrass meadows as global CO₂ sinks may be answered in the coming years. The effects of global warming on seagrass metabolism also require further research, particularly concerning the effects of respiration. Additionally, ocean acidification with the concomitant CO₂ increase is expected to positively affect seagrass photosynthetic rates, but experimental studies are scarce (Palacios & Zimmermann 2007). Another interesting aspect to explore related to ocean acidification is the interaction between seagrass photosynthesis and the calcification rates of other organisms within the community (e.g. Semesi et al. 2009). We thus expect future research to bridge the gap between the photosynthetic behaviour of individual plants and the various aspects of community-level metabolism.

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Discussion

One of *Halophila stipulacea*’s outstanding features is its wide depth distribution. In the summer time, its plant meadows can extend from the intertidal down to a depth of ~50 m. Along this gradient, the irradiance changes from high levels of PAR and UV radiation in shallow water to very dim and spectrally blue-shifted light at depth. In order to grow under such differences in irradiance, we hypothesised that *Halophila stipulacea* must use mechanisms in its light utilization and, accordingly, in its photosynthetic apparatus that are adapted to the specific conditions at which it grows.

In shallow waters, the ability to withstand high irradiances may be facilitated by the ability of the plant's chloroplasts to move into clumps during the day and the apparently efficient mechanism for non-photochemical quenching (Chapter 1). While chloroplast movement was previously described for *Halophila stipulacea* in drawings (Drew, 1979), the present PhD work (Chapter 1) shows for the first time this phenomenon in photographs, as well as its diurnal oscillations in high-light grown plants. We observed that clumping of chloroplasts occurred only in plants growing in shallow water, and the importance of such self-shading of the chloroplasts should be seen especially against the background that the Gulf of Aqaba-area of the Red Sea may experience one of the highest irradiances on earth on a yearly basis (Winters et al. 2003).

In addition to the obvious photoprotective role of chloroplasts movements, the latter was also shown to have an effect on photosynthetic parameters. Midday chloroplast clumping decreased the light absorbed by the plant leaves and, thus, decrease the co-called absorption factor (AF) and, accordingly, influenced the calculation of electron transport rates (ETR). When using PAM fluorometry and using ETR as an estimator for photosynthetic rates we therefore suggested the use of a corrected AF, as a result of changes in leaf absorption: The latter changes diurnally in shallow-growing plants and affects the calculation of
photosynthetic rates differently along the day depending on the degree of chloroplast clumping at a certain time of the day, and we devise a practical way of taking this into account when calculating such rates (Chapter 1).

In addition to high photosynthetically active radiation (PAR, 400-700 nm), coastal waters, especially clear tropical waters such as in the Red Sea, also feature high levels of UV radiation (UVR, 280-400 nm) in their upper layers (Chapter 4). It was found that chloroplast movements that cause their midday clumping under high-PAR conditions was present only in plants acclimated on a water-table to control-filters that allowed UV to pass through them: The clumping phenomenon was absent in plants that were protected from UV by UV-filters but exposed to high PAR levels. This strongly indicated that UVR (rather than PAR), or UVR in the presence of PAR, was the trigger of chloroplast movements in Halophila stipulacea. As mentioned previously, the surface irradiance characteristics of the studied region (northern Red Sea) are among the highest in the world (Winters et al. 2003). We found high UVR doses similar to those on the shaded water table also in situ at 6 m depth (where >50% of surface-UV doses remained). Indeed, 6-m growing plants featured midday chloroplast clumping as verified by us (Chapters 2 and 4). Thus, apparently, the successful adaptation of this plant to shallow waters (<6 m at the sites studied) may be partly based on the ability of its chloroplasts to shade one another (and perhaps the nucleus too) in order to protect them from high irradiances and/or UVR.

While the reduction in midday ETRs were largely an indirect effect of UVR via the decrease leaf absorption caused by the clumping of chloroplasts, thus causing a lower AF, UVR also affected photosynthesis directly by reducing quantum yields. The daily reduction in effective quantum yield ($\Delta F/Fm'$) can be seen as a dynamic form of photoinhibition (or down-regulation of PSII) since values in the evening returned to morning values. One mechanism that can explain this midday reduction in effective quantum yields is the operation of the
xanthophyll cycle, in which excess light energy is dissipated as heat (Demmig-Adams and Adams 2006), as indeed shown in Chapters 1 and 2. Thus, as part of the more general negative effects of UVR on phytoplankton (Agusti and Liabres 2007) and other marine photosynthetic organisms (Sinha et al. 1998), including seagrasses (Meng et al. 2008), the photosynthetic apparatus of some seagrasses is also influenced by UVR. Logically, this radiation activates mechanisms such as chloroplast clumping and a dynamic down-regulation of photosystem (PS) II that protect at least *Halophila stipulacea* against it.

PAM fluorometry has been suggested as a method to measure photochemical and non-photochemical rates back in 1986 (Schreiber et al. 1986). Since then, an in situ approach (via the DivingPAM, Walz, Germany) to study marine photosynthesis in macrophytes was established and tested also for seagrass photosynthesis (Beer et al. 1998, Ralph et al. 1998). Nowadays, in situ PAM fluorometry is widely use as a robust method to study seagrass photosynthesis and metabolism (reviewed by Silva et al. 2009). While quantifying the quantum yield using PAM fluorometry is under broad agreement among researchers, the calculation of true ETR values is controversial (Evans 2009, Dan Tchernov, personal communications). Indeed there are several challenges and potential errors when calculating ETRs, especially when failing to measure accurate AF and FII values. The current work suggested a few methods and applications to overcome these errors (Sharon and Beer 2008, Sharon et al. 2011; Chapters 1 and 5). (It should be mentioned that another possible inaccuracy when using fluorescence to study photosynthesis is the possibility of electron radiation from PSII [while Walz have developed the PAM 2000 which should overcome this problem, there still isn't a submergible device available on the market]).

In addition to the need of using correct parameters to calculate ETRs, it is recommended, in some cases, to test whether the studied organism (*Halophila stipulacea* in our study) is showing reasonable correlation between calculated ETRs and oxygen evolution
(Beer et al. 1998, Dan Tchernov, personal communications). This is a challenging fundamental question since oxygen evolution tests net photosynthesis while measuring quantum yields and calculating ETR examines gross photosynthesis (this could be amended partly by calculating and subtracting dark-respiration). Nevertheless, this correlation was examined for several seagrass species and PAM fluorometry was suggested as a robust method to assess photochemical study in *Halophila stipulacea* after gaining a strong correlation coefficient when using a third degree polynomial function ($r^2=0.95$, Beer et al. 1998) when comparing oxygen evolution to ETR calculations. Initially, it was suggested that photorespiration was the cause of the oxygen evolution deviation from linearity for *Halophila stipulacea* at high irradiances. In addition, the authors explain the correlation deviation by 1) an experimental flaw causing different PPF (photosynthetic photon flux) reaching the plants leafs when using both methods and 2) differences in the calculation of AF's.

In all, the molar ration of O$_2$/ETR in *Halophila stipulacea* is close to the theoretical 0.25, and it was concluded that the Diving-PAM is suitable for measurements of photosynthetic ETRs in seagrasses (Beer et al. 1998). Nevertheless, there is a need to expand our knowledge in gaining a more exact O$_2$/ETR values in future research. Evans (2009) questions the use of fluorescence for ETR determinations since several fluorescence signals are emitted from chlorophyll in the multilayer leave which might cause false measurements. We believe that future fluorescence studies can contribute and improve our understating while still, for in-situ seagrass photosynthesis research, PAM fluorometry is a favourable method.

Deep-growing plants of the seagrass *Halophila stipulacea* received at their depth limit in the summer (~50 m) a down-welling PAR of ~5% of that at the surface, UVR was virtually absent and the spectrum narrowed to blue-green light. At this depth chloroplast clumping is not induced and the chloroplasts are evenly dispersed in the cytoplasm volume to maximize light absorption along the day. In addition to wider and longer leafs at depth
(Schwartz and Hellblom 2002), the current PhD work found some biochemical and physiological adaptations that may explain the successful growth of *Halophila stipulacea* under dim irradiance at its lower growth limit as follows:

Changes in Chl concentration are a known response of photosynthetic organisms, including seagrasses, in their acclimation to variations in the light environment. Here (Chapter 5), it was found that in deep-growing *Halophila stipulacea* leaves both Chl a and b were increased, as was also previously reported in our studies for this species (Chapter 1, 3 and 4), as well as for other seagrasses (Wiginton and McMillan 1979, Dennison 1990, Abal et al. 1994). However, unlike in most other studies, the ratio of chlorophyll *a:b* did not change between the different depths (1 and 48 m), and therefore cannot explain the adaptive mechanism that enables *Halophila stipulacea* to grow under very dim irradiances.

Unlike for the chlorophyll *a:b* ratios, there was a marked change of almost three times in PSI : PSII ratio in the deep-growing *Halophila stipulacea* plants. This change was a result of changes in the PSI content (Chapter 5), similar to previous studies in various photosynthetic organisms (Fujita 1997, Yamazaki et al. 2005, Levitan et al. 2011). Yamazaki et al. (2005) suggested that the higher ratios of PSI:PSII may be derived from the need for a higher ATP:NADPH ratio, which can be obtained by cyclic electron flow around PSI, increasing ADP phosphorylation around the cytochrome b6/f complex. In addition we found, similarly to those authors, a higher level of fluorescence emission in PSI than is PSII after excitation as measured by 77K fluorescence. The high PSI:PSII ratio found here for *Halophila stipulacea* growing at its depth limit, together with the higher functionality of PSI as indicated by the 77K fluorescence emission, thus suggest an 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 how the favour of a higher ATP:NADPH production provides and adaptive advantage for extremely deep-growing *Halophila stipulacea* plants.

The increase in Rubisco in shallow-growing *Halophila stipulacea* plants correlates with the higher photosynthetic ETR under high-light conditions. 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 on extremely deep-growing plants (Chapter 5) is to emphasize the need of using correct FII (the probability of a photon to be absorbed by PSII) values when calculating ETRs, especially for plants featuring a wide depth-distribution pattern such as exemplified for *Halophila stipulacea*. When using FII values based on our protein analysis (both protein concentration and functionality), the maximum photosynthetic rate (Pmax) is higher in the shallow-growing plants as compared when calculated using the commonly accepted value of 0.5. However, the initial slope ($\alpha$) of the rapid light-curve (RLC) 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. Based on our finding from Chapter 5, we conclude that one should be cautious when using 0.5 as the FII value as 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.

Once we found and explained different physiological mechanisms enabling *Halophila stipulacea* to grow under high light and UV radiation in shallow waters and under dim environments at depth, we were curious to question whether these traits are plastic or evolve from two ecotypes at the two depths. Our results indicate that *Halophila stipulacea* is a highly
plastic seagrass that can acclimate to various light environments quickly (Chapter 3). This was shown before for plants grown under high-light conditions, where chloroplast movements were suggested as a means to both capture photons under diurnal low-light periods (when chloroplasts are dispersed) and apparently protect the leaves from photodamage during midday (by clumping the chloroplasts, Chapter 1). Later we showed that the photosynthetic apparatus also features plasticity and can acclimate to various irradiances along a depth gradient according to classical adaptation strategies of high- and low-light plants (e.g. both chlorophyll contents and $P_{\text{max}}$ changed according to dogma within a week). Further, this plasticity is in line with the thought that specimens of *Halophila stipulacea* growing at various depths in a meadow are not ecotypes, although the maximal irradiance during midday spans at least one order of magnitude.

The apparent lack of ecotype formation at various depths within a *Halophila stipulacea* meadow may stem from its largely vegetative growth pattern (through rhizome elongation) resulting in large areas belonging to the same clone. Further, we have observed that the vertical extension of the meadow differs between seasons. During the summer, the studied meadow extends from 5 m to a depth of 50 m, while during the winter the meadow’s lower limit only extends to ~30 m. This may be due to irradiance and/or temperature; the lowest irradiance in the Gulf of Aqaba is in January and the lowest temperature in March (Winters et al. 2006).

Chapter 3 suggests that the plasticity in the response to irradiance levels would allow for the successful acclimation to the different depths as observed throughout the year. This would then explain the dynamic seasonal meadow extensions as based on clonal growth, although *Halophila stipulacea* meadows were found to be highly polymorphic between depths in the Mediterranean (Procaccini et al. 1999). On the cellular level, the fast (within 6 d) adjustment of $P_{\text{max}}$ to irradiance points towards rapid changes in the carbon fixation
reactions during the acclimation to high or low midday irradiances. These changes could involve key enzymes of the Calvin cycle, e.g. Rubisco, or other reactions following those of the primary photochemistry. The fact that Pmax values in the transplanted seagrasses became similar to those of the plants growing originally at the transplant depths shows that these acclimations are not only qualitative but also quantitative. In summary, it is proposed that the plant’s adaptive abilities to change its photosystem compositions, light utilisation, pigment accumulation and carboxylation rates may be involve also in its acclimation process to changed light environments as shown in Chapter 3. While chl a + b content also showed plasticity, the response in the plants transplanted from the high- to the low-light environment was slower than in the low- to high-light transplants, indicating that a longer time is required for the production of new chlorophyll than its loss. In addition, it is possible that maintaining a lower concentration of light-absorbing pigments in shallow water may decrease the potential for photodamage or/and the damage from reactive oxygen species.

The lack of changes in chlorophyll a:b ratios in all treatments of the transplantation experiment, together with the finding from Chapter 5 (for both shallow- and deep-growing plants), suggests that this parameter has no role in *in situ* acclimation processes, in contrast to what was reported by Durako et al. (2003) for 2 other *Halophila* species. While terrestrial shade-adapted plants often increase chlorophyll b contents relative to chlorophyll a, it may be that the spectral changes with depth prevent such a change in *Halophila stipulacea*.

For *Halophila stipulacea*, some previous studies have reported changes in light utilisation, through pigments concentration and leafs size, yet, in neither case was it verified whether those changes could be due to acclimation processes or were the outcome of longer-term adaptations possibly resulting in ecotype formations. Olesen et al. (2002) have suggested that some seagrasses (i.e. *Cymodocea nodosa*) can acclimate and respond differently to changing light conditions than others (i.e. *Posidonia oceanica*). While *Cymodocea nodosa*
displayed reduced light requirements with depth, both species acclimated mainly at the population structure level by reducing self-shading in deeper waters.

This is the first work reporting that *Halophila stipulacea*’s wide depth distribution across a broad range of irradiances (from ~2000 to <100 μmol photons m⁻² s⁻¹) can be explained by its plastic photosynthetic abilities allowing for successful acclimation both spatially and seasonally. These plastic abilities include chloroplasts movements, changes in light absorbing pigments, photosystems composition and functionality and Rubisco concentration. Still, there remain some unanswered issues, like the energetic advantage of favouring ATP vs. NADPH production and other accessories pigments’ involvements in the plant’s light utilisation under extreme light environments.
4. Conclusions

The present research provided several novel findings regarding *Halophila stipulacea*'s adaptations and accordingly acclimations to the wide light gradient in which it grows:

(i) We found a rationale for the observation of diurnal chloroplast movements in high-light growing *Halophila stipulacea* plants;

(ii) It was shown that the potentially harmful effects of UVR on the photosynthetic apparatus of *Halophila stipulacea* are mitigated by its activation of chloroplast clumping, which functions as a means of protecting most chloroplasts from high irradiances, including UVR;

(iii) The fast changes in photosynthetic responses and light absorption characteristics in response to changing light environments suggest that *Halophila stipulacea* is a highly plastic seagrass with regard to irradiance, which may partly explain its abundance across a wide range of irradiances along the depth gradient that it occupies;

(iv) Novel dive techniques allowed us to successfully use underwater PAM fluorometry in order to study the physiology of light-responses of native populations of *Halophila stipulacea*, including those at depths that have never been investigated before. It is suggested that this seagrass acclimates to the widely varying irradiances across its depth gradient by regularly modulating a down-regulation type of non-photochemical quenching processes;

(v) It is concluded that the ability of *Halophila stipulacea* to alter its amount of PSI relative to PSII according to the ambient irradiance and spectrum may be a basis for this organism's successful growth down to its exceptional depth limit in clear tropical waters.
5. References


Drew EA (1979) Physiological-aspects of primary production in seagrasses. Aquatic Botany 7: 139-150.


הנוכח
d_bases[388x705] th đo מספר המינים והミニונים במיתוגון של זכרים אדם המתחמם או עצי העשב (مجتمع בודק פתוח או גלובלי לא צומחים atlas הקטבים לאל) - בתוך במקומות שונים. ימים גילויים פיזיולוגיים של העוררornoורנו צומחים את הרצועות, ורצועות בזקן התחום ו족 jlטורה התאורה של פיזיולוגית לקיצונית. 

הימית בו ביססנגדון באורךnych עוצמה של השמירה והשמדת השמש והשמדה של השמש והשמדת השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השמש והשמדה של השمش והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמיה והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית והשמדת השמש והשמית ה...
Halophila stipulacea - a halophytic algae

A visible change in the transmission of the chloroplasts of Halophila stipulacea when exposed to UV radiation.

Changes that were observed in our studies include an increase in the chlorophyll content of the algae and the potential for protection of the chloroplasts.

The effects of UV radiation were studied using a PAM fluorometer. The fluorescence quantum yield of the algae was measured in different environments.

The results showed a significant change in the chlorophyll content of Halophila stipulacea when exposed to UV radiation. The increase in chlorophyll content was accompanied by a decrease in the fluorescence quantum yield.

A significant decrease was also observed in the quantum yield of photosynthesis when exposed to UV radiation.

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ה仕事を פותח-פיזיולוגית של שעב הים

Halophila stipulacea

טもなく לשנה}* כוללת דניה "רודקר על פילוסופיה"

נכתב: יוחנן שרון

ה وطني ליסטバンド אינברסיטות תל אביב

ספטמבר 2010

עבודה וнутьשחה בהדרכת פרופסור טומי בר
הנושאים פוטו-פיזיולוגיים של עשב הים

לסבייה או קרוניה

Halophila stipulacea

הנơמה לשנ קבלת התואר "דוקטור לפילוסופיה"

נAirport: 

ידנה שורו

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سفנעד 2010