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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 (O_2) evolution and chlorophyll fluorescence measurements. O_2-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 O_2 optodes, underwater CO_2 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 photosynthesise 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 · O_2 evolution · Chlorophyll *a* fluorescence · Carbon uptake · CO_2 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 O_2 values, or by following continuous O_2 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

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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 CO_2 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 CO_2 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

O_2 measurements

Measuring the O_2 evolved during photosynthesis is one of the oldest and simplest ways of quantifying the photosynthetic activity of plants. In the aquatic environment, O_2 concentration can be determined: (1) chemically by Winkler titration, (2) polarographically using O_2 electrodes, or (3) optically using O_2 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 O_2 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 O_2 concentrations, arguably better than most commercial field-going O_2 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 O_2 before and after whole-community incubations); (2) the difficulty in maintaining adequate homogenization of the medium during incubations, leading to increased boundary layers and resulting in underestimations of photosynthetic rates (Koch 1994); (3) the likelihood of O_2 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 O_2 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 O_2 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 oxymeters 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 oxymeters, with better performance, have been used in conjunction with customized incubation chambers, allowing for versatile and tailor-made apparatuses (Smith et al. 1984, Fourqurean & Zieman 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*; Ramírez-García 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 O_2 release with electron transport rates in the same species; and Cayabyab & Enríquez (2007) determined the photosynthetic light responses of *T. testudinum* plants submitted to 3 distinct light treatments. These integrated O_2 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 HCO_3^- by seagrasses. Using Clark-type O_2 electrode-based experimental setups, the use of HCO_3^- 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 & Rehnberg 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 CO_2 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 HCO_3^- 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 HCO_3^- utilization, one of them firstly reported for seagrasses based on extruded protons-mediation of HCO_3^- uptake. Mercado et al. (2003) investigated the affinity for DIC and the presence of different mechanisms of HCO_3^- use in 2 morphotypes of intertidal *Z. noltii* from southern Portugal. They found that this species had a low affinity for HCO_3^-, 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 O_2 release and electron transport have been elucidated using such O_2 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 O_2 electrode to demonstrate and quantify the transport of O_2 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 O_2 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 O_2 that is channelled down internally to below-ground tissues and eventually leaked to ensure aerobiosis in the rhizosphere. Fourqurean & Zieman (1991) used a custom-made transparent acrylic chamber with a coupled Clark-type polarographic O_2 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. O_2 evolution was continuously monitored by a Clark-type O_2 electrode installed at an intermediate point of the system. Masini et al. (1995) and Masini & Manning (1997) determined photosynthetic light responses in *Posidonia 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 O_2 sensor, recording O_2 values every 15 s.

Some researchers brought high precision O_2 electrodes to the field, connecting them to benthic incubation chambers to monitor the whole-community O_2 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 O_2 evolution within the chambers was followed using a high-resolution dissolved O_2 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 O_2 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 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 hyperboria* 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, Q_A). 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_o or F_s (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 $\Phi_{PSII} = F_m - F_o/F_m$ or simply F_v/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 $Y = F_m' - F_s/F_m' = \Delta F/F_m'$ (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, 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. 1995, see however Grzymski et al. 1997 for alternative distribution values in some forms of macroalgae). Depending on the research-specific goals, or if AF cannot be obtained, it may not always be necessary or relevant to use absolute values of ETR. In such cases, relative ETRs (rETR), expressed as the product of the effective quantum yield by irradiance, can also be used (for a full review of the optical properties and absorptances of seagrass leaves see Durako 2007). Absolute ETRs are mandatory, for instance, when comparisons of ETR and gross photosynthetic O_2 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 fluorometry 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_v/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 *in situ* measurements of effective quantum yields on plants exposed to natural conditions. Novel, automated, multichannel 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 O_2 production in the search for an expedient and field-going methodology to estimate photosynthetic rates. In these experiments, O_2 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 O_2 produced, there is a theoretical molar ratio of 0.25

between O_2 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 O_2 production and ETR, and a 0.25 O_2/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 O_2 evolution and ETRs in seagrasses. The authors examined the O_2/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 O_2 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*. Enríquez & Rodríguez-Román (2006) verified a linear relationship and a ratio of 0.25 for *Thalassia testudinum*, but only for irradiances <170 µmol $m^{-2}s^{-1}$. 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 O_2 response curves, and (3) the fact that ETR tends to saturate at higher irradiances than O_2 evolution. Putative explanations for problems concerning ETR calculations are electron-cycling around PSI and non-photochemical quenching in PSII reaction centres at saturating light levels (Franklin & Badger 2001). Uncertainties in O_2 evolution may be related to photorespiration, which can be an important O_2 sink (e.g. Flexas & Medrano 2002 and references therein), or the Mehler reaction, in which O_2 is photoreduced 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 F_v/F_m 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 & Enríquez (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 pachyrhizu* (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 capricorni*, *Syringodium isoetifolium*, Campbell et al. 2006), osmotic stress (*H. ovalis*, Ralph 1998), herbicide toxicity (*H. ovalis*, *Z. capricorni*, *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 CO_2 flux measurements have rarely been used to estimate seagrass productivity; 4 distinct types of approaches have been described.

(1) Direct measurement of CO_2 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 O_2 and decreasing CO_2 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 O_2 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 CO_2 (pCO_2) 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 CO_2 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 CO_2 fluxes have also been used to estimate the net community production of seagrass-dominated coastal systems (Gazeau et al. 2005b). The air–sea CO_2 flux is computed from the air–sea gradient of pCO2, the gas transfer velocity and the solubility coefficient of CO_2. These fluxes can be determined either by following pCO_2 evolution inside a floating bell system (Frankignoulle & Distèche 1984) or by simultaneous measurements of both air and water pCO_2 (Gazeau et al. 2005a, 2005b).

Other methods

Radioactively labelled carbon ($^{14}CO_2$ or $H^{14}CO_3^-$) was one of the first (Beer et al. 2001) methods to determine CO_2 uptake. The ^{14}C 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 communitiy production. Hermand et al. (2001) and Hermand (2006) summarized the applicability of geo-acoustic inversion techniques to monitor photosynthetic O_2 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 O_2 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 O_2. The general operating principle lies in the simultaneous measurement of vertical water column velocity and O_2 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. O_2 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. O_2 electrodes are also usually employed in the laboratory, with obvious limitations related to the removal of plants from their natural environments. Measurements of CO_2 employing IRGA are much more sensitive than O_2 measurements and thus require lower incubation times. While the IRGA technique is probably the best for measurements of intertidal seagrasses exposed to the air, its recent adaptation for measuring dissolved CO_2 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_v/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 CO_2 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 CO_2 fixation and O_2 production rates. In fact, although it may represent more than half of the whole-plant O_2 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 O_2 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 O_2 leakage by seagrass roots and rhizomes, using O_2 micro-electrodes. O_2 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 photobiological 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

Method	Applications	Advantages	Disadvantages
O_2 titration (Winkler)	• Photosynthesis and dark respiration of whole plants or leaf cuts incubated in bottles (laboratory or *in situ*) • O_2 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 O_2 concentrations only • Cumbersome
O_2 electrodes coupled to small reaction chambers	• Photosynthesis and dark respiration of leaf cuts (laboratory)	• High resolution • High accuracy • Continuous O_2 measurements • Highly controlled conditions • Possibility to manipulate the incubation medium	• Intrusive • Highly artificial • Spectral quality of artificial light sources
O_2 microelectrodes	• O_2 consumption by belowground tissues (*in situ*) • O_2 leakage into the rhizosphere (*in situ*) • O_2 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
O_2 optodes	• O_2 mapping of seagrass rhizosphere (*in situ*)	• Not very intrusive • 2-dimensional measurements • Very sensitive at low O_2 concentrations • Does not consume O_2 • 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
CO_2 evolution	• *In situ* measurements of community uptake and release of CO_2, in incubation chambers	• Non-intrusive • Integration of whole-community metabolism • Highly reliable in air-exposed conditions	• Possibility of underestimating CO_2 uptake in underwater conditions • Problems related to containment in closed chambers
Geo-acoustics	• *In situ* large-scale estimation of community O_2 production	• Large-scale application, suitable for ecosystem level studies • Continuous measurements	• Underdeveloped technique
Eddy correlation	• *In situ* sediment–water fluxes of dissolved O_2 • Community level O_2 fluxes metabolic studies	• Non-intrusive • Autonomous underwater equipment • Good surface integrating capacity • Continuous measurements	• Underdeveloped technique

pertaining to seagrass meadows as global CO_2 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 CO_2 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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