

Evaluation of biophysical and optical determinations of light absorption by photosystem II in phytoplankton

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Abstract

Fast repetition rate (FRR) fluorescence can provide highly resolved estimates of light absorption by photosystem II (PSII), a variable that is critical to bio-optical determinations of phytoplankton productivity. We compared estimates of chlorophyll *a*-specific light absorption by PSII, $a_{\text{PSII}}^{\text{chl}}$, using both biophysical (FRR) and optical (chlorophyll *a*-specific light absorption coefficient, a^{chl}) techniques on cultures of phytoplankton from diverse taxa. Biophysical determinations of $a_{\text{PSII}}^{\text{chl}}$ were obtained from the product of the effective light absorption cross-section of PSII (σ_{PSII}), measured by FRR fluorescence, and the ratio of PSII reaction centers to chlorophyll *a* (n_{PSII}), measured by oxygen flash yields. Both parameters were highly variable between individual taxa. In mixtures of algae, the estimates of σ_{PSII} were largely determined by the taxon that dominated fluorescence intensity. Independent optical estimates of $a_{\text{PSII}}^{\text{chl}}$ were obtained by weighting the light absorption spectra of photosynthetic pigments by the fluorescence excitation spectra. The biophysical and optical estimates of $a_{\text{PSII}}^{\text{chl}}$ were highly correlated ($r^2 = 0.94$) with a slope that was not significantly different from 1 and an intercept of 0. Estimates of productivity using biophysical or optical absorption measurements should therefore be comparable when the latter are adjusted to account for the proportion of light provided for photochemistry into both photosystem I and II. Finally, we show how simultaneous measurements of σ_{PSII} and $a_{\text{PSII}}^{\text{chl}}$ can be used to derive n_{PSII} where flash-yield measurements are impractical, as is almost universally the case in field measurements.

Oceanographers and limnologists desire rapid, in situ measurements of primary productivity to address fundamental and applied research questions. The ability of fast repetition rate (FRR) fluorometry (Kolber et al. 1998) to provide measurements of primary productivity has been examined by a number of groups (Suggett et al. 2001, 2003; Moore et al. 2003; Raateoja et al. 2004). Discrepancies between $^{14}\text{CO}_2$ assimilation, or O_2 evolution, and FRR fluorescence estimates of photosynthetic electron transfer of $\pm 100\%$ to 200% have been reported (Suggett et al. 2001, Moore et al. 2003, Raateoja et al. 2004). However, it is not clear that these discrepancies

can be attributed exclusively to shortcomings of the FRR technique (or limitations in its application) because (1) measurements of CO_2 and O_2 exchange may also be flawed, (2) the comparison may be based on parallel rather than simultaneous measurements, or (3) key variables required for a rigorous intercomparison may not have been measured. In other words, there is no gold standard against which estimates of photosynthetic electron transfer rates provided by the FRR technique can be compared. The best that these previous studies provide is an intercomparison, whereas a completely independent evaluation of the assumptions of the FRR technique is desired.

In this paper, we step back from the direct comparison of independent measurements of photosynthesis rates by FRR fluorescence and ^{14}C assimilation, both of which may be subject to error, to an evaluation of one of the key assumptions of the FRR fluorescence technique. In this way, we hope to resolve a fundamental methodological issue concerning the application of the FRR technique. Specifically, we evaluate whether FRR measurements of the effective cross-section of photosystem II are consistent with completely independent bio-optical measurements. To our knowledge, this is the first time that such a test of the FRR technique has been reported. This is a necessary

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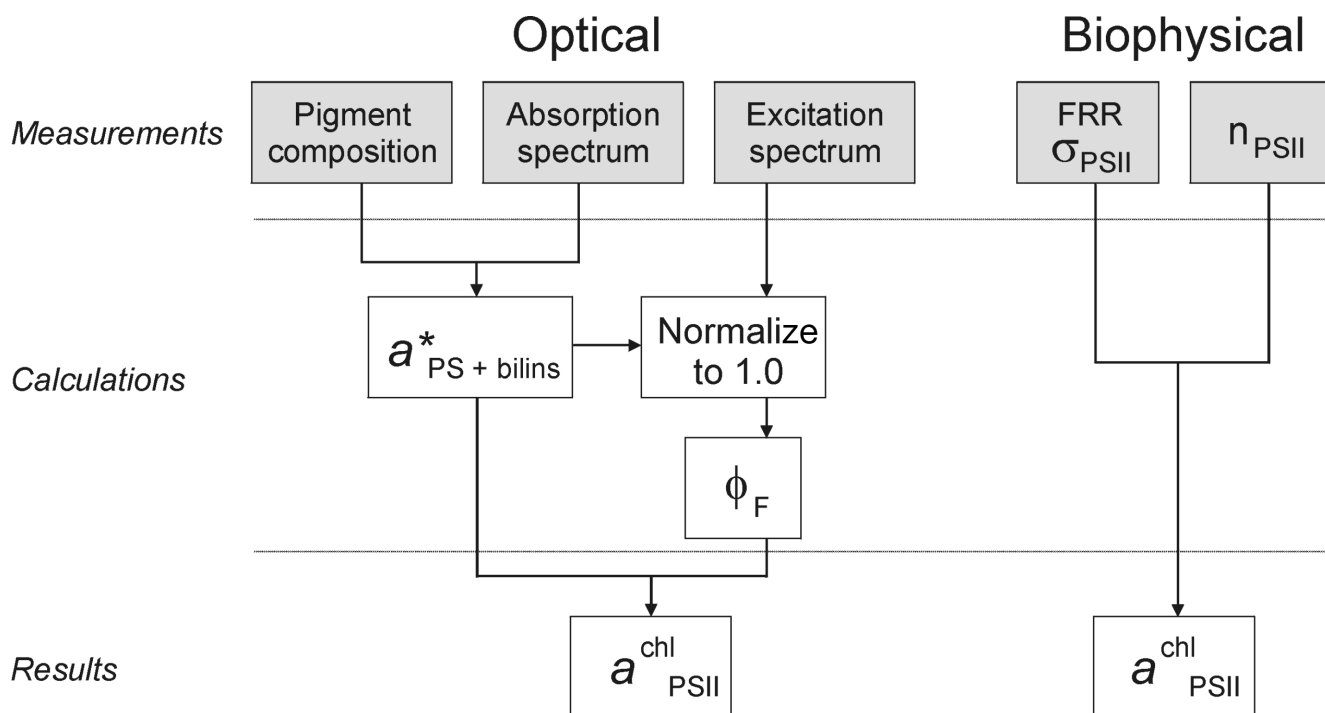


Fig. 1. Steps involved in the comparison of optical and biophysical determinations of light absorption by PSII— $a^{\text{chl}}_{\text{PSII}}$, $\text{m}^2 (\text{mg chl } a)^{-1}$. Optical and biophysical absorption are independently measured with units of m^{-1} and quanta^{-1} , converted to per RCII $^{-1}$ as 6.023×10^{23} quanta $(\text{mol RCII})^{-1}$, but are normalized to the chlorophyll a concentration. All measurements and calculations are described in the main text.

step in the validation of the FRR technique. The functional PSII cross-section is one of the two variables needed to calculate photosynthetic electron transfer rates from FRR fluorometry. The other variable is the quantum efficiency of electron transfer through photosystem II, which will be addressed elsewhere.

Background—Active fluorescence provides rapid in situ quantification of the efficiency with which absorbed light is used for photochemical electron transport through photosystem II (PSII) (Laws et al. 2002; Kromkamp and Forster 2003). Some techniques, such as pulse amplitude modulation fluorescence (Schreiber et al. 1993), rely upon independent measurements of the chlorophyll a -specific light absorption coefficient, a^{chl} , to obtain absolute rates of photosynthetic electron transfer (Kromkamp and Forster 2003; Suggett et al. 2003). Although a^{chl} can be measured optically, additional measurements or assumptions are required to estimate the proportion of a^{chl} solely directed to PSII photochemistry, $a^{\text{chl}}_{\text{PSII}}$ (Fig. 1, Kromkamp and Forster 2003). Furthermore, a^{chl} is typically measured from discrete water samples constraining the scales at which fluorometric estimates of algal productivity can be made.

In contrast, fast repetition rate (FRR) fluorescence (Kolber et al. 1998) enables a rapid biophysical measure of both photochemical efficiency and light absorption in situ, from which photosynthetic electron transfer rates can be calculated (Falkowski and Kolber 1995). However, these rates are expressed per photosynthetic unit, and the measure of light

absorption is a cross-section for functional photosystem II reaction centers, σ_{PSII} . Importantly, σ_{PSII} is not directly proportional to a^{chl} .

The FRR fluorescence technique induces a simultaneous, single turnover (closure) event of the majority of PSII reaction centers (RCIIs). The rate of reaction center closure depends upon the rate of light absorption, the efficiency of excitation transfer to the PSII reaction center, the yield of charge separation, and the pool size of functional PSII reaction centers (Mauzerall and Greenbaum 1989) and, therefore, yields an effective rate of light absorption (σ_{PSII}) (Dubinsky et al. 1986; Falkowski and Kolber 1995; Falkowski and Raven 1997). σ_{PSII} has units of $\text{\AA}^2 \text{ quantum}^{-1}$ (or $\text{m}^2 \text{ mol RCII}^{-1}$) and can be considered to be a functional absorption cross-section or target.

The rate of PSII reaction center closure is primarily controlled by the ratio of light-harvesting pigments to PSII reaction centers. σ_{PSII} may be reduced at high photon flux densities by photoprotective mechanisms operating over multiple time scales. These range from minute-scale associated with xanthophyll cycling (Olaizola et al. 1994) to hours associated with xanthophyll acclimation (Babin et al. 1996). σ_{PSII} can also change following state transitions (Falkowski and Raven 1997; Behrenfeld and Kolber 1999). A loss of reaction center functionality may also increase σ_{PSII} (Falkowski and Kolber 1995) in proportion to the fraction of reaction centers lost and the probability of excitation transfer among the remaining functional reaction centers. This

Table 1. Table of definitions for biophysical and optical determinations of the chlorophyll *a*-specific absorption by PSII ($\sigma_{\text{PSII}}^{\text{chl}}$)

Parameter	Definition	Units
Biophysical		
σ_{PSII}	Effective absorption by photosystem II (PSII)	$\text{\AA}^2 (\text{quanta})^{-1}$
n_{PSII}	Photosynthetic unit size of PSII	$\text{mol RCII} (\text{mol chl } a)^{-1}$
[RCII]	Concentration of functional PSII reaction centers	mol RCII m^{-3}
$\sigma_{\text{PSII}}^{\text{chl}}$	Chlorophyll <i>a</i> -specific absorption by PSII ($\sigma_{\text{PSII}} \times n_{\text{PSII}} \times 0.00675$)	$\text{m}^2 (\text{mg chl } a)^{-1}$
Optical		
[chl <i>a</i>]	Concentration of chlorophyll <i>a</i>	mg (m)^{-3}
F_{730}	Fluorescence excitation at 730 nm	Instrument units
F_{730}'	F_{730} spectra scaled to a value of 1 ($\lambda = 400$ to 700 nm)	Dimensionless
<i>a</i>	Optical absorption coefficient by phytoplankton	m^{-1}
σ^{chl}	Chlorophyll <i>a</i> -specific optical absorption coefficient	$\text{m}^2 (\text{mg chl } a)^{-1}$
$\sigma', \sigma^{\text{chl}'}$	<i>a</i> and σ^{chl} spectra scaled to a value of 1 ($\lambda = 400$ to 700 nm)	Dimensionless
C_i	Concentration of pigment <i>i</i>	mg (m)^{-3}
a_i	Weight-specific absorption coefficient of pigment <i>i</i>	$\text{m}^2 (\text{mg})^{-1}$
σ_{PS}^* , σ_{PP}^* , σ_{bilins}^*	Chlorophyll <i>a</i> -specific absorption reconstructed from photosynthetically (PS and bilins) and nonphotosynthetically active (PP) pigments (Eq. 2, 4)	$\text{m}^2 (\text{mg chl } a)^{-1}$
$\bar{\sigma}_{\text{PS+bilins}}^{\text{chl}'}$	Chlorophyll <i>a</i> -specific absorption of only photosynthetically active pigments (Eq. 7)	$\text{m}^2 (\text{mg chl } a)^{-1}$
σ_{pack}	Intracellular pigment packaging (Eq. 3)	Dimensionless
<i>E</i>	Excitation PPF of FRR fluorometer LEDs	$\mu\text{mol (m)}^{-2} (\text{s})^{-1} (\text{nm})^{-1}$
PPFD	Photosynthetically active photon flux density	$\mu\text{mol (m)}^{-2} (\text{s})^{-1}$
\bar{a}	Effective <i>a</i> weighted to the excitation spectra of the FRR fluorometer LEDs	m^{-1}
\bar{a} , \bar{a}^{chl} , \bar{a}_{PS}^*	Effective <i>a</i> , σ^{chl} , σ_{PS}^* , σ_{PP}^* and σ_{bilins}^* weighted to the excitation spectra of the FRR fluorometer LEDs (Eq. 5, 6)	$\text{m}^2 (\text{mg chl } a)^{-1}$
\bar{a}_{PP}^* , $\bar{a}_{\text{bilins}}^*$		
<i>P</i>	Proportion of total light absorbed and transferred to PSII (Eq. 9)	Dimensionless
$\sigma_{\text{PSII}}^{\text{chl}}$	Chlorophyll <i>a</i> -specific absorption by PSII ($\sigma_{\text{PS+bilins}}^{\text{chl}} \cdot P$) (Eq. 8)	$\text{m}^2 (\text{mg chl } a)^{-1}$

Direct σ_{PSII} measurements are converted to units of $\text{m}^2 (\text{mol RCII})^{-1}$, $\sigma_{\text{PSII}} \times 6023$, and n_{PSII} measurements are converted to units of $\text{mol RCII} (\text{mg chl } a)$, $n_{\text{PSII}}/892500$, to yield the factor 0.00675 for $\sigma_{\text{PSII}}^{\text{chl}}$ (Suggett et al. 2003). Optical absorption coefficients (*a*) were measured directly or reconstructed from algal pigments and are both chlorophyll *a* (weight)-specific, σ^{chl} and σ^* , respectively, unless otherwise indicated. Wavelength-adjusted weight-specific absorption coefficients were taken from Bidigare et al. (1990) for algal pigments (*i*) that are photosynthetically active (PS) or photoprotective (PP, nonphotosynthetically active) (Bidigare et al. 1990; Babin et al. 1990). Absorption by phycobilins (bilins) was estimated indirectly (see main text).

may occur, for example, under conditions of nutrient limitation (Greene et al. 1994; Berges et al. 1996) and photoinhibition (Vasiliev et al. 1994; Babin et al. 1996; Gorbunov et al. 2001). As a result, σ_{PSII} may vary in situ by a factor of about 1.5 to 3 across a range of temporal and spatial scales (Greene et al. 1994; Vasiliev et al. 1994; Behrenfeld et al. 1996; Behrenfeld and Kolber 1999; Gorbunov et al. 2001). However, few measurements of σ_{PSII} have been obtained from individual taxa under controlled conditions (Dubinsky et al. 1986; Berges et al. 1996; Koblížek et al. 2001; Raateoja and Seppälä 2001), and it is not known to what extent σ_{PSII} is determined by physiological or taxonomic shifts within and across phytoplankton communities.

A measurement of the ratio of PSII reaction centers to the chlorophyll *a* concentration (n_{PSII} , mol RCII: mol chl *a*) is required to convert σ_{PSII} into a chlorophyll *a*-specific rate of light absorption for PSII photochemistry ($\sigma_{\text{PSII}}^{\text{chl}}$, Fig. 1, Table 1):

$$\sigma_{\text{PSII}}^{\text{chl}} = \sigma_{\text{PSII}} \cdot n_{\text{PSII}} \quad (1)$$

n_{PSII} changes as a result of photoacclimation (Falkowski et al. 1981; Dubinsky et al. 1986), photoinhibition (Barlow and Alberte 1985) and nutrient limitation (Herzig and Falkowski 1989; Berges et al. 1996). Measurements of n_{PSII} by the oxygen flash yield (Falkowski et al. 1981) require high concentrations of living phytoplankton, and the technique is largely impractical for natural samples (Falkowski and Kolber 1995). Therefore, n_{PSII} in situ has been estimated assuming a “typical” value of n_{PSII} and using a proxy of PSII reaction center functionality (Kolber and Falkowski 1993; Babin et al. 1996). This indirect approach has been employed in several investigations (Kolber and Falkowski 1993; Babin et al. 1996; Boyd et al. 1997; Suggett et al. 2001; Moore et al. 2003; Raateoja et al. 2004) but has not been validated. Furthermore, laboratory observations of n_{PSII} upon which the “typical” values are based come from a small range of phytoplankton taxa.

Objectives—To have confidence in the FRR biophysical estimates of $\sigma_{\text{PSII}}^{\text{chl}}$ requires that the following pivotal questions be addressed. (1) Can biophysical determinations of $\sigma_{\text{PSII}}^{\text{chl}}$ be

Table 2. Summary of phytoplankton species and culture conditions at Horn Point (HP) or the University of Essex (ES)

Species	Clone reference	Laboratory	Growth conditions				Batch volume
			PPFD	L:D	Temperature	Media	
<i>A. anophagefferens</i>	CCMP 1790	HP	18, 80	Continuous	20	Artificial SW + f/2	1500
<i>C. muelleri</i>	CCAP 1010/3	ES	50, 300	14:10	25	Filtered SW + f/2	250
<i>D. tertiolecta</i>	CCMP 1320	HP	18, 300	Continuous	20	Artificial SW + f/2	1500
<i>E. huxleyi</i>	CCAP 920/6	ES	50, 250	14:10	18	Filtered SW + f/2	250
<i>P. minimum</i>	<i>Choptank isol.</i>	HP	18, 300	Continuous	20	Artificial SW (15 PSU) + f/2	1500
<i>P. provasolii</i>	CCMP 1203	HP	18, 300	Continuous	20	Artificial SW + f/2	1500
<i>T. weissflogii</i>	CCMP 1047	HP	18, 80	Continuous	20	Artificial SW + f/2	1500
<i>R. salina</i>	CCAP 978/27	ES	75, 375	14:10	18	Filtered SW + f/2	75
<i>S. major</i>	<i>Choptank isol.</i>	HP	18, 300	Continuous	20	Artificial SW (15 PSU) + f/2	1500
<i>Synechococcus</i> spp.	CCAP 1479/9	ES	75, 375	14:10	18	JM (freshwater)	75
<i>Synechococcus</i> spp.	CCMP WH7803	ES	50, 400	14:10	25	Filtered SW + f/2	250

Growth conditions are photosynthetic photon flux density (PPFD, $\mu\text{mol photon m}^{-2} \text{s}^{-1}$), light:dark (L:D, hours) cycle, temperature ($^{\circ}\text{C}$), and media in which phytoplankton were grown. The media were Jaworski's medium (JM), filtered seawater (SW), or artificial seawater (ASW, Keller et al. 1987). SW and ASW were enriched with f/2 (Guillard and Ryther 1962), 107 μM silicate, and selenous acid (Keller et al. 1987). All species were grown in semicontinuous culture at 30 PSU (practical salinity units) except *P. minimum* and *S. major*, which were grown at 15 PSU. Cultures were obtained from The Culture Collection of Algae and Protozoa (CCAP), Dunstaffnage Marine Laboratory, Scotland, U.K., and The Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), Boothbay Harbour, Maine, USA. *Prorocentrum minimum* and *Storeatula major* were isolated from the Choptank River, Maryland, USA, by Alan Lewitus and have been maintained at Horn Point.

reconciled with optical determinations of $a^{\text{chl}}_{\text{PSII}}$ for fluorometric estimates of PSII electron transport? (2) To what extent does σ_{PSII} vary between taxa? (3) To what extent can variations in σ_{PSII} be attributed to measurable variables that affect the rate of light absorption used for photochemical electron transport? (4) Can n_{PSII} be estimated indirectly for accurate biophysical determinations of $a^{\text{chl}}_{\text{PSII}}$? Evaluating these questions will allow more confidence to be placed in FRR fluorescence biophysical measurements of σ_{PSII} and $a^{\text{chl}}_{\text{PSII}}$ for natural phytoplankton communities. Therefore, we determined $a^{\text{chl}}_{\text{PSII}}$ in the laboratory from corresponding biophysical (σ_{PSII} and n_{PSII}) and optical (light absorption and spectral fluorescence) estimates (Fig. 1). This was performed for a variety of algal taxa with different light-harvesting mechanisms and pigment compliments. The results are discussed in the context of collection and interpretation of parameters required to determine $a^{\text{chl}}_{\text{PSII}}$ from natural waters.

Materials and procedures

Phytoplankton were grown at Horn Point Laboratory, Maryland, USA (HP, the pelagophyte *Aureococcus anophagefferens*, the chlorophyte *Dunaliella tertiolecta*, the dinoflagellate *Prorocentrum minimum*, the prasinophyte *Pycnococcus provasolii*, the cryptomonad *Storeatula major*, and the diatom *Thalassiosira weissflogii*) and the University of Essex, UK (ES, the diatom *Chaetoceros muelleri*, the prymnesiophyte *Emiliania huxleyi*, the cryptomonad *Rhodomonas salina*, the cyanobacteria *Synechococcus* spp. CCAP 1479/9 and WH7803) (Table 2). All species were grown at two different photon flux densities

(PPFD, $\mu\text{mol m}^{-2} \text{s}^{-1}$), referred to as LL and HL throughout (Table 2). Cell growth was monitored from daily measurements of fluorescence and each alga was maintained and harvested in exponential growth phase. Triplicate measurements were obtained from sequential dilutions.

FRR fluorometry—A Chelsea Instruments FRR fluorometer (FAST^{track} 182027) was programmed to give single turnover saturation of PSII (ST) from 100 flashlets of 1.1 μs (as described previously, Suggett et al. 2003) applied at 1-s intervals. Other ST protocols have been employed to alter the saturation profile of PSII (Prásil et al. 1996; Kolber et al. 1998; Laney 2003; Suggett et al. 2003). However, the protocol we employ here currently provides the fastest rate of closure of PSII reaction centers using the commercially available FRR fluorometer and represents the minimum requirement to yield a saturation plateau for algae with small cross-sections (Fig. 2). Other instruments may deliver a different intensity and/or spectral quality of excitation and directly affect the level of PSII saturation and hence σ_{PSII} . Therefore, it is essential to assess the ST protocol of each instrument before employing a generalized or recommended setting. The minimum (F_0) and maximum (F_m) fluorescence, the effective absorption cross-section (σ_{PSII}) and the degree of excitation energy transfer between PSII reaction centers (connectivity, ρ) were determined by fitting the model of Kolber et al. (1998) to the mean of 15 ST sequences using software provided by Sam Laney (v4, <http://picasso.oce.orst.edu/ORSOO/FRRF/>). Values of σ_{PSII} are directly output with units of \AA^2 (quanta^{-1}) but expressed as m^2 (mol RCII^{-1}) (see Table 1).

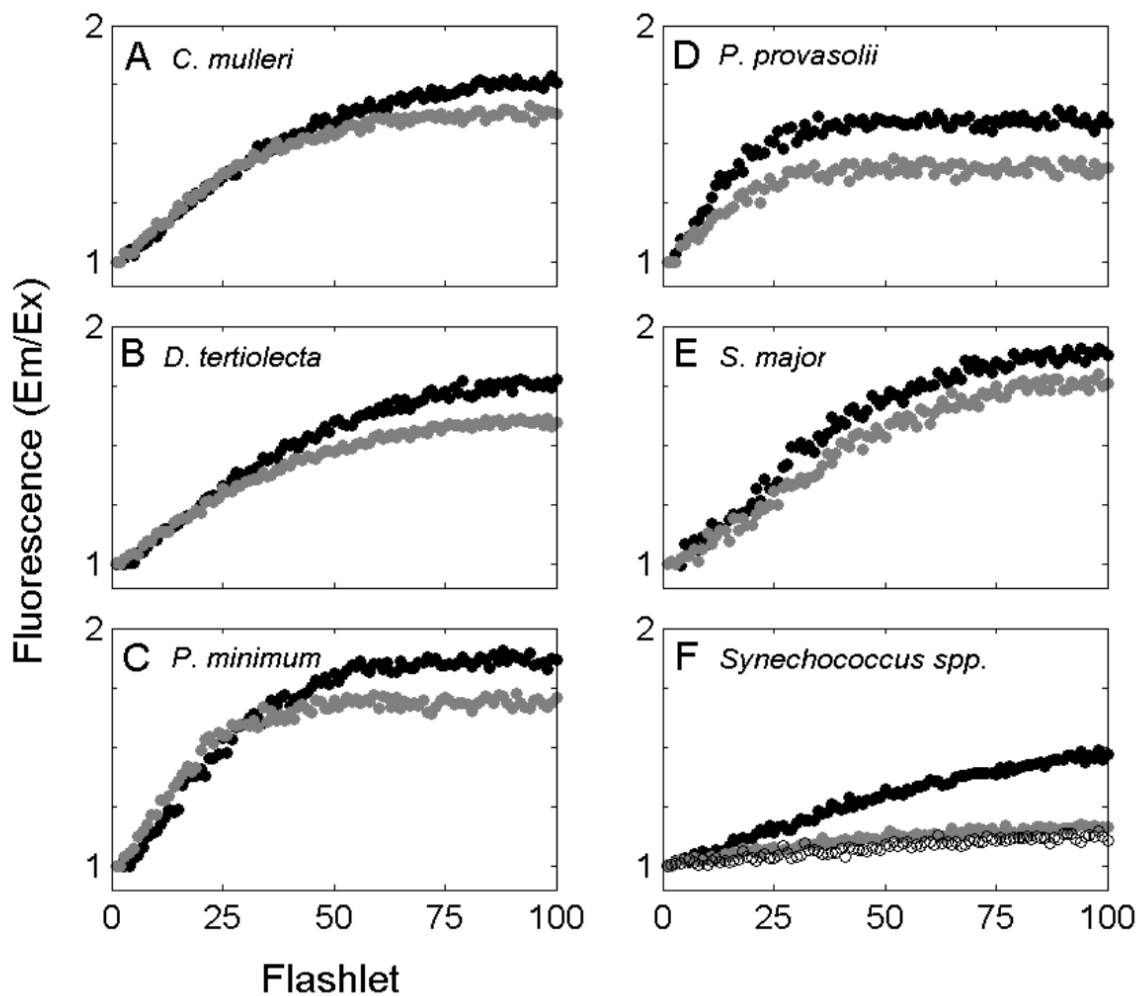


Fig. 2. Fluorescence arising from cumulative PSII excitation using the FRR fluorescence protocol. The emission to excitation ratio (Em/Ex) yields the value of fluorescence at each of 100 flashlets. Em/Ex increases from minimum (F_o) to maximum (F_m) levels according to the effective absorption cross-section (σ_{PSII}) and the degree of energy transfer between PSII reaction centers (ρ) (see main text). Em/Ex are shown for a variety of dark-adapted phytoplankton grown under LL (●) and HL (◐). Data in Panel F are for *Synechococcus* WH7803 under LL (●) and HL (◐) and for *Synechococcus* 1479/9 under LL (○). Each Em/Ex is the mean of 15 excitation sequences and is normalized to a value of 1 at flashlet 1.

FRR fluorescence measurements were made once algal samples had been dark-acclimated for 30 to 40 min. Algal samples were placed into a quartz cuvette positioned in the FRR open chamber—excitation of 1.93×10^{12} quanta $(\text{m}^2)^{-1}$ $(\text{count})^{-1}$ —and were maintained at growth temperature using a Peltier temperature control probe (Suggett et al. 2003). Blanks were obtained by gravity-filtering cultures through GF/F filters (Whatman). Each blank measured with the FRR fluorometer was typically less than 1% of the algal fluorescence. All fluorescence parameters were adjusted for instrument-specific response, scatter, and baseline functions (Laney 2003), while processed.

Each ST sequence gives rise to a characteristic FRR fluorescence induction curve (Fig. 2). σ_{PSII} and ρ are interrelated as the rate and shape, respectively, of the fluorescence rise from F_o to F_m . However, for the purpose of this investigation, we wished

to evaluate σ_{PSII} independent of variations in ρ . The mean value of ρ (\pm standard deviation) estimated from the dark-adapted FRR measurements from all cultures was 0.32 ± 0.08 (dimensionless) with highest and lowest values of 0.38 ± 0.11 and 0.26 ± 0.05 recorded for *S. major* and *P. provasolii*, respectively. Therefore, all ST sequences were subsequently reprocessed using a fixed value for ρ of 0.3 ($\sigma_{\text{PSII}}^{\rho 0.3} = 1.01 \times \sigma_{\text{PSII}}^{\text{pfree}}$, $r^2 = 0.994$, $n = 34$). Values of σ_{PSII} were lower when ST sequences were processed using a ρ of 0 rather than of 0.3 ($\sigma_{\text{PSII}}^{\rho 0.3} = 1.05 \times \sigma_{\text{PSII}}^{\text{pzero}}$, $r^2 = 0.976$, $n = 26$).

Photosynthetic unit size—Algal suspensions were concentrated using gravity filtration onto 47 mm GF/F filters and resuspended in 4 to 5 mL of filtrate. Spectrophotometric measurements of chlorophyll *a*-specific light absorption (a^{chl} , see light absorption and pigments section) were made both before and after concentration to ensure that a^{chl} within

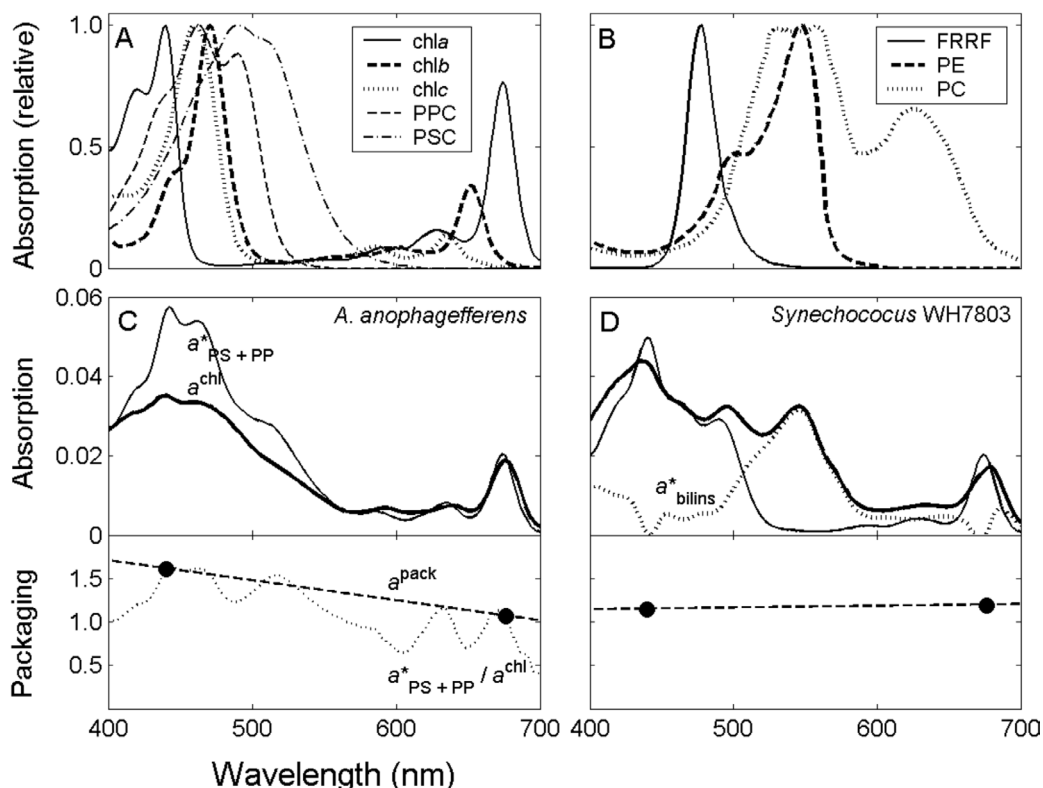


Fig. 3. (A and B) Absorption spectra of photosynthetic (chlorophyll *a*, chl *a*; chlorophyll *b*, chl *b*; chlorophyll *c*, chl *c*; fucoxanthin, PSC; phycoerythrin from WH7803, PE; and phycocyanin from WH8103, PC) and nonphotosynthetic (β -carotene, PPC) pigments. All spectra are from Bidigare et al. (1990) except PC, which has been redrawn from Falkowski and Raven (1997). The excitation spectrum from the FRR fluorometer LEDs (see Suggett et al. 2003, Raateoja et al. 2004) are also shown. Spectra are normalized to a value of 1 at the wavelength of peak absorption. (C and D) Measured and reconstructed absorption spectra (a^{chl} and $a^*_{\text{PS+PP}}$, respectively, $\text{m}^2 \text{mg chl } a^{-1}$) are shown for *A. anophagefferens* and *Synechococcus* WH7803. Packaging (Dimensionless) was determined as $a^*_{\text{PS+PP}}/a^{\text{chl}}$ at 440 and 676 nm (filled circles) and using Eq. 3 (a^{pack} , dashed line) at other wavelengths. Absorption for phycobilins, a^*_{bilins} , was determined from $a^*_{\text{PS+PP}}$, a^{chl} and a^{pack} (Eq. 4).

the suspension had not changed significantly. Similarly, concentrating the suspension did not alter FRR measures of PSII photochemical efficiency ($(F_m - F_o)/F_m (= F_v/F_m)$) of the algae. Oxygen concentrations were measured using a Clark-type electrode housed within a DW1 liquid-phase oxygen electrode chamber (Hansatech Instruments). An ST saturation flash system (see Suggett et al. 2003 for details) was positioned around the reaction chamber. Flash yields of O_2 were constant for flash frequencies between 10 and 50 flashes s^{-1} and measurements were performed routinely at 20, 30, and 40 flashes s^{-1} . Photosynthetic unit size is expressed as the O_2 flash yield relative to total chlorophyll *a* content. n_{PSII} is reported as the product of mol O_2 (mol chl *a*) $^{-1}$ and 4 mol RCII (mol O_2) $^{-1}$ i.e., mol RCII (mol chl *a*) $^{-1}$ throughout.

Light absorption and pigments—Optical density was measured on each culture and corresponding growth media (Table 2) in matched 10 mm quartz cuvettes using a U-3000 spectrophotometer fitted with an integrating sphere ($\phi 60$) (Hitachi High-Technologies) (ES and HP). Absorption was calculated from optical density after correcting for pathlength and residual

scattering at 750 nm. The chlorophyll *a*-specific absorption coefficient (a^{chl}) was obtained by normalizing absorption to the chlorophyll *a* concentration, [chl *a*]. Pigments were measured by high-performance liquid chromatography as described previously (for ES, Suggett et al. 2003; for HP, Van Heukelem et al. 1992; Van Heukelem and Thomas 2001). Samples for high-performance liquid chromatography were collected on Whatman GF/F filters, frozen in liquid nitrogen, and held at -80°C until analysis.

Chlorophyll *a*-specific absorption coefficients for photosynthetic (PS) and nonphotosynthetic (PP) pigments (a^*_{PS} and a^*_{PP} , respectively) were determined as

$$a^*_{\text{PS(PP)}} = \sum_{\text{PS(PP)}} \left(\frac{\sum_{400}^{700} a_i(\lambda) \cdot C_i}{[\text{chl } a]} \right) \quad (2)$$

where a_i is the weight-specific absorption coefficient ($\text{m}^2 \text{mg}^{-1}$, Bidigare et al. 1990; Fig. 3), and C_i is the concentration (mg m^{-3}) of each pigment (*i*) for all wavelengths (λ) between 400

Table 3. Summary of mean (and standard deviation) measurements of the effective absorption cross-section— σ_{PSII} , $\text{A}^2 (\text{quanta})^{-1}$ —from the FRR fluorometer, n_{PSII} —mol RCII (mol chl a) $^{-1}$ —from O_2 flash yields and biophysical estimates of the chlorophyll a -specific absorption of PSII— $a_{\text{PSII}}^{\text{chl}}$, $\text{m}^2 (\text{mg chl } a)^{-1}$ —under LL and HL growth PPFDs (Table 2)

Species	Growth PPFD	σ_{PSII}	n_{PSII}	$a_{\text{PSII}}^{\text{chl}}$
<i>A. anophagefferens</i>	18	1451 (80)	1.05^{-3} (4.83 $^{-5}$)	1.02^{-2} (4.61 $^{-4}$)*
	80	1114 (73)	1.38^{-3} (6.26 $^{-5}$)	1.03^{-2} (8.12 $^{-5}$)*
<i>C. muelleri</i>	50	351 (39)	1.69^{-3} (8.88 $^{-5}$)	3.95^{-3} (8.85 $^{-5}$)
	300	472 (55)	1.92^{-3} (1.15 $^{-4}$)	6.07^{-3} (9.23 $^{-4}$)
<i>D. tertiolecta</i>	18	285 (52)	1.35^{-3} (6.29 $^{-5}$)	2.57^{-3} (9.86 $^{-5}$)
	300	345 (46)	1.86^{-3} (1.97 $^{-4}$)	4.29^{-3} (1.81 $^{-4}$)
<i>E. huxleyi</i>	50	495 (48)	1.54^{-3} (8.78 $^{-5}$)	5.11^{-3} (2.22 $^{-4}$)
	250	579 (61)	1.86^{-3} (1.03 $^{-4}$)	7.21^{-3} (1.58 $^{-4}$)
<i>P. minimum</i>	18	601 (47)	1.87^{-3} (9.04 $^{-5}$)	7.51^{-3} (3.77 $^{-4}$)
	300	909 (71)	2.32^{-3} (1.94 $^{-4}$)	1.41^{-2} (4.14 $^{-4}$)
<i>P. provansolii</i>	18	1257 (120)	1.07^{-3} (4.04 $^{-5}$)	8.99^{-3} (3.03 $^{-4}$)
	300	1005 (132)	1.61^{-3} (1.32 $^{-4}$)	1.01^{-2} (5.89 $^{-4}$)
<i>T. weissflogii</i>	18	270 (49)	1.71^{-3} (8.21 $^{-5}$) *	2.99^{-3} (4.00 $^{-4}$)
	80	344 (38)	1.80^{-3} (1.03 $^{-4}$) *	4.15^{-3} (3.77 $^{-4}$)
<i>R. salina</i>	75	409 (46)*	1.96^{-3} (1.02 $^{-4}$) *	5.37^{-3} (1.22 $^{-4}$)*
	375	370 (52)*	2.12^{-3} (9.98 $^{-5}$) *	5.26^{-3} (1.93 $^{-4}$)*
<i>S. major</i>	18	351 (47)	1.93^{-3} (1.20 $^{-4}$)	4.50^{-3} (2.78 $^{-4}$)*
	300	291 (44)	2.25^{-3} (2.23 $^{-4}$)	4.39^{-3} (2.15 $^{-4}$)*
<i>Synechococcus</i> 1479/9	75	153 (49)	3.58^{-3} (3.38 $^{-4}$)	3.68^{-3} (3.33 $^{-4}$)
	375	234 (66)	4.12^{-3} (5.27 $^{-4}$)	6.45^{-3} (2.23 $^{-4}$)
<i>Synechococcus</i> WH7803	50	268 (56)	3.41^{-3} (1.98 $^{-4}$)	6.13^{-3} (9.01 $^{-5}$)
	400	375 (69)	4.23^{-3} (4.76 $^{-4}$)	1.14^{-2} (2.28 $^{-4}$)

$a_{\text{PSII}}^{\text{chl}}$ was calculated following Eq. 1 ($\sigma_{\text{PSII}} \times n_{\text{PSII}} \times 0.00675$, Table 1). Values were significantly different (t test, $P < 0.05$) between LL and HL unless otherwise indicated (*). Species are separated as (upper) chromophytes and chlorophytes, (middle) cryptophytes, and (lower) cyanobacteria.

and 700 nm. These absorption reconstructions were then normalized to [chl a] (Table 1).

Determination of a_{PS}^* was not possible for the phycobiliprotein containing cryptophytes and cyanobacteria because phycobiliproteins were not measured. Instead, phycobilin absorption was estimated by assuming that the proportion of chlorophyll a -specific absorption, a^{chl} , not accounted for by $a_{\text{PS+PP}}^*$ was from phycobilins, a_{bilins}^* .

The difference between a^{chl} and $a_{\text{PS+PP}}^*$ in nonphycobilin-containing species indicates that packaging was spectrally dependent (Fig. 3C). This nonlinear dependence of packaging on wavelength across the photosynthetically active spectrum presumably reflects different extents of packaging for the various pigments (Lorenz et al. 2003). Estimation of a_{bilins}^* required that the proportion of packaging was determined where phycobilins did not absorb. We treated packaging ($a_{\text{PS+PP}}^*$ vs. a^{chl}) as a linear function of wavelength between 440 and 676 nm because absorption by chlorophylls and carotenoids is

highest while absorption by phycobilins is lowest (Fig. 3) at 440 and 676 nm:

$$a^{\text{pack}}(\lambda) = \left[\frac{\left(\frac{a_{\text{PS+PP}}^*}{a^{\text{chl}}} \right)_{440} - \left(\frac{a_{\text{PS+PP}}^*}{a^{\text{chl}}} \right)_{676}}{676 - 440} \right] \cdot \lambda \quad (3)$$

a^{pack} (dimensionless, Eq. 3) for the entire photosynthetically active spectrum yielded estimates of packaging that were $16 \pm 6\%$ of $a_{\text{PS+PP}}^*/a^{\text{chl}}$ for nonphycobilin species (data not shown). a_{bilins}^* was subsequently calculated as (see Fig. 3)

$$a_{\text{bilins}}^*(\lambda) = a^{\text{chl}}(\lambda) - \left(\frac{a_{\text{PS+PP}}^*(\lambda)}{a^{\text{pack}}(\lambda)} \right) \quad (4)$$

The lower values of a^{pack} than of $a_{\text{PS+PP}}^*/a^{\text{chl}}$ for nonphycobilin-containing taxa suggest that use of a^{pack} may result in an overestimate of a_{bilins}^* for the cryptophytes and cyanobacteria. However, this source of error is likely to be minimal for

cyanobacteria because they exhibit relatively low levels of pigment packaging (Fig. 3D).

Finally, the effective chlorophyll *a*-specific absorption coefficients of PS, PP, and phycobilins were weighted to the spectrum of light emitting diodes (LED) excitation from the FRR fluorometer used to generate $\sigma_{\text{PSII}}(\bar{a}_{\text{PS}}^*, \bar{a}_{\text{PP}}^*, \bar{a}_{\text{bilins}}^*)$

$$\bar{a}_{\text{PS (PP) (bilins)}}^* = \sum_{\text{PS (PP) (bilins)}} \left(\frac{\sum_{700}^{400} a_{\text{PS (PP) (bilins)}}^*(\lambda) \cdot E(\lambda)}{\sum_{700}^{400} E(\lambda)} \right) \quad (5)$$

where *E* is the irradiance provided by the FRR fluorometer LEDs (Suggett et al. 2003) for all wavelengths between 400 and 700 nm (Fig. 3).

Spectrofluorescence—Fluorescence excitation spectra were measured in 10 mm quartz cuvettes between 400 and 800 nm using a Perkin Elmer LS50B (ES, Perkin-Elmer) or a Shimadzu RF-5301 (HP, Shimadzu Scientific Instruments). The emission monochromator was set to 730 nm (F_{730}) because 730 nm fluorescence has been shown to correspond with O_2 evolution, and therefore PSII action spectra (Neori et al. 1988), but also minimizes potential reabsorption of fluorescence (Hofstraat et al. 1992). Each sample was treated with 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU, <1% ethanol, to a final concentration of 20mM) and pre-illuminated for 5 minutes to penetrate and saturate PSII reaction centres (Johnsen and Sakshaug 1993). Triplicate spectra of each sample were made. Blanks consisted of filtrate (see FRR fluorometry section).

The LS50B (ES) was set to correct the raw F_{730} excitation spectra for distortions produced by the excitation xenon light source. This procedure was verified using (1) an independent measure of the excitation light incident on the sample position of the Perkin-Elmer LS50B (using an SR9000 spectroradiometer) to correct the raw excitation spectra and (2) corresponding F_{730} and a^{chl} measurements of chlorophyll *a* standard (Sigma) in 90% acetone (Lutz et al. 1998, 2001). The excitation light of the Shimadzu RF-5301 (HP) was not measured and therefore F_{730} and a^{chl} measurements of chlorophyll *a* standard in 90% acetone spectra were relied upon to provide a shape correction to subsequent F_{730} measurements.

Assessment

Comparison of biophysical estimates of $a^{\text{chl}}_{\text{PSII}}$ with optical estimates of a^{chl} —The biophysical estimate of the chlorophyll *a*-specific absorption coefficient for PSII ($a^{\text{chl}}_{\text{PSII}}$) was calculated as the product of σ_{PSII} and n_{PSII} (Table 1). Values of $a^{\text{chl}}_{\text{PSII}}$ varied by a factor of 5 between species and were highest for *A. anophagefferens* and lowest for *D. tertiolecta* (Table 3).

Biophysical estimates of $a^{\text{chl}}_{\text{PSII}}$ were compared with the chlorophyll *a*-specific absorption coefficient (a^{chl}) following Suggett et al. (2003). This comparison requires that we specify the proportion of a^{chl} that contributes excitation energy to PSII. First, a^{chl} was weighted to the spectrum of LED excitation from the FRR fluorometer,

Table 4. Mean (and standard deviation) chlorophyll *a*-specific absorption of photosynthetic pigments (PS and phycobilins, $\bar{a}_{\text{PS+bilins}}^{\text{chl}}$) and proportion of total light absorbed by PSII (*P*, dimensionless)

Species	Growth		<i>P</i>
	PPFD	$\bar{a}_{\text{PS+bilins}}^{\text{chl}}$	
<i>A. anophagefferens</i>	18	2.9 ⁻² (3.35 ⁻⁴)*	0.36 (0.010)
	80	3.1 ⁻² (2.48 ⁻⁴)*	0.34 (0.009)
<i>C. muelleri</i>	50	6.4 ⁻³ (8.22 ⁻⁵)	0.58 (0.012)*
	300	1.0 ⁻² (3.15 ⁻⁴)	0.57 (0.021)*
<i>D. tertiolecta</i>	18	6.1 ⁻³ (8.06 ⁻⁵)	0.42 (0.014)*
	300	8.2 ⁻³ (9.11 ⁻⁵)	0.43 (0.009)*
<i>E. huxleyi</i>	50	8.6 ⁻³ (9.38 ⁻⁵)	0.55 (0.008)*
	250	1.4 ⁻² (1.11 ⁻⁴)	0.56 (0.011)*
<i>P. minimum</i>	18	1.6 ⁻² (2.71 ⁻⁴)	0.53 (0.011)*
	300	2.6 ⁻² (1.97 ⁻⁴)	0.54 (0.006)*
<i>P. provasolii</i>	18	1.4 ⁻² (2.58 ⁻⁴)	0.53 (0.011)*
	300	1.7 ⁻² (3.06 ⁻⁴)	0.53 (0.009)*
<i>T. weissflogii</i>	18	7.3 ⁻³ (1.26 ⁻⁴)*	0.52 (0.010)*
	80	7.7 ⁻³ (1.33 ⁻⁴)*	0.51 (0.022)*
<i>R. salina</i>	75	9.4 ⁻³ (1.04 ⁻⁴)	0.51 (0.017)
	375	1.2 ⁻² (8.99 ⁻⁵)	0.54 (0.009)
<i>S. major</i>	18	1.0 ⁻² (1.60 ⁻⁴)	0.49 (0.007)*
	300	1.4 ⁻² (1.21 ⁻⁴)	0.48 (0.012)*
<i>Synechococcus</i> 1479/19	75	1.7 ⁻² (1.74 ⁻⁴)	0.25 (0.012)*
	375	2.6 ⁻² (2.49 ⁻⁴)	0.27 (0.008)*
<i>Synechococcus</i> WH7803	50	1.8 ⁻² (2.02 ⁻⁴)	0.32 (0.017)
	400	4.1 ⁻² (1.94 ⁻⁴)	0.30 (0.007)

Both $\bar{a}_{\text{PS+bilins}}^{\text{chl}}$ (Eq. 7) and *P* (Eq. 9) are weighted to the excitation from the FRR fluorometer LEDs (*E*). Species separation and (*) as for Table 3.

$$\bar{a}^{\text{chl}} = \frac{\sum a^{\text{chl}}(\lambda) \cdot E(\lambda)}{\sum E(\lambda)} \quad (6)$$

The spectrally weighted values of \bar{a}^{chl} varied by a factor of 10 between species and were 5% to 10% lower than values of a^{chl} observed solely at the peak wavelength of FRR excitation (478 nm) (data not shown).

Second, because some absorbed light is not directed to photochemistry, a further correction was applied to account for the proportion of light absorption by photosynthetically active pigments (see Babin et al. 1996) ($\bar{a}_{\text{PS+bilins}}^{\text{chl}}$),

$$\bar{a}_{\text{PS+bilins}}^{\text{chl}} = \bar{a}^{\text{chl}} \cdot \left(\frac{\bar{a}_{\text{PS+bilins}}^*}{\bar{a}_{\text{PS+bilins+PP}}^*} \right) \quad (7)$$

Values of $\bar{a}_{\text{PS+bilins}}^{\text{chl}}$ varied by a factor of 6 between species and were highest for *A. anophagefferens* and lowest for *D. tertiolecta* (Table 4).

Third, because light-harvesting pigments are not distributed evenly between photosystems I and II, another correction was applied to account for the proportion of light absorbed by photosynthetic pigments, which is directed toward PSII (*P*)

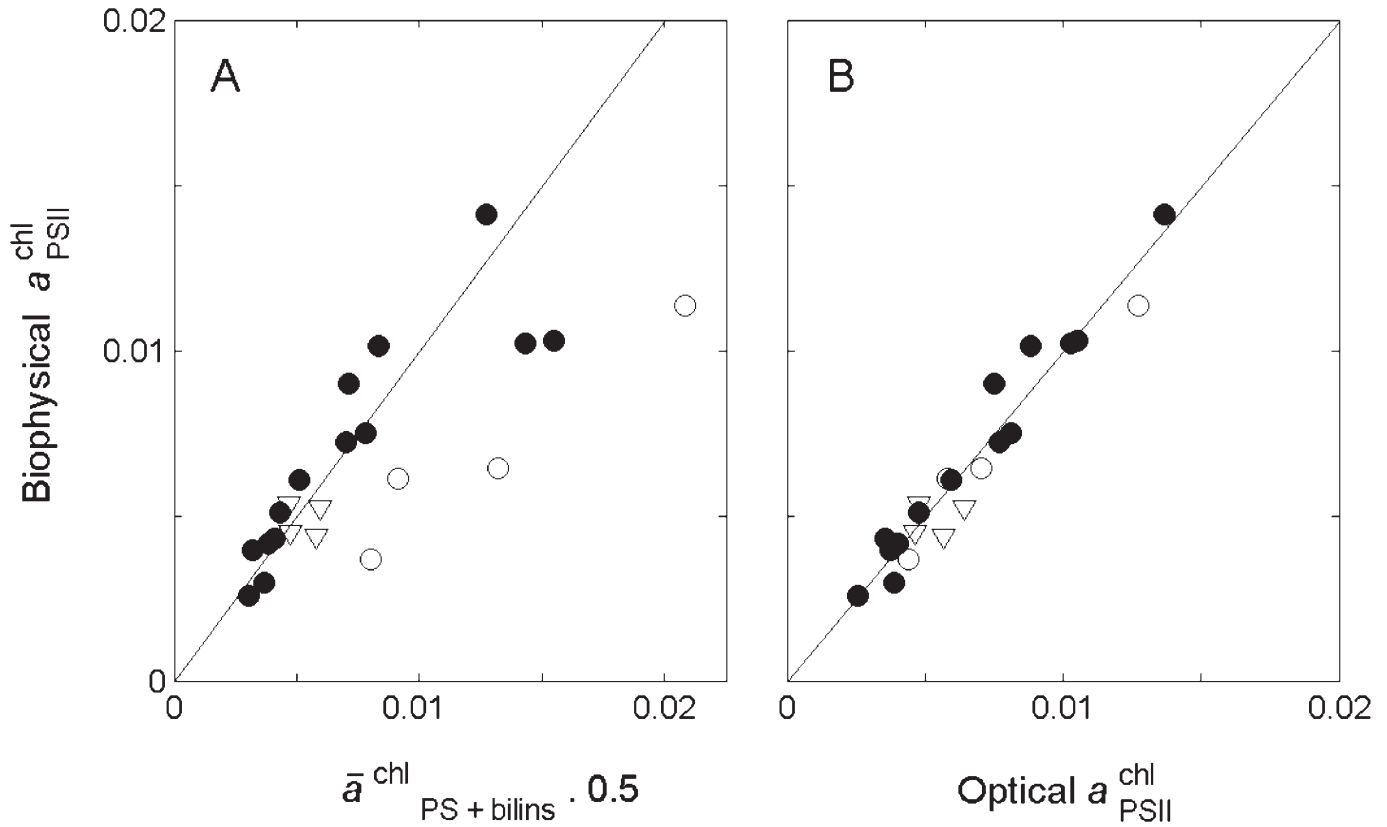


Fig. 4. Comparison of the chlorophyll a -specific absorption for PSII— $a_{\text{PSII}}^{\text{chl}}$, $\text{m}^2 (\text{mg chl } a)^{-1}$ —estimated biophysically (Eq. 1, see Table 1) with that estimated optically (Eq. 8) for chromophytes and chlorophytes (●), cryptophytes (▽), and *Synechococcus* species (○). For A, the proportion of light absorbed by PSII for Eq. 8 is assumed to be 0.5. For B, P was estimated using the ratio of 730 nm fluorescence excitation to chlorophyll a -specific light absorption by photosynthetically active pigments (Eq. 9, Fig. 5). Regression equations for (A) non-phyco bilin containing species, biophysical $a_{\text{PSII}}^{\text{chl}} = 0.910 \times (\text{optical} \times 0.5)$ ($r^2 = 0.684$, $n = 14$, $P < 0.001$); all species, biophysical $a_{\text{PSII}}^{\text{chl}} = 0.756 \times (\text{optical} \times 0.5)$ ($r^2 = 0.427$, $n = 22$, $P < 0.001$); (B) all species, biophysical $a_{\text{PSII}}^{\text{chl}} = 0.987 \times \text{optical } a_{\text{PSII}}^{\text{chl}}$ ($r^2 = 0.937$, $n = 22$, $P < 0.001$). For data in panel B, the standard error of the slope was ± 0.022 and not significantly different from a value of 1.0 (one-sided t test). All points are the mean from triplicate measurements.

$$\text{Optical } a_{\text{PSII}}^{\text{chl}}(\lambda) = \bar{a}_{\text{PS+bilins}}^{\text{chl}}(\lambda) \cdot P(\lambda) \quad (8)$$

Previously, P has been chosen to be 0.5 on the assumption that 50% of excitation energy is delivered to PSII and 50% to PSI for linear electron flow (Gilbert et al. 2000; Kromkamp and Forster 2003). Consistent with this assumption, we found that biophysical estimates of $a_{\text{PSII}}^{\text{chl}}$ (Eq. 1, Table 1) were correlated with $\bar{a}_{\text{PS+bilins}}^{\text{chl}} \cdot 0.5$ (Fig. 4A). This relationship could be significantly improved upon removal of data for the cryptophyte and cyanobacteria (Fig. 4A legend).

We estimated P for all taxa using a scaling approach appropriate to the FRR fluorometer excitation spectrum, as follows. The areas under corresponding fluorescence excitation (730 nm emission), F_{730}' , and absorption spectra were set equal to unity (F_{730}' , a^{chl} , respectively). Both spectra would have identical shapes only if the efficiency of excitation energy transferred to PSII and, by implication PSI, was the same at all wavelengths. Preferential absorption and transfer of energy into PSI at any given wavelength is indicated when $F_{730}'(\lambda) < a^{\text{chl}}(\lambda)$ (Fig. 5).

$F_{730}'(\lambda)$ is the result of light absorbed and transferred to PSII and should be compared with $\bar{a}_{\text{PS+bilins}}^*$ as opposed to $a^{\text{chl}}(\lambda)$ to account for the proportion of light absorbed solely by photochemically active pigments of PSII (Fig. 5). Therefore, we calculated P as follows:

$$P = 0.5 \cdot \left(\frac{\sum_{700}^{400} \left(\frac{F_{730}'(\lambda)}{a_{\text{PS+bilins}}^*(\lambda)} \right) \cdot E(\lambda)}{\sum_{700}^{400} E(\lambda)} \right) \quad (9)$$

where the area under F_{730}' and $a_{\text{PS+bilins}}^*$ spectra between 400 and 700 nm were set equal to 1 (F_{730}' , $a_{\text{PS+bilins}}^*$). P was weighted by the excitation spectrum of the FRR fluorometer LEDs, $E(\lambda)$. The coefficient 0.5 is based on the assumption that the total excitons absorbed for photochemistry (400 to 700 nm) are divided equally between PSI and PSII. This assumption would be the case for linear electron flow. However, any cyclic electron flow around PSI would lead us to overestimate P .

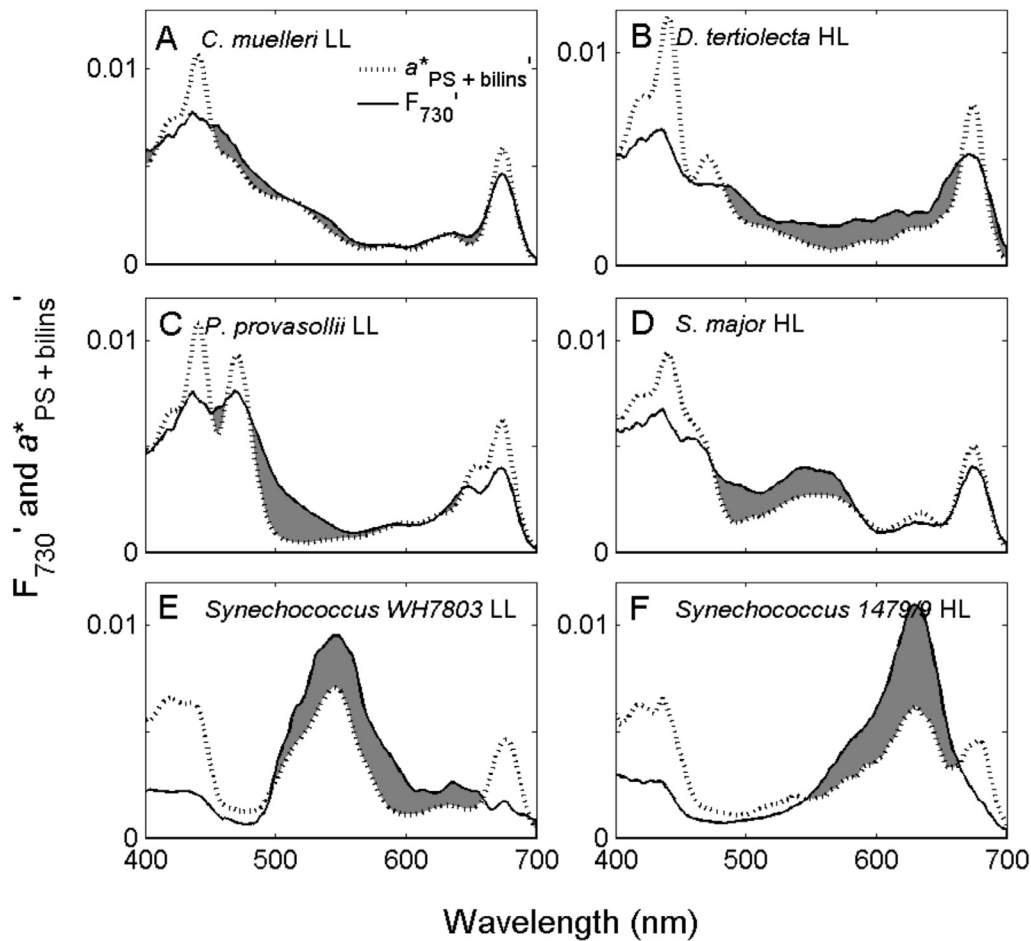


Fig. 5. Mean spectra of 730 nm fluorescence excitation (F_{730} , solid line) and absorption reconstructed from photosynthetically active pigments ($a_{PS+bilins}^*$, dotted line, Eq. 2 and 4). The areas under both F_{730} and $a_{PS+bilins}^*$ spectra were set to unity (F_{730}' and $a_{PS+bilins}'$, respectively). Wavelengths at which fluorescence exceeds absorption are shaded and indicate preferential (>50%) excitation of PSII over PSI. In contrast, wavelengths at which absorption exceeds fluorescence are left unshaded and indicate preferential (>50%) excitation of PSI over PSII. Examples are given from measurements made at Horn Point (HP) and the University of Essex (ES) and under LL and HL growth (Table 2).

P varied from 0.34 to 0.58 for nonphycobilin-containing species and 0.27 to 0.32 for phycobilin-containing species (Table 4, Fig. 5). The lowest values of P , were observed for *Synechococcus* species, and reflect the low proportion of absorption associated with PSII relative to PSI at 480 nm (Johnsen and Sakshaug 1996; Lutz et al. 2001). Values of P were not significantly different between LL and HL growth treatments for the majority of species (Table 4).

Once all corrections were applied, biophysical estimates of a_{PSII}^{chl} (Eq. 1, Table 1) were highly correlated with optical estimates of a_{PSII}^{chl} (Eq. 8) with a slope close to unity and zero intercept (Fig. 4B). This relationship was weaker when our preferred determination of P (Eq. 9) was replaced by alternative scaling approaches that have been described previously (see Appendix I).

σ_{PSII} from mixtures—Measurements of σ_{PSII} were made following mixing of individual, nutrient-replete cultures of *P. minimum* HL, *R. salina* HL, *D. tertiolecta* LL, and *E. huxleyi* LL (Fig. 6). As expected, we found that the maximum fluores-

cence yield observed varied according to the proportion of mixing of two populations within a sample

$$\text{Expected } F_m = x_1 \cdot F_{m1} + x_2 \cdot F_{m2} \quad (10)$$

where x_1 and x_2 are the proportions of populations 1 and 2 in the mixture and F_{m1} and F_{m2} are the maximum fluorescence yields of populations 1 and 2 prior to mixing. Analogously, the expected σ_{PSII} for the mixture was obtained by weighting the fluorescence signal from each population following mixing as

$$\text{Expected } \sigma_{PSII} = \frac{x_1 \cdot F_{m1} \cdot \sigma_{PSII1} + x_2 \cdot F_{m2} \cdot \sigma_{PSII2}}{x_1 \cdot F_{m1} + x_2 \cdot F_{m2}} \quad (11)$$

where σ_{PSII1} and σ_{PSII2} are the initial effective absorption cross-sections prior to mixing.

A proportional change of σ_{PSII} was observed when cultures were mixed, provided that the two populations had approximately equivalent initial maximum fluorescence yield, F_m

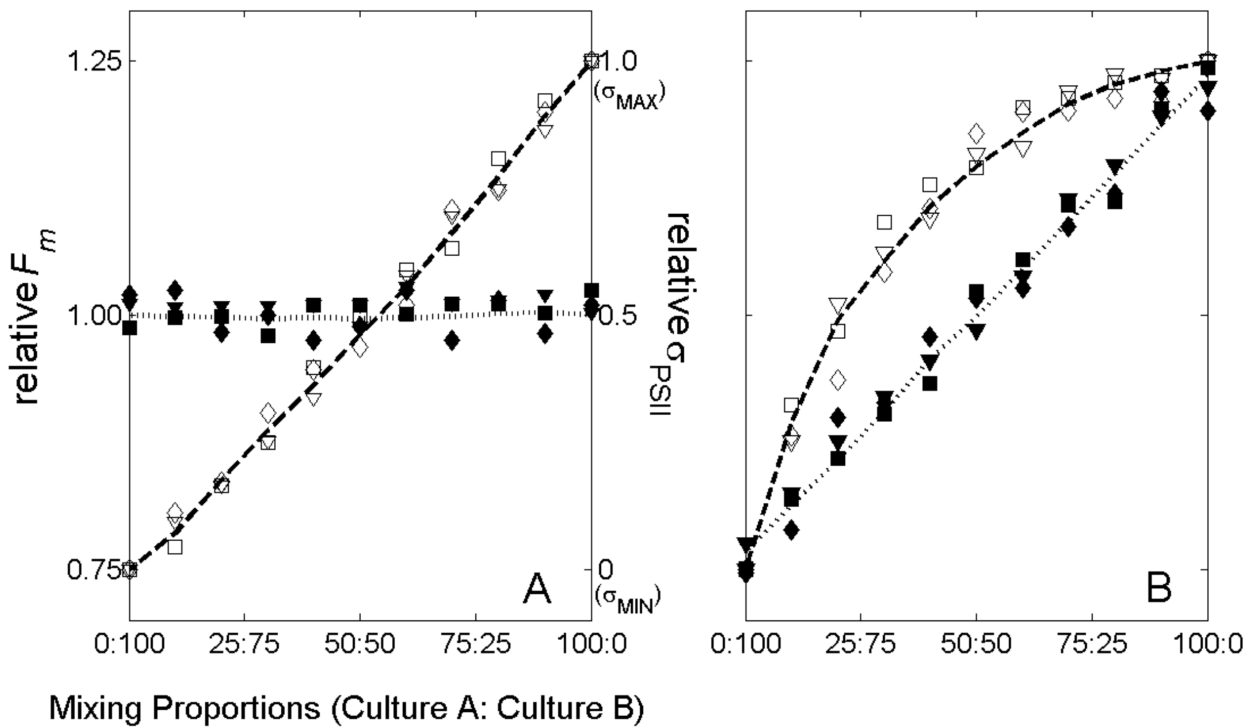


Fig. 6. F_m (closed symbols) and σ_{PSII} (open symbols) from mixed algal cultures. Culture A: B mixtures were *P. minimum* HL:*R. salina* HL (\diamond), *P. minimum* HL:*D. tertiolecta* LL (\square) and *P. minimum* HL:*E. huxleyi* LL (∇). Mixing was performed with (A) initial F_m that were equivalent (to within 2% to 5%) for both cultures or (B) initial F_m for culture A (always *P. minimum*) was twice that of culture B (F_o was unaltered). In either case, only one of the cultures was diluted with fresh media. 15 ST were collected for each mixture and averaged to 1 Ex/Em (see Materials and methods). ρ was set to zero for fitting the Kolber et al. (1998) model. Dotted and dashed lines are expected values of F_m and σ_{PSII} following Eq. 10 and 11, respectively. F_m and σ_{PSII} are expressed as relative values to account for differences in absolute values between experiments. All mixing proportions were made up to a 10 mL suspension.

(Fig. 6A). Conversely, a disproportionate response of σ_{PSII} was observed when populations with different initial F_m were mixed (Fig. 6B). In the latter case, σ_{PSII} changed little from the value exhibited by the population with the higher initial F_m until the mixing ratio approached an approximately equivalent fluorescence yield for the two populations. This simple exercise demonstrates that the mixing of populations will be a crucial determinant of σ_{PSII} in situ.

Indirect determination of n_{PSII} —The close relationship between biophysical and optical absorption by PSII (Fig. 4, Table 6) indicates that n_{PSII} can be derived from independent measures of σ_{PSII} , a^{chl} , F_{730} and pigment concentrations. Rearrangement of Eq. 1 and 8 yields:

$$\sigma_{\text{PSII}} \cdot n_{\text{PSII}} \cdot 0.00675 = a^{\text{chl}}_{\text{PSII}} = \frac{\bar{a}^{\text{chl}}_{\text{PS+bilins}} \cdot P}{\sigma_{\text{PSII}} \cdot 0.00675} \quad (12)$$

$$n_{\text{PSII}}^{\text{derived}} = \frac{\bar{a}^{\text{chl}}_{\text{PS+bilins}} \cdot P}{\sigma_{\text{PSII}} \cdot 0.00675}$$

where 0.00675 is a unit conversion constant (Tables 1 and 3). $n_{\text{PSII}}^{\text{derived}}$ (Eq. 12) compared well with n_{PSII} measured from O_2 flash yields (Fig. 7A). We found that $n_{\text{PSII}}^{\text{derived}}$ was within $\pm 2\%$ to 25% of the measured n_{PSII} .

Our direct approach to deriving n_{PSII} (Eq. 12) is more robust than the approach that is currently employed to estimate n_{PSII} (Falkowski and Kolber 1995; Babin et al. 1996; Suggett et al. 2001)

$$n_{\text{PSII}}^{\text{estimated}} = \left(\frac{F_v/F_o}{1.8} \right) \cdot n_{\text{PSII}}^* \quad (13)$$

where F_v/F_o ($= F_m - F_o/F_o$) is a variable fluorescence factor indicative of the proportion of reaction centers that are capable of evolving oxygen, and 1.8 is the maximum value measured for F_v/F_o (see Babin et al. 1996). n_{PSII}^* is assumed to equal either 0.0020 or 0.0033 mol O_2 (mol chl a^{-1}) respectively for eukaryotes or cyanobacteria (Falkowski and Kolber 1995). In Eq. 13, the term $(F_v/F_o)/1.8$ provides an estimate of reaction center functionality for an assumed n_{PSII} . Values of $n_{\text{PSII}}^{\text{estimated}}$ were $\pm 5\%$ to 50% (eukaryotes) and -40% to 90% (cyanobacteria) of the measured n_{PSII} and were not significantly correlated with n_{PSII} measured from the O_2 flash yields (Fig. 7B).

Discussion

Biophysical determination of $a^{\text{chl}}_{\text{PSII}}$ —Use of FRR fluorescence to estimate primary productivity relies on an estimate of light absorption that is specific to PSII photochemistry. This estimate is obtained from the effective cross-section (σ_{PSII}) and size

Table 5. Summary of forward stepwise regression performed between the effective absorption cross-section measured by the FRR fluorometer, σ_{PSII} ($\text{\AA}^2 \text{ quanta}^{-1}$, dependent variable), and several variables known to influence light absorption ($n = 22$, $F = 26.88$, $P < 0.001$)

Variable	Coefficient	<i>t</i>	<i>P</i>	<i>r</i> ²
$\bar{a}^*_{\text{PS+bilins}}; \bar{a}^*_{\text{PP}}$	2.692	0.83	0.319	0.335
$1/n_{\text{PSII}}$ (mol chl <i>a</i> : mol RCII ⁻¹)	1.503	6.46	0.000	0.214
Packaging (a^{pack}_{478} Dimensionless)	-200.67	-5.16	0.000	0.269
Constant	162.1	1.41	0.177	
<i>r</i> ² total (adjusted)				0.788

The chlorophyll *a*-specific effective absorption was determined for photosynthetic, nonphotosynthetic, and phycobilin and pigments (Eq. 5). Photosynthetic pigments (PS) were chlorophylls *b* and *c*, 19'-hexanoyloxyfucoxanthin, fucoxanthin, and peridinin; nonphotosynthetic pigments (PP) were alloxanthin, β -carotene, diadinoxanthin, diatoxanthin, lutein, prasinoxanthin, and zeaxanthin (Bidigare et al. 1990; Babin et al. 1996). n_{PSII} was measured directly by O₂ flash yields and a^{pack} was estimated from corresponding optical and reconstructed absorption spectra (Eq. 3, see main text).

(n_{PSII}) of the photosynthetic unit (Falkowski and Kolber 1995). σ_{PSII} and n_{PSII} varied by factors of 9.5 and 4, respectively, between algal taxa. σ_{PSII} was highest for *A. anophagefferens* and *P. provasolii* and lowest for the *Synechococcus* species whereas the opposite trend was observed for n_{PSII} (Table 3).

σ_{PSII} is determined by factors affecting the functional absorption 'target' (Fig. 8, Table 5). Both the inverse of the PSII reaction center pool size ($1/n_{\text{PSII}}$) and pigment packaging (a^{pack}) were significantly related to σ_{PSII} . However, the ratio of absorption by photosynthetic to nonphotosynthetic pigments, accounted for 33% of the variability of σ_{PSII} and was the strongest single predictor of σ_{PSII} (Table 5). Additional factors, for example, pigment-specific excitation energy transfer efficiencies (Sukenic et al. 1987, Mauzerall and Greenbaum 1989)

and possible measurement errors may also contribute to the observed variability of σ_{PSII} .

$\bar{a}^*_{\text{PS+bilins}}$; \bar{a}^*_{PP} and a^{pack} can be determined from natural waters. In contrast, routine determination of n_{PSII} from natural waters is largely precluded by the insensitivity of the flash-yield approach. Instead, n_{PSII} has been estimated from constant n_{PSII} and $(F_{730}/F_o)/1.8$ (Eq. 13) (Falkowski and Kolber 1995; Babin et al. 1996; Boyd et al. 1997; Suggett et al. 2001; Moore et al. 2003; Raateoja et al. 2004). Our data suggest that this estimate is subject to considerable uncertainty and hence will contribute to large errors in fluorescence-based determinations of productivity. This uncertainty arises from limitations in use of the assumed values of n_{PSII} (n_{PSII}) for prokaryotes and eukaryotes and the use of F_{730}/F_o of 1.8 to assess reaction center functionality. Maximum values of F_{730}/F_o may vary between species, reflecting differences in xanthophyll acclimation, transthylakoid (Δ)pH potential, spill-over from PSII to PSI, and reaction center versus antenna quenching. In particular, fluorescence from phycocyanin-containing *Synechococcus* species is known to contaminate the chlorophyll *a* fluorescence emission and cause an artefactual increase in chlorophyll *a*-specific F_o (Campbell et al. 1998) and, therefore, a reduction in F_{730}/F_o .

Smallest values of σ_{PSII} are typically observed for *Synechococcus* species (Table 3; see also Koblížek et al. 2001; Raateoja et al. 2001). Compared to eukaryotic phytoplankton, cyanobacteria have a high n_{PSII} (Barlow and Alberte 1985) and much lower ratios of PSII to PSI (Barlow and Alberte 1985; Berges et al. 1996; Falkowski and Raven 1997). In addition, most chlorophyll *a* is associated with PSI. Phycobiliproteins dominate the pigment assembly of PSII in cyanobacteria and absorb light at longer wavelengths than most carotenoids. Therefore, absorption coefficients (Bidigare et al. 1990; Falkowski and Raven 1997) and/or transfer efficiencies (Mauzerall and Greenbaum 1989; Hu et al 1999) for phycobilipigments are less effective than chlorophylls and accessory carotenoids for saturating PSII with energy provided by the blue excitation waveband of

Table 6. Regression statistics from comparisons of biophysical and optical $\alpha^{\text{chl}}_{\text{PSII}}$

Estimation of <i>P</i> for optical $\alpha^{\text{chl}}_{\text{PSII}}$	Normalization of spectra	Regression of biophysical (y) versus optical (x) $\alpha^{\text{chl}}_{\text{PSII}}$	
		Coefficient	<i>r</i> ²
Eq. 9	$[F_{730} \alpha^*_{\text{PS+bilins}} = 1]_{\lambda, 400-700\text{nm}}$	0.987	0.937
0.5 · $\left(\frac{\sum_{700}^{400} \left(\frac{F_{730}'(\lambda)}{a^{\text{chl}}(\lambda)} \right) \cdot E(\lambda)}{\sum_{700}^{400} E(\lambda)} \right)$	$[F_{730} \alpha^{\text{chl}} = 1]_{\lambda, 400-700\text{nm}}$	1.188	0.838
	$F_{730} = \alpha^{\text{chl}}$ at 676 nm	0.678	0.820
	$F_{730} = \alpha^{\text{chl}}$ at 545 nm	0.806	0.843

Different approaches were used to estimate *P* for the optical determination of $\alpha^{\text{chl}}_{\text{PSII}}$ ($\bar{a}^*_{\text{PS+bilins}}$, *P*, Eq. 8). In all cases, *P* is the difference between F_{730}' and $\alpha^*_{\text{PS+bilins}}$ (Eq. 9), or F_{730}' and α^{chl} , weighted to the FRR fluorometer LEDs and following normalization of spectra. Normalization is by (1) setting the area under both spectra to unity (our method); (2) setting F_{730} equal to α^{chl} at 676 nm (Sakshaug et al. 1991; Johnsen and Sakshaug 1993; Sosik and Mitchell 1995); (3) setting F_{730} equal to α^{chl} at 545 nm (Lutz et al. 1998, 2001). For all regressions, $n = 22$, $P < 0.001$. The intercept was set to zero throughout.

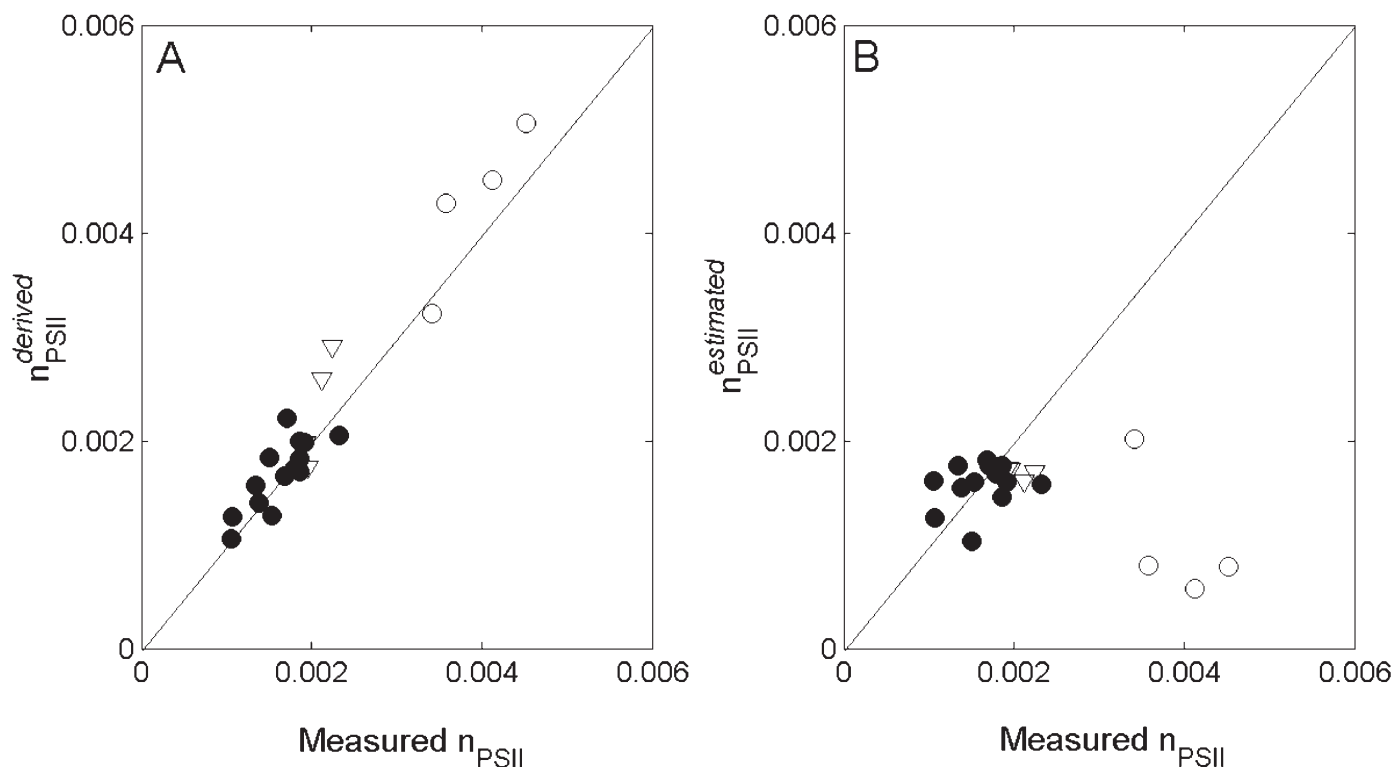


Fig. 7. Comparison of n_{PSII} (mol RCII, mol chl a^{-1}) measured from the O_2 flash yields with (A) $n_{PSII}^{derived}$ from measurements of σ_{PSII} , a^{chl} , F_{730} , and pigment concentrations (Eq. 12) and (B) $n_{PSII}^{estimated}$ from the approach of Falkowski and Kolber (1995), Babin et al. (1996) (Eq. 13). For A, $n_{PSII}^{derived} = 1.066 \times n_{PSII}$ ($r^2 = 0.937$, $n = 22$, $P < 0.001$); values of $n_{PSII}^{estimated}$ were not significantly related with measured n_{PSII} . Symbols are as Fig. 4.

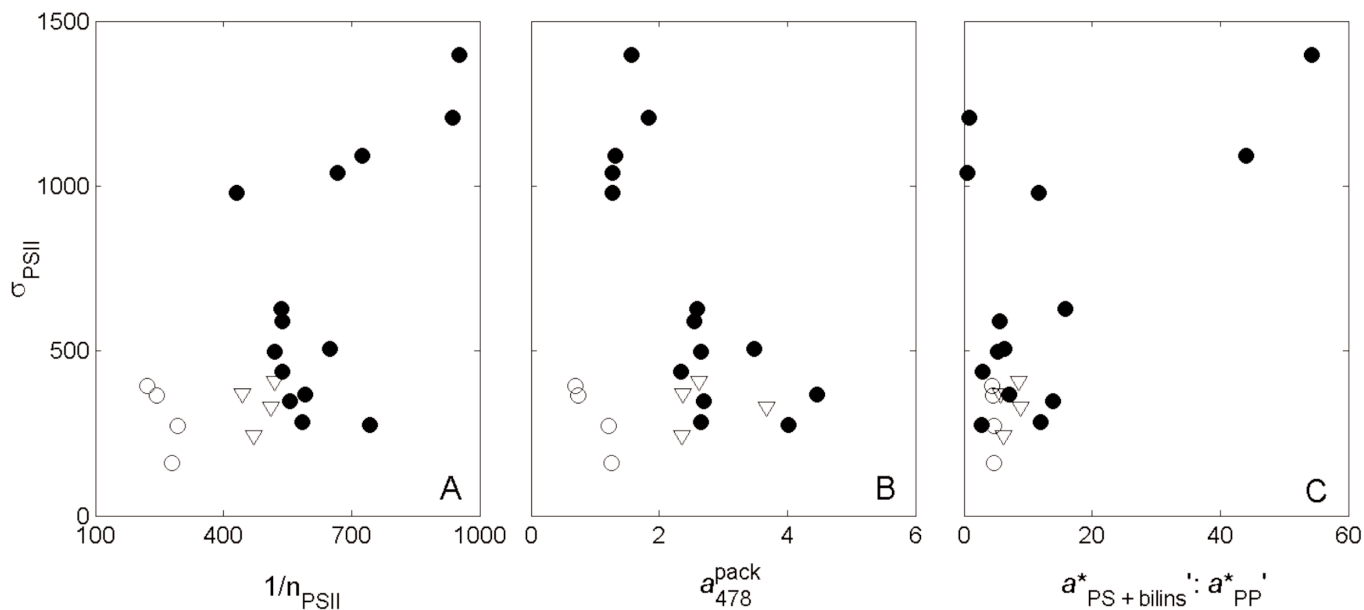


Fig. 8. Comparison of the absorption cross-section of PSII, σ_{PSII} , \AA^2 (quanta) $^{-1}$, from the FRR fluorometer with (A) n_{PSII} , mol RCII (mol chl a^{-1}), measured from O_2 flash yields. $\sigma_{PSII} = 1.234 \times 1/n_{PSII} - 116$ ($r^2 = 0.457$, $n = 22$, $P < 0.001$); (B) a_{478}^{pack} at the peak wavelength of FRR excitation (478 nm). $\sigma_{PSII} = -115 \times a_{478}^{pack} + 815$ ($r^2 = 0.187$, $n = 22$, $0.05 > P > 0.02$). (C) $a_{PS+bilins}^* : a_{PP}^*$, the ratio of chlorophyll a -specific effective absorption by photosynthetic pigments (PS, chlorophylls b and c , 19'-hexanoyloxyfucoxanthin, fucoxanthin, and peridinin) and phycobilins to nonphotosynthetic pigments (PP, alloxanthin, β -carotene, diadinoxanthin, diatoxanthin, lutein, prasinoxanthin, and zeaxanthin). $\sigma_{PSII} = 15.4 \times a_{PS+bilins}^* : a_{PP}^* + 393$ ($r^2 = 0.335$, $n = 22$, $0.005 > P > 0.002$). Symbols are as Fig. 4.

the FRR fluorometer. It is also possible that cyanobacterial σ_{PSII} were underestimated throughout experimentation since, unlike eukaryotic phytoplankton, they can exhibit a state transition-related decrease in σ_{PSII} in the dark (see Behrenfeld and Kolber 1999).

Observations of σ_{PSII} in situ arise from a complicated mix of photoadaptive and photoacclimative (*sensu* Falkowski and Raven 1997) modifications to the light-harvesting complexes. Our laboratory observations indicate that dark-adapted σ_{PSII} is more variable between taxa ($\sim 150\text{--}1450 \text{ \AA}^2 \text{ quanta}^{-1}$) than between growth PPFs for any given species (at most, $\pm 50\%$) (Table 3). Although dark-adapted measurements minimize the extent of variability of σ_{PSII} , we nonetheless conclude that changes of σ_{PSII} in situ cannot be interpreted solely from a physiological viewpoint (Greene et al. 1994; Behrenfeld et al. 1996; Behrenfeld and Kolber 1999; Moore et al. 2003). σ_{PSII} in situ will largely be determined by the fluorescence yield contributed by the dominant species within a mixed algal community. This yield contains signatures of the environment upon physiology (acclimation, stress, and adaptive properties) and pigment biomass. However, algal physiology and biomass are inextricably linked across communities and are both forced by environmental gradients. Therefore, the future key to interpreting σ_{PSII} in situ will rest on both taxonomic and physiological information for the algal community.

a^{chl}_{PSII} and derivation of n_{PSII}—Biophysical and optical estimates of $a^{\text{chl}}_{\text{PSII}}$ were highly correlated with a slope close to 1.0 when P was determined using Eq. 9 (Fig. 4B, Table 6). When $a^*_{\text{PS+ bilins}}$ was replaced with a^{chl} for P in Eq. 9 (Table 6), optical estimates of $a^{\text{chl}}_{\text{PSII}}$ exceeded biophysical estimates by $\sim 20\%$. This indicates that the proportion of light absorbed by nonphotosynthetically active pigments was relatively small for the majority of taxa tested here. However, the ratio of F_{730} to the two absorption parameters is not the same at every excitation wavelength (data not shown) reflecting differences in the contribution of specific pigments to excitation energy transfer to PSII and PSI. Furthermore, the similarity observed when using $a^*_{\text{PS+ bilins}}$ or a^{chl} may reflect difficulties in defining nonphotosynthetically active pigments, for example, the use of a^*_{pp} assumes zero transfer of energy to the chlorophyll pigment-protein complex. This assumption is unlikely to be correct given measured transfer efficiencies for PPs (see Appendix I).

The most basic approach for estimating P is to assume a constant value of 0.5. This yields a relatively close relationship between biophysical and optical $a^{\text{chl}}_{\text{PSII}}$ for eukaryotes (Fig. 4A). Therefore, it is possible to argue that use of any further scaling only accounts for a relatively small proportion of variability for these algae ($\sim 15\%$ to 25% , Fig. 5, Table 6). Fluorescence excitation spectra provide a more accurate means for accounting for the proportion of light absorbed by PSII, in particular for cyanobacteria.

Few direct measurements of absorption by PSII relative to PSI have been made for phytoplankton. Previous indirect

determinations from F_{730} and a^{chl} spectra have shown that cyanobacteria have the lowest proportion of total light absorption by PSII (Johnsen and Sakshaug 1996; Lutz et al. 2001). Berges et al. (1996) measured the effective absorption and concentration of reaction centers for both PSI and PSII in *T. weissflogii* under nutrient replete growth conditions and demonstrated that 46% of total absorption was from photosynthetic pigments associated with PSII. This value is very similar to the value that we report for *T. weissflogii*. However, it should be noted that both values of n_{PSII} and σ_{PSII} measured by Berges et al. (1996) were lower than those measured from the clone used in our investigation. It is difficult to explain these differences in n_{PSII} , in particular, because the values from Berges et al. (1996) are also lower than those reported for the same clone by Dubinsky et al. (1986). The differences in σ_{PSII} may reflect differences between the wavebands used for PSII excitation.

The relationship between biophysical and optical $a^{\text{chl}}_{\text{PSII}}$ and the ability to obtain $n_{\text{PSII}}^{\text{derived}}$, will be affected by the errors associated with individual measurements. Biophysical estimates of $a^{\text{chl}}_{\text{PSII}}$ (Eq. 1) require reliable measurements of σ_{PSII} and, therefore, an accurate description of connectivity (ρ) between PSII reaction centers (Laney 2003). Conversion of n_{PSII} measured as mol O₂:mol chl *a* to mol RCII:mol chl *a* must assume that 4 electrons are always used to produce 1 mol O₂. However, this assumption may be incorrect where the proportion of cyclic to linear electron flow is significant. Furthermore, measurements of n_{PSII} are based on net O₂ evolution rates and may be influenced by cellular O₂ consumption processes, such as photorespiration and Mehler activity. Additions of NaHCO₃ (see Dubinsky et al. 1986) did not change the n_{PSII} that we measured (data not shown), so it is unlikely that photorespiration was significant. However, we cannot discount the possible contribution of Mehler activity.

Artifacts attributable to light scattering and related to factors such as cell size, shape, or lith coverage in coccolithophores may lead to overestimates of optical absorption measurements (Ciotti et al. 2002; Suggett et al. 2003). Optical absorption measurements are further complicated when algal suspensions are taken from natural waters as a result of low algal biomass and the presence of nonalgal particulates. Various methods to compensate for these limitations have been proposed (summarized by Mitchell et al. 2000) but add to the difficulty and resolution with which accurate measurements are made.

Spectral reconstruction techniques can yield a first-order estimate of the differential contribution of photosynthetic (PS) and photoprotectant (PP) pigments to total absorption (Lutz et al. 2001, Lohrenz et al. 2003). However, the accuracy with which this technique can be used is limited as a result of (1) differences of pigment absorption (magnitude and spectral shifts) in solvents compared to absorption by pigment-protein complexes in vivo, (2) the use of a limited number of representative pigment-specific absorption coefficients, and (3) lack of data

on phycobiliprotein concentrations (Sosik and Mitchell 1991, 1995; Johnsen and Sakshaug 1993; Lutz et al. 2001).

Inherent losses due to pigment packaging in reconstructed spectra can be accounted for by normalizing the proportion of absorption by either PS or PP against a^{chl} (Babin et al. 1996; Lutz et al. 2001; Eq. 7) but still assumes that the extent of packaging is equal for all pigments. The variation in absorption between various accessory pigments is not large at the wavelength of peak absorption (~5 nm for either PS or PP; Babin et al. 1996; Lohrenz et al. 2003) but may increase across the entire spectrum and yield significant differences in total absorption by different pigments (Sosik and Mitchell 1991, 1995). This error may also be enhanced when normalizing reconstructed absorption to the narrow band of excitation delivered by the FRR fluorometer. Finally, our estimate of phycobilin absorption (Eq. 4) likely yielded some error, in particular for the cryptophytes. However, optical and biophysical estimates of $a^{\text{chl}}_{\text{PSII}}$ were more divergent from unity and less highly correlated when a^*_{bilins} was excluded (data not shown). Actual phycobiliprotein measurements may help to explain some of the variability associated with optical measurements of $a^{\text{chl}}_{\text{PSII}}$ but also aid the interpretation of σ_{PSII} .

Comments and recommendations

The FRR fluorometer cannot be used as a stand-alone instrument for estimating primary productivity. Additional measurements of chlorophyll concentration and n_{PSII} are required to obtain volumetric rates from the reaction center specific rates provided by the FRR fluorometer (Kolber and Falkowski 1993; Suggett et al. 2001; Moore et al. 2003). Additional measurements are also required to extrapolate from σ_{PSII} at 478 nm to σ_{PSII} across the photosynthetically active band (Suggett et al. 2001; Raateoja et al. 2004). Based on the validation of FRR determinations of σ_{PSII} described in this paper, we make the following recommendations for additional bio-optical measurements that should be made in conjunction with FRR fluorometry to obtain more accurate biophysical estimates of primary productivity.

We recommend that the concentration of PSII reaction centers, designated [RCII] (Table 1), be obtained by dividing the rate of light absorption corrected for nonphotosynthetic pigments and the proportion of excitation energy directed to PSII (P), by σ_{PSII} .

$$[\text{RCII}] = \left(\frac{\bar{a}_{\text{PS+bilins}} \cdot P}{\sigma_{\text{PSII}} \cdot 6023} \right) \quad (14)$$

where [RCII] is the concentration of reaction centers per unit volume of water, $\bar{a}_{\text{PS+bilins}}$ is the effective optical absorption by phytoplankton (m^{-1} , Table 1), and substitutes for the effective chlorophyll a -specific optical absorption, $\bar{a}^{\text{chl}}_{\text{PS+bilins}}$, of Eq. 6 and 7. P is estimated using Eq. 9, and the factor 6023 converts σ_{PSII} from $\text{\AA}^2 \text{ quanta}^{-1}$ to $\text{m}^2 \text{ mol RCII}^{-1}$ (Table 1).

We recognize that one difficulty in applying Eq. 14 in situ is the determination of absorption spectra for photosynthetically active pigments ($a^*_{\text{PS+bilins}}$) because this procedure requires

discrete pigment samples. Therefore, calculating [RCII] using Eq. 14 would still limit a truly independent determination of PSII productivity using the FRR fluorometer. A simpler bio-optical approach would be to replace $\bar{a}_{\text{PS+bilins}}$ with \bar{a} and to determine P from the difference between F_{730}' and the optical absorption, a' (m^{-1} , Table 1),

$$[\text{RCII}] = \left(\frac{\bar{a} \cdot P}{\sigma_{\text{PSII}} \cdot 6023} \right) \text{ with } P = 0.5 \cdot \left(\frac{\sum_{700}^{400} \left(\frac{F_{730}'(\lambda)}{a^{\text{chl}}(\lambda)} \right) \cdot E(\lambda)}{\sum_{700}^{400} E(\lambda)} \right) \quad (15)$$

Estimates of [RCII] using Eq. 15 were within $\pm 2\%$ to 35% of [RCII] measured from the O_2 flash yields ($n_{\text{PSII}} \times [\text{chl } a]$) ([RCII] Eq. 15 = $0.921 \times [\text{RCII}]_{\text{measured}}$, $r^2 = 0.722$, $n = 22$, $P < 0.001$) (data not shown). Therefore, while Eq. 15 is the most practical to employ, it may result in a general underestimate of [RCII] (see also Table 6).

We also recommend that σ_{PSII} in situ be extrapolated from the value at 478 nm measured by the FRR fluorometer to other wavelengths as follows:

$$\sigma_{\text{PSII}}^{\text{abs}} = \frac{\sigma_{\text{PSII}}(478)}{F_o(478)} \cdot \left(\frac{\sum_{400}^{700} F_o(\lambda) \cdot \text{PPFD}(\lambda)}{\sum_{400}^{700} \text{PPFD}(\lambda)} \right) \delta\lambda \quad [16]$$

where F_o is the minimum fluorescence and PPFD is the actinic excitation at wavelengths throughout the photosynthetically active waveband ($\delta\lambda = 1 \text{ nm}$).

Given our laboratory observations, further validation of the various approaches for estimating P in situ should be considered. Particular attention should be given to the development of methods to directly measure the absolute quantum yield for fluorescence (ϕ_f) (Falkowski and Raven 1997). As such, in situ optical techniques may prove a better focus for future determinations of ϕ_f for natural populations. Further validation of the relationship between n_{PSII} (or [RCII]), σ_{PSII} and P should also be undertaken for natural waters. n_{PSII} is difficult to ascertain in situ, and it not known to what extent this parameter will actually vary. However, determination of n_{PSII} is currently limited by the sensitivity with which O_2 evolved from ST flash frequencies can be measured. Recent improvements to oxygen sensors may help to overcome this limitation.

Finally, from this study, we can conclude that (1) biophysical and optical determinations of $a^{\text{chl}}_{\text{PSII}}$ appear to be equally valid, and (2) a high degree of confidence can be placed on the rapid FRR measures of light absorption (σ_{PSII}). Therefore, variability of σ_{PSII} in situ will reflect changes in phytoplankton taxonomy and physiology, both of which must be independently characterized to fully interpret σ_{PSII} . Quantifying this variability using rapid approaches in situ is fundamental toward improving bio-optical measurements and models of

primary productivity (Laws et al. 2002). We have demonstrated that using a combination of bio-optical and biophysical measurements can potentially improve the FRR technique for quantifying light absorption and hence light-dependent rates of productivity.

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