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Experimental conditions accompanying the measurements of photosynthesis

Introduction

A successful comparison of primary production measurement techniques demands carefully-controlled conditions and accurate recording of relevant parameters at all steps between sample collection and final calculation of results. Sakamoto et al. (1984), in the proceedings of the 1st Group for Aquatic Productivity Workshop in Konstanz, noted that differences observed between different techniques could have been due to "...the handling of the experimental samples, especially in the care which was taken in shielding them from excess or stray light". A recommendation of the GAP authors was that "Attention to methodological details must be emphasised if accurate assessments of parameters such as I_k and compensation depth are to be made".

The following article describes the experimental conditions accompanying the comparison of photosynthesis measurements during "Pri-Pro 1997". Relevant parameters are described, together with the main causes of error, and suggestions are made for future workshops of this kind. The summary is divided into three parts describing

(1) collection of the water sample and subsequent handling before the measurements
(2) design of the experimental chambers used for the incubations, with reference to control of temperature and irradiance
(3) measurement of the additional optical parameters which are required for comparisons of photosynthesis measurements made under different spectral irradiance conditions.

Sample collection, handling and characterisation

Collection and handling

The collection depth should be clearly defined for field samples e.g. by use of Niskin bottles with automatic opening and closure, alternatively an integrated water column sample can be taken by means of a long plastic cylinder. For the Zingst workshop, a large experimental mesocosm containing natural phytoplankton was deployed (Schubert *et al.* 1998, this volume). The mesocosm container was continually mixed during the whole day by means of small centrifugal pumps situated at the bottom of the tank. Additional mixing was performed manually shortly before the water samples were removed for measurements, thus the sample was representative of the whole container. The volume of sample collected from the population should be large in order to provide sufficient amounts for all groups, and to minimise container effects and changes in temperature during the time between collection and the start of measurements. The sample may be filtered during collection to remove larger zooplankton. At the Zingst workshop, the water had already been filtered through 200 µm gauze during the filling of the 1000 l mesocosm. The time between collection from the water body, or in this case 1000 l mesocosm, and use in experiments should be kept as short as possible, with the sample stored under controlled irradiance and temperature conditions. At Zingst, a 10 l subsample was taken at each time point and stored in a plastic bucket in dim light at room temperature (approx. 10 °C higher than the mesocosm temperature). The sub-samples used by each group in the intercomparison should be taken after a thorough mixing, then introduced into the various measurement chambers as quickly as possible, and not allowed to stand under uncontrolled conditions in the laboratory where they may be subject to warming. The start time for the photosynthesis-irradiance curves should be synchronised for all groups, and should ideally be within 1 h for all. The actual start times of P-I measurements in "Pri-Pro 1997" varied from 0.5 to 4 h, as considerable time was needed to concentrate samples for some of the experients.

Photosynthesis measurements using Clark-type oxygen electrodes, or with certain types of fluorescence detection equipment may not be sensitive enough to function with water samples containing algal populations with less than 100 µg chl a l^{-1}. Concentration is necessary in

order to ensure a sufficient signal-to-noise ratio when measuring P-I curves over short time periods. The method used for concentration of the sample should ideally be tested beforehand in separate experiments to ensure that photosynthetic rates are not altered by the treatment. Samples in Zingst for the Light Pipette (HRO) and LED-PAM fluorimeter (FTZ) were concentrated by repetitive centrifugation at moderate speed, without cooling, until chl a concentrations of > 500 µg l^{-1} were achieved. The Light Pipette was also used to measure a P-I curve of an unconcentrated mesocosm sample. A run time of 120 min was used and the rates obtained were similar to those of the concentrated samples.

Nutrient concentrations and algal biomass.

Large difference in nutrient concentrations between the original water body (mesocosm) and the experimental subsamples are not likely to occur if zooplankton are absent and the precautions listed above for handling samples are observed e.g. storage time is kept short and handling stress is avoided. Measurement of the macronutrient concentrations is desirable, and may assist in the calculation of carbon fixation from fluorescence-based electron flow rates (nitrate assimilation is an important sink for electrons), and in the interpretation of photosynthetic and respiratory quotients (Davies & Williams 1984). Nutrient concentrations in the 1000 l mesocosm were not measured at Zingst, but measurements in the water column adjacent to the field station on the date that the compartment was filled showed values of 0 µg l^{-1}, 0.31 µg l^{-1}, 0.85 µg l^{-1} and 2.31 µg l^{-1} for nitrate, nitrite, ammonium and phosphate respectively.

Chlorophyll concentration was used as a proxy measurement for algal biomass in the subsamples. Measured volumes of sample were filtered at ~400 mbar suction pressure onto Whatman GFF filters. The filters were extracted at room temperature overnight in 100% dimethylformide (DMF). In a recent comparison by Wright et al. (1997), DMF was found to be the most effective solvent for phytoplankton extraction. (N.B. 100% methanol was however recommended for general use due to the toxicity of DMF). The equations of Porra et al. (1989) were used to calculate concentrations of chl a and chl b. Concentrations are given in Table 2.1.1 of Domin (1998, this volume). To date, no extinction coefficients for chl c in DMF have been published, so the possible contribution of this pigment cannot be estimated. Additionally, no distinction is made in this method between chlorophyll and its breakdown products such as phaeophytin. A recommendation for future

workshops is to use HPLC for pigment analysis rather than spectrophotometric methods (e.g. Jeffries et al. 1997).

Design of the experimental chambers and measurement of P v I curves

Control of temperature

Both respiration (R) and the dark reactions of photosynthesis (which determine P_{max}) are enzymatic and are strongly dependent upon temperature. The temperature setting for the P-I curves should be agreed on by the investigators before the experiments, and then be controlled accurately by use of suitable water baths, Peltier elements etc. It is advantageous to have online measurements of temperature in the cuvettes during fluorescence and oxygen measurements, especially when small volumes of sample are exposed to strong actinic irradiances. In the case of *in situ* bottle experiments, the temperature in the water column should be recorded for comparison with the laboratory measurements. Temperature differences ranging from 13 °C (in situ bottle experiments) to 23 °C (fluorescence cuvettes) existed between the various measurements systems used in Zingst. In the absence of temperature control, the results of samples measured at two different temperatures should be related using suitable Q_{10} values from the literature.

Control of irradiance

Accurate measurements of irradiance are essential before any attempts are made to critically compare the methods used for generating P-I curves. In addition, the estimation of carbon fixation rates from fluorescence parameters alone (e.g. Kolber & Falkowski 1993) also depends upon the accuracy of the irradiane measurements.

As a first step in determining the actinic irradiances used in such a workshop, the irradiance meters and spectroradiometers used by each group should be directly compared against a stable light source of known output. Previous experience of such intercalibrations has shown that irradiance meters may vary considerably, with deviations of up to 50% for machines of the same model being occasionally reported (results of Hanse Tag in Hamburg, 1995). Irradiance meters which deviate by more than 20% from the group mean could be excluded from measurements. The spectral and angular distribution of the light source should be known, and

the calibration of the standard light source should be confirmed in advance with a spectroradiometer operated by qualified optical specialists (e.g. Deutsche Wetterdienst). Sufficient time (1 day) should be allowed for these initial comparisons, for measurement of the irradiance incident upon the various photosynthesis cuvettes, and for subsequent discussions, before the start of the primary production measurements.

One problem which emerged during irradiance measurements at Zingst was that some cuvette set-ups were illuminated with fibre-optic cables which produced narrow, concentrated light beams. The full light-collecting surface of the available irradiance meters (Li-Cor LI-192) or spectroradiometer (Macam) were only partially illuminated by these beams, and thus could not be used for measurements. Use of miniaturised flat (cosine) and spherical (4 ^{1}) sensors (such as the instruments produced by Zemoko, Koudekerke, NL) together with micromanipulators is recommended to alleviate this problem and also to allow the measurement of light gradients within algal suspensions in the cuvettes.

Ideally, experimental vessels should be designed in order to minimise the problems listed above, e.g. good temperature control is essential, together with a stirring mechanism and a simple construction which allows the actual irradiance inside the cuvette to be measured. Use of cylindrical cuvettes containing internal focal points, or reflective side walls or mirrors which cause multiple pathlengths through the cuvette should be avoided.

Increasing the chlorophyll concentration of the sample is often necessary to improve the signal-to-noise ratio, but a serious disadvantage of using concentrated algal samples is that strong attenuation of irradiance within the cuvette occurs. In extreme situations, e.g. with chl concentrations of over 5000 µg l^{-1} the incident irradiance (I_o) may be reduced by over 50% within a 10 mm cuvette. The spectral quality is also shifted under these conditions to favour wavelengths which are not strongly absorbed by the algal pigments. Furthermore, the algae are subject to a rapidly fluctuating light field instead of constant conditions. A correction must be applied to determine the mean irradiance within the cuvette. The following equation can be used for monochromatic irradiation of algal suspensions:

Van Liere & Walsby (1982)

$$I_m = \frac{(I_o - I_b)}{\ln(I_o / I_b)} \qquad \text{(Equation 1)}$$

where I_m = mean irradiance in the chamber, I_o = irradiance at the front face of the chamber, I_b = irradiance at the back of the chamber. This equation can also be used with very little error for estimating the mean broadband PAR in a photosynthesis chamber (Bright & Walsby 1998). Irradiance values for all photosynthesis measurements made with concentrated samples at Zingst were corrected by using a spectrally-resolved version of Equation 1. $I_{o(l)}$ was measured for all actinic light sources with a spectroradiometer at a position just in front of the cuvette. $I_{b(l)}$ was calculated indirectly using spectral attenuation coefficients for the concentrated algal samples measured with a spectrophotometer, and assuming that the irradiance was attenuated exponentially in accordance with the Lambert-Beer law. In future, it would be advisable to avoid these uncertainties either by using concentrated algal suspensions only in combination with short optical pathlength cuvettes, or by making direct measurements of the light gradient within the algal suspension. No correction was made for measurements on unconcentrated samples at Zingst (for a 10 mm cuvette, and 35 µg chl a l^{-1}, $I_b > 0.95*I_o$).

I have given prime importance in this chapter to accurate measurement of irradiance, but there are also other factors to consider when comparing P-I curves. The number of irradiance steps, and the spacing of the irradiance steps, are important factors in the experimental design. Too few irradiance steps in the light-limited part of the curve will reduce the accuracy with which alpha can be determined. Sufficient high irradiance points must be included to clearly show that light saturation has been reached. Recommendations for designing P-I curves and mathematical fitting of the data are given in a review by Henley (1993). The length of each irradiance step may also influence the photosynthetic rates. Longer irradiance steps at the higher irradiances will increase the likelihood of photoinhibition being observed, but too short a step length may also alter photosynthetic rates e.g. by incomplete activation of RUBISCO if the sample has been dark-acclimated (Macintyre & Geider 1996). Comparisons of P-I curves obtained with widely different incubation times should only be done with caution (Macedo et al. 1998)

Optical parameters required for comparison of photosynthesis-irradiance curves

In addition to the variations in cuvette design, and the problems with strong attenuation within the cuvette mentioned above, there were also large differences in the spectral distribution of the various actinic light sources used at the workshop (Figure 1).

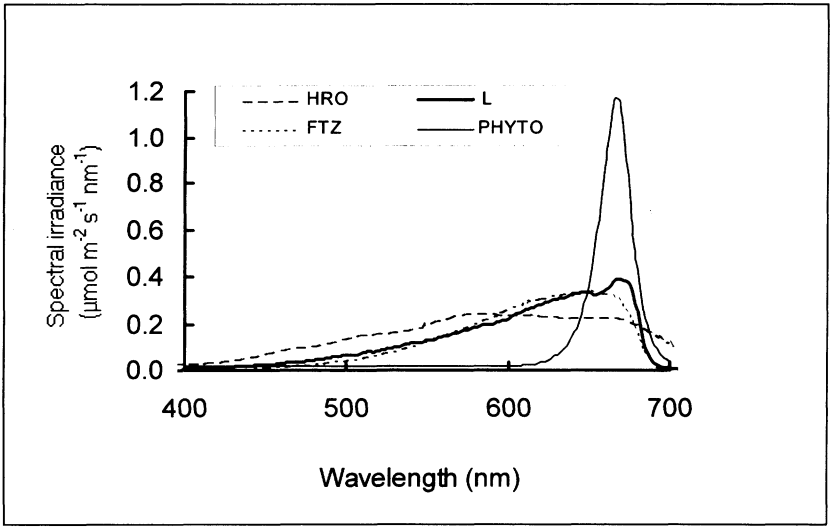

Fig. 1 Comparison of four different actinic light spectra at low irradiance. **HRO** = halogen lamp of the Light Pipette (Uni. Rostock) at 49 µmol m^{-2} s^{-1}; **L** = halogen lamp of a Schott KL-1500 (Uni. Leipzig) at 43 µmol m^{-2} s^{-1}; **FTZ** = halogen lamp of a Schott KL-1500 (FTZ Busum) at 40 µmol m^{-2} s^{-1}; **PHYTO** = red LED (Büro für Umweltmesstechnik) at 35 µmol m^{-2} s^{-1}. Spectra were measured at the I_0 position with a Macam SR-9910 spectroradiometer. The shape of the **HRO** and **PHYTO** spectra was constant at all irradiances, whereas the **L** and **FTZ** spectra were blue-shifted at higher irradiances. When working with concentrated algal suspensions, the mean spectral irradiance would differ from these spectra due to attenuation within the cuvette.

The spectra produced by the various light sources are also different in shape to natural solar radiation and to the *in situ* light climate of the mesoscosm. These spectral differences can potentially affect photosynthetic rates, as photosynthesis in the light-limited range of the curve is influenced by the rate at which algae absorb light:

(Kirk 1994) $$\alpha = \phi_{max} . a^*$$ (Equation 2)

where a = photosynthetic efficiency as measured by the initial slope of the P-I curve, f_{max} = maximal quantum efficiency of photosynthesis, and a^* = absorbtion cross-section of the photosynthetic apparatus.

Of these parameters, a^* is spectrally dependent, whereas f_{max} is generally assumed not to depend upon the wavelength of the actinic irradiance (but see Schofield et al. 1996). Differences in the photosynthetic efficiency, a, between different actinic light sources e.g. between a laboratory cuvette and *in situ* water column incubations, can be predicted if both the actinic irradiance spectra and absorption spectra of the algae are known:

(Arrigo & Sullivan 1992): $$\alpha_a = \alpha_b . \frac{\bar{a}^*_a}{\bar{a}^*_b}$$ (Equation 3)

where a_a = initial slope in light field *a*, a_b = initial slope in light field *b* , $\bar{a}_a^*$ = spectrally-weighted absorption in light field *a*, $\bar{a}_b^*$ = spectrally-weighted absorption in light field *b*. The usefulness of this correction method was confirmed by Schofield et al. (1996).

The mean spectrally-weighted absorption coefficient is a measure of the effectiveness of algae at absorbing quanta from a given spectral irradiance distribution, and is an important component of bio-optical models of productivity. It is derived from a combination of the algal absorption spectra and the spectral distribution of the actinic irradiance:

(Bannister 1974; Berner et al. 1987) $$\bar{a}^* = \frac{\sum_{400}^{700} (a^*_{(\lambda)} * I_{(\lambda)} * \Delta\lambda)}{\sum_{400}^{700} (I_{(\lambda)} * \Delta\lambda)}$$ (Equation 4)

where $a_{(l)}^*$ is the optical cross-section of the algae, and $I_{(l)}$ is the scalar irradiance at wavelength l. Normally, cross-sections are expressed per unit chlorophyll, but may equally well be expressed per cell, or per unit C if accurate measurements of these are available. In the following calculations I have chosen chlorophyll as the biomass parameter for calculating the amount of light absorbed by the algae under different actinic irradiance conditions with Equation 4. This assists our comparisons with the literature, as virtually all published data for phytoplankton is in the chl-specific form. The actual amount of quanta absorbed at any moment in time from a light stream is given by the upper part of Equation 4:

(Kroon et al. 1993) $$AQ = \sum_{400}^{700}(a^*_{(\lambda)} * I_{(\lambda)} * \Delta\lambda)$$ (Equation 5)

The optical cross-section referred to in Equations 4 and 5 can be either the total light absorbed, or the light absorbed which is used in photosynthesis. I will differentiate between these two forms of cross-section by adding subscripts as in Sosik & Mitchell (1995):

a^*_{ph} refers to the total photons absorbed by the algae, and a^*_{ps} is in most cases more useful, and refers only to the photons absorbed by photosynthetically-active pigments.

a^*_{ph}, the total absorption cross-section of the algae can be estimated either by concentrating the algae on a filter and scanning the filter in a suitable designed spectrophotometer [Quantitative Filter Technique developed by Kiefer & SooHoo (1982), Mitchell & Kiefer (1984)], or by scanning the sample inside, or at the inlet port of an integrating sphere (Maske & Haardt 1987; Nelson & Prezelin 1993). Spectra obtained with the QFT method must be corrected for the increase in pathlength caused by multiple scattering within the wet filter (ß-correction; Mitchell 1990). True phytoplankton absorption is derived by correcting scans for the absorption due to dissolved organic material, and for non-phytoplankton detrital material (Kishino et al. 1985, Roesler et al. 1989). Figure 2 shows the a^*_{ph} spectra for the concentrated phytoplankton samples used at Zingst,

with distinctive peaks and shoulders at 438 nm (chl a), 460-500 nm (carotenoids, chl b), 630 nm (phycocyanin) and 680 nm (chl a). The mean unweighted absorption was 0.012 m^2 (mg chl a)$^{-1}$, which is at the lower end of values typically reported for field samples. The signal-to noise ratio with unconcentrated samples was too low to give an accurate spectra.

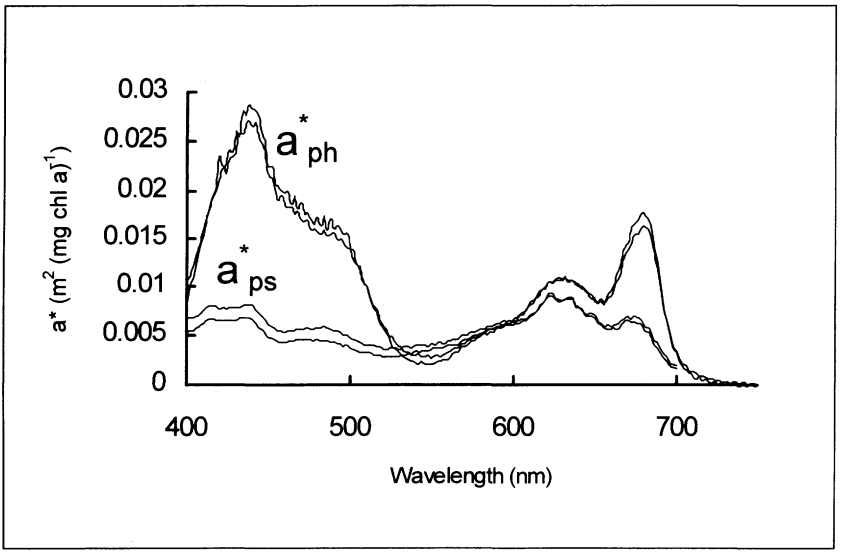

Fig. 2 Chl-specific *in vivo* absorption spectra (a^*_{ph}) measured for concentrated phytoplankton samples compared to scaled, quantum-corrected, DCMU-enhanced fluorescence excitation spectra (a^*_{ps}) of the same samples. Samples were collected from the mesocosm and concentrated by centrifugation before use in photosynthesis experiments at 10:30 and 16:30. The absorption spectra were measured in the integrating sphere attachment of a Lambda 2 spectrophotometer, and are corrected for absorption due to dissolved organic material, but not for detrital absorption. Residual scattering effects have been removed by subtracting the absorption value at 750 nm
The F_{DCMU} spectra, measured in a Hitachi 4010 spectrofluorimeter, were first quantum-corrected, then scaled to 35% of the red absorption peak.

Several methods exist for estimating a^*_{ps}, the absorption cross-section of only the photosynthetically-active pigments. One solution is to use HPLC either to measure the amount of absorption due to non-photosynthetic

pigments (such as zeaxanthin and diadinoxanthin) and then subtract the contribution to these from the total absorption spectra, another is to completely „reconstruct" the *in vivo* absorption of only the photosynthetically-active pigments. These methods have been pioneered by Prezelin and co-workers (Nelson & Prezelin 1990; Johnsen et al. 1994, but have been criticised due to the difficulties of converting from *in vitro* to *in vivo* absorption. The alternative approach is to use the *in vivo* excitation spectra for photosystem II fluorescence to estimate the shape of the photosynthetically-active absorption spectra for this photosystem (Neori et al. 1988). PSII fluorescence emission is measured in the far-red region (> 720 nm) in order to include the whole range of photosynthetically-active pigments in the scan (350-700 nm). It is recommended to add DCMU to the algal suspension in order to close all PSII reaction centres, and to raise the fluorescence yield of "active" chlorophyll relative to "dead" chlorophyll breakdown products (Johnsen & Sakshaug 1993). Before use, the raw F_{DCMU} spectra require correction for changes in the quantum output of the excitation lamp. Examples of quantum-correction techniques are given by Mitchell & Kiefer (1984), and Lutz et al. (1998).

The fluorescence method has the advantage of being relatively insensitive to scattering, but has the disadvantage that the F_{DCMU} spectra have no units, and cannot be used directly in models of algal absorption. A scaling procedure is used to solve this problem, in which the F_{DCMU} spectra is matched to the corresponding a^*_{ph} spectra. Techniques and problems associated with scaling of the F_{DCMU} spectra, in particular the problems associated with phycobilin-containing algae, are discussed by Johnsen & Sakshaug (1996), and Lutz et al. (1998). The F_{DCMU} spectra measured in Zingst showed activity at 420-440 nm (chl a), 460-500 nm (chl b and carotenoids), 620 nm (phycocyanin), and 680 nm (chl a). In this example, the F_{DCMU} spectra of the concentrated samples were scaled so that the red peak of the F_{DCMU} spectra was equal to 35% of the a^*_{ph} spectra. This is an empirical factor which I have chosen to account for the fact that most of the chlorophyll in this mixed algal sample is in cyanobacteria, and is therefore associated with the non-fluorescent PSI. Use of a higher scaling factor would have resulted in the a^*_{ps} spectra "overshooting" the a^*_{ph} spectra, which is not physiologically possible. Scaled in this way, a^*_{ps} has a mean unweighted value of 0.005 m^2 (mg chl a)$^{-1}$, or 45% of the total absorption. The remaining 55% of the absorption would be accounted for by PSI and photoprotective pigments (zeaxanthin is known to be present in high

concentrations in Bodden samples). Clearly, the scaling of the F_{dcmu} spectra to obtain cross-sections in absolute units is highly subjective and is an area of considerable uncertainity. Nevertheless, in cases where only the relative shape of the algal spectra are needed, e.g. with Equation 3, then the quantum-corrected fluorescence excitation spectra can be used with confidence.

I have calculated $\tilde{a}^*$ the mean spectrally-weighted algal absorption for the 4 actinic irradiances shown in Figure 1, using both types of a^* spectra (Table 1), together with the percentage difference in comparison with a perfect "white" light source (e.g. weighted with a value of 1 throughout the spectrum).

Tab. 1 Variation in mean spectrally-weighted absorption ($\tilde{a}^*$) arising from the differences in actinic light spectra shown in Fig. 1, and compared to a an unweighted "white" light (=100%). Absorption cross-sections are calculated for both total phytoplankton absorption (a^*_{ph}) and for PSII-specific photosynthetically-active pigment absorption (a^*_{ps}) as in Figure 2. The effective absorption in each actinic light spectra is given as a percentage of the unweighted spectra. Actinic light source abbreviations and descriptions are given in the legend to Figure 1. "Lo" = irradiance settings between 35 and 49 µmol m^{-2} s^{-1} ; "Hi" = irradiance settings between 400 and 800 µmol m^{-2} s^{-1}.

Name	Light source		a^*_{ph}		a^*_{ps}	
"White"	Equal quanta at all wavelengths	-	0.0115	100%	0.0052	100%
HRO	Light Pipette	Hi	0.0092	80%	0.0058	112%
	Halogen lamp	Lo	0.0092	80%	0.0058	112%
L	Halogen lamp	Hi	0.0094	82%	0.0061	117%
		Lo	0.0093	81%	0.0064	123%
FTZ	Halogen lamp	Hi	0.0085	74%	0.0061	117%
		Lo	0.0086	75%	0.0064	123%
PHYTO	Red LED	Hi	0.0117	102%	0.0064	123%
		Lo	0.0117	102%	0.0064	123%

For the a^*_{ph} spectra, the three halogen lamp spectra produced a mean effective absorption which was between 19% and 26% lower than that with theoretical "white" light. This is because these spectra contained substantial amounts of quanta at wavelengths which were minimally

absorbed by the Zingst algae. The red LED of the PHYTO-PAM resulted in higher effective absorption, as it matched the red absorption band of chl a and b. Increasing the power supply of the halogen lamps (L, FTZ) caused a blue-shift of the spectrum, but there was only a small effect on effective absorption. The results obtained with the spectra for a^*_{ps} differ in that all 4 actinic light sources showed 12-23 % higher effective absorption in comparison to "white" light, and that there was very little difference between the effectivity of all 4 light sources.

The large contribution of orange-red absorbing pigments to the a^*_{ps} spectra matched better the distribution of the actinic spectra. In this case, the blue shift caused by changing the L and FTZ halogen lamps from low irradiance (approx 40 µmol m^{-2} s^{-1}) to high irradiance (approx. 400-800 µmol m^{-2} s^{-1}) caused a 4% drop in the absorption efficiency. Thus, assuming that the a^*_{ps} is most appropriate weighting spectrum for measurements of PSII activity via fluorescence and oxygen evolution, then very minor differences in α would be expected from the use of different actinic light sources at the workshop.

As intercomparisons of the optical methods listed here have rarely been reported in the literature, one productive area for future "Pri-Pro" workshops would be to include a series of such tests e.g. determination of a^*_{ph} spectra with the filter technique compared to with an integrating sphere.

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