Vol. 11: 127–138, 2010
doi: 10.3354/ab00298

AQUATIC BIOLOGY
Aquat Biol

Published December 14

Microalgal pigment ratios in relation to light intensity: implications for chemotaxonomy

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ABSTRACT: Pigment-based chemotaxonomy relies upon the assertion that the pigment arrays of mixed microalgal communities (phytoplankton, periphyton, microphytobenthos) can be deconvoluted in ways to provide an estimate of taxon-specific chlorophyll *a* (chl *a*) as a proxy for biomass. This relies on having valid chl *a*:biomarker pigment ratios. However, pigment ratios change for a variety of reasons. We examined the effects of only light intensity (photon flux density, PFD) on the pigment ratios of 10 species in the phyla Cyanobacteria, Chlorophyta, Chromophyta (Bacillariophyceae), Haptophyta, and Pyrrophyta. Chl *a*:marker pigment versus PFD (30–45 through 1800 µmol quanta m^{-2} s^{-1}) data revealed distinct trends for photosynthetic accessory pigments (PAP) versus photoprotective pigments (PPP). Chl *a*:PAP class pigments (chl *b*, fucoxanthin, peridinin, echinenone) exhibited relative stability (possibly stoichiometry) with increasing PFD, while chl *a*:PPP class pigment trends revealed large increases in the relative amounts of the PPP pigments (zeaxanthin, myxoxanthophyll, canthaxanthin, scytonemins). Total chl *a* $cell^{-1}$ often decreases in concert with increasing PFD and partially or totally explains the decrease in chl *a*:PPP ratios. We verified that all 'xanthophyll cycle' pigments expressed large and erratic ratios and therefore did not predict chl *a* levels. We conclude that light (400 to 700 nm) measurements taken in the field can offer valuable ancillary data for adjusting pigment-based chemotaxonomic formulae for the study of microalgal communities.

KEY WORDS: Chemotaxonomy · Light · Pigments · Biomarker ratios · Chlorophylls · Carotenoids · Photoprotection

INTRODUCTION

The main principle of pigment-based chemotaxonomy rests with the ability to dissect the pigment assemblages of natural microalgal communities, relating taxon-specific biomarker pigments to the amount of chlorophyll *a* (chl *a*), as a proxy for biomass, contributed by each group to the total community chl *a* (Millie et al. 1993, Mackey et al. 1996, Louda 2008). Advances in high performance liquid chromatography (HPLC) have greatly facilitated the separation, identification, and quantitation of chlorophylls and carotenoids (Mantoura & Llewellyn 1983, Wright & Jeffrey 1997) and have fostered this methodology.

Taxon-specific pigments, or biomarkers, must be present in discernable ratios to biomass or a proxy for biomass, here chl *a*, for the method to yield ecologically useful results (cf. Descy & Metens 1996). The field of pigment-based chemotaxonomy uses multiple linear regression (MLR) formulae (Gieskes & Kraay 1983, Wilhelm et al. 1991, Barlow et al. 1993), inverse simultaneous equations (Vidussi et al. 2000, Louda 2008) and advanced algorithms, such as the matrix factorization program CHEMTAX (Mackey et al. 1996) or the recent Bayesian Compositional Estimator (BCE) method of biomarker-based taxonomy (Van den Meersche et al. 2008), for calculating taxon-specific chl *a*.

The easiest assumption is that chl *a*:marker pigment ratios do not change throughout a data set or from site to site, with depth and/or day to day. However, as seen from the literature (Gieskes & Kraay 1983, Wilhelm et al. 1991, Barlow et al. 1993, Mackey et al. 1996, refer-

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ences in Jeffrey et al. 1997, Wright & Jeffrey 2005) and the data herein, pigment ratios do change in response to various environmental factors. Data from field samples are entered into a multiple regression / multiple simultaneous equation formula or matrix wherein taxon-specific chl *a* values are the dependent variables and the concentrations of specific marker pigments are the independent measured variables. Chl *a*:marker pigment molar ratios (Louda 2008) or marker pigment: chl *a* weight ratios (i.e. CHEMTAX; Mackey et al. 1996, Eker-Develi et al. 2008) are then used as multipliers for calculating the contribution of each major algal group to total phytoplankton biomass, expressed as a function of chl *a*. Errors stem from the fact that pigment ratios change for a number of reasons, such as growth rate (Goericke & Montoya 1998, Pinckney et al. 2001), variant species composition (Schlüter et al. 2000, Wright & Jeffrey 2005), light availability (Wright & Jeffrey 2005, Leonardos & Harris 2006), temperature (Greisberger & Teubner 2007) and the nutritional state of phytoplankton (Mackey et al. 1996, Goericke & Montoya 1998), amongst others (cf. Eker-Develi et al. 2008 and references therein). The sunscreen pigment scytonemin is also reported to be affected not only by light but by the state of desiccation in certain cyanobacteria (Fleming & Castenholz 2007). Given the above, the use of pigment ratios needs to be standardized for each system (Lewitus et al. 2005) to provide homogeneous datasets (Mackey et al. 1998). Relative to light, this also can include splitting samples into low versus high light samples (Descy et al. 2000, Rodriguez et al. 2006) or similar depth strata in the sampled area (Mackey et al. 1998).

Pigments that have a light-harvesting function (photosynthetic accessory pigments; PAP) in photosynthesis tend to covary with chl *a* (Green & Durnford 1996), while those that appear to have a photoprotective function (photoprotective pigments; PPP) can be expected to increase in high light intensities (Goericke & Montoya 1998).

Xanthophyll cycle pigments change rapidly in concert with photic flux and light:dark cycles. These include carotenoid ↔ carotenoid epoxide conversions in chlorophytes (zeaxanthin, ZEA ↔ antheraxanthin, ANTH ↔ violaxanthin, VIOLA; Demmig-Adams 1990) and chromophytes (diatoxanthin, DX ↔ diadinoxanthin, DD; Hager 1980). Given the rapid changes in these series, it can then be argued that none of these pigments can exist in reliable or useable quantitative relationships with chl *a*. However, the sum of xanthophyll cycle pigments (ZEA+ANTH+VIOLA or DD + DX) may yield meaningful information as to the chemotaxonomic and/or light history of microalgal communities and indicate specific biomarker ratio values to be used when a certain photic history is inferred. The 'epoxidation state' (EPS) of the xanthophylls cycle (EPS = [VIOLA + 0.5 ANTH] / [VIOLA + ANTH + ZEA]; Thayer & Bjorkman 1992) may also provide insight as to light history over the short (~ hours to days) term.

Additional complications in pigment interpretations arise if senescent, dead, or grazed materials are present in the natural seston, periphyton, or microphytobenthos being analyzed. In these cases, the presence of pheopigments may confound analysis of the data (Louda 2008) as pigments such as peridinin (PERI; dinoflagellates) and fucoxanthin (FUCO; chrysophytes, diatoms) degrade faster than xanthophylls, such as ZEA and lutein (LUT; Leavitt & Carpenter 1990, Louda et al. 1998, 2002, Patoine & Leavitt 2006).

Variations of pigment content within various taxa, even at the species level, are often ignored in biomass estimations. Thus, a need exists to define ratios and especially their variability in ways that will allow reliable/verifiable estimates of chl *a* contributions for each taxon (Peeken 1997, Jeffrey et al. 1999) or 'plankton functional group' (PFG; Paerl et al. 2003), as summarized in this quote: 'The biggest problem remaining for all such techniques is the paucity of data available for pigment ratios of major species over a wide range of light and nutrient regimes' (Jeffrey et al. 1999, p. 890). This is especially critical since past studies (Carreto et al. 2008) have shown a logarithmic decrease in pigments (e.g. chl *a* per cell) and decreasing chl *a*:marker pigment ratios with increasing irradiance.

In the present study, we evaluated irradiance, an easily measured laboratory and field parameter, as a driver for changes in chl *a*:biomarker pigment ratios. Data were collected on pigment ratios and, where possible, pigment contents cell^{-1}. Ten algal species (3 cyanobacteria, 2 chlorophytes, 2 diatoms, 2 dinoflagellates, and 1 haptophyte) were investigated under photosynthetically active radiation (PAR) irradiances of 30–45, 108–120, 300, and 1600–1800 µmol quanta m^{-2} s^{-1}. These we classify as low, moderate, high, and intense light conditions, respectively. Other growth parameters were not altered, and harvesting occurred during nutrient replete conditions.

MATERIALS AND METHODS

Experimental organisms. Freshwater and marine microalgal species were purchased (Carolina Biological Supply Company).

Cyanobacteria: *Lyngbya* sp. (fresh), *Scytonema hoffmanni* (fresh), *Anacystis nidulans* (also known as *Synechococcus* sp.: marine).

Chlorophyta: *Closterium acerosum* (fresh), *Cosmarium turpinii* (fresh).

Chrysophyta, Bacilliariophyceae: *Navicula* sp. (fresh), *Phaeodactylum* sp. (marine).

Chrysophyta, Prymnesiophyceae: *Isochrysis galbana* (also known as 'T-ISO': marine).

Pyrrophyta, Dinophyceae: *Gymnodinium* sp. (marine), *Amphidinium carteri* (marine). Cross contamination of the *Gymnodinium* sp. with *A. carteri* (PERI) as supplied precluded any meaningful interpretation of biomarker (fucoxanthin) data for *Gymnodinium* sp.

Culture conditions. Algae were cultured in 50 ml lots within 125 ml baffled bottom flasks (low light) or tissue culture flasks (moderate to intense). Light levels are given here as low (30–44.5 µmol quanta m^{-2} s^{-1}), moderate (108–120 µmol quanta m^{-2} s^{-1}, high (300 µmol quanta m^{-2} s^{-1}), and intense (1600–1800 µmol quanta m^{-2} s^{-1}). Light sources included sunlight quality (Verilux Instant Sun™), Full Spectrum™ (ValuTek), 'aquarium' (Phillips), and UVA fluorescent tubes (Sylvania Gro-Lux™). Low light illumination (30 to 44 µmol quanta m^{-2} s^{-1}) of the 125 ml flasks in a rotating (60 rpm) water bath was from overhead using 1 each sunlight and aquarium tubes set on a 12:12 h light:dark cycle. Low light illumination of the flat (tissue culture) flasks was from above and below using sunlight and aquarium quality 20 W bulbs, 1 each above and below a flat wire mesh rack. Moderate (110–120 µmol quanta m^{-2} s^{-1}) and high (300 µmol quanta m^{-2} s^{-1}) light conditions were achieved within a temperature controlled (25°C) Revco-Harris growth chamber in which 6 sunlight quality fluorescent plus 6 Full Spectrum™ fluorescent bulbs were placed around the shelf holding the flasks (maximum of 3 bulbs each, top, bottom, and both sides). Intense illumination (1600–1800 µmol quanta m^{-2} s^{-1}) was achieved within a Percival Model E36HID growth chamber equipped with full spectrum halogen lights to which 4 UVA fluorescent tubes were added. We could not directly measure the UVA irradiance within the flat NUNCLON Δ polystyrene culture flasks (#153732). However, both the published UV/Vis transmission spectrum of NUNCLON Δ polystyrene (www.nuncbrand.com) and that which we recorded (200–800 nm) revealed 52 %T (percent transmission) at 315 nm, 56 % at 320 nm, and 87 % at 400 nm, covering the range of UVA. Transmission of visible light from 400 to 800 nm was 90 %T. All growth was at 25 ± 1.5°C. Light (PAR radiation: 400–700 nm) was measured with a 4π spherical radiometer and Li-Cor LI-250 light meter.

Algal culturing. Freshwater algae were cultured in media prepared from the Carolina Biological Supply Company materials: 20 ml of Alga-Gro (*f*/2) concentrate (#153751) was added to 980 ml of spring (karst) water (Zephyrhills, Nestle) and autoclaved (122°C, 2 atm, 45 min). This was used for the culture of all freshwater species except the following 2. In the case of the diatom *Navicula* sp., 20 mg l^{-1} of sodium silicate were added prior to sterilization. For the culture of the cyanobacterium *Scytonema hoffmanii*, sterile soil water extract (# 153785) was added to the pre-sterilized media given above.

Marine species, except as given below, were cultured in Carolina Biological Supply Alga-Gro (*f*/2) seawater medium (#153754). The marine cyanobacterium *Anacystis nidulans* (~*Synechococcus* sp.) was cultured in Allen's medium (#153745). Alga-Gro medium is Guillard's (1975) *f*/2 medium (Basch 1996).

Growth monitoring. Cell densities were monitored every 2 to 4 d by aseptically removing a small aliquot (~0.5 to 1.0 ml) of the culture, following a thorough but gentle mixing of the culture, and then measuring absorbency (AU, ~ optical density, OD) at 430 nm versus time (days of growth); this allowed sampling during early to mid logarithmic growth. Cross checks of the OD method of growth monitoring were performed using either electronic cell counting with a Coulter Model ZM cell counter or microscopically with a hemocytometer for single-celled or filamentous forms, respectively. Growth periods ranged between 5 and 26 d, depending upon species, and varied inversely with light intensities. As cultures were never taken to the retardation or stationary phases, we consider sampling to have occurred during nutrient replete conditions.

Replicate analyses (N = 5 to 13) of each species/light level represent separate cultures, not samples from the same culture. This was done in order to allow growth and pigment variability to be expressed. Additionally, this allowed cultures to not become overly dense, ensuring both nutrient replete conditions as well as precluding significant self-shading while still providing excellent pigment yields when the entire culture was harvested.

Pigment extraction and analyses. All sample handling and pigment analyses were carried out under dim yellow light conditions to prevent photo-oxidative alteration/isomerization of pigments. Extraction and HPLC conditions followed those previously published (Louda et al. 1998, 2000, 2002, Hagerthey et al. 2006).

HPLC data handling. Pigment identities were determined based on retention time and spectral absorbance from a photodiode array (PDA) detector. The retention times and spectral absorbance of the pigments were compared to those from >90 standards (Louda et al. 2002, Louda 2008) for final confirmation. Pigment standards were obtained through partial synthesis or derivatization (Louda 1993), and many were purchased from DHI Water and Environment and Sigma-Aldrich as single compounds or known qualitative mixtures.

Quantification relied on the Beer-Lambert relation wherein peak areas at an appropriate wavelength (440 nm = chlorophylls, carotenoids; 410 nm = pheo-

pigments, 394 nm = internal standard) were divided by an extinction coefficient from the literature (e.g. Britton 1995, Jeffrey 1997 and references in each). Extinction coefficients that were used but did not correspond to the exact maximum for which they were reported were adjusted by the ratio of absorbance at the HPLC monitoring/integrating wavelength to that at the wavelength of the reported coefficient. This quantitation protocol was verified using solutions of authentic known pigments that were prepared using standard spectrophotometric techniques.

Chl *a*-epi (epimer, chl *a*′) and ECHIN partially coelute from the 300 mm C-18 column used here (cf. Louda et al. 1998, 150 mm column). ECHIN and chl *a*′ were 'colorimetrically separated' by manual integration at 460 and 666 nm, respectively, thus eliminating their spectral overlap at 440 nm and allowing valid individual quantitation using wavelength adjusted extinction coefficients. The extremely small absorbance of chl *a*′ at 460 nm was ignored during the calculation of echinenone. Tests with known mixtures confirmed this approach. LUT and ZEA were baseline separated on our HPLC system (Louda et al. 2002).

The chl *a*:biomarker pigment ratios of the 10 algal species were calculated from the concentrations of individual chlorophylls and carotenoids in cultures grown at low, moderate, high, and intense irradiance levels. Replicate analyses of each species at the various light intensities were made as follows: low (N = 7), moderate (N = 11), high (N = 5) and intense (N = 13). Data were plotted as mean ± SD. All trends were initially plotted using linear, log normal, power, and exponential trend lines. In all cases, except chl *a*:DD (power), log normal trends gave significantly higher R^2 values.

Herein we report molar, not weight, ratios for all pigment data presented, as they are biochemically more appropriate than simple weight ratios. For the w/w ratios and their inverses (i.e. CHEMTAX), see Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/b011p127_supp.pdf. Further, when we quote or compare our ratios to literature values, we have converted the reported inverse w/w ratios to our molar chl *a*:marker pigment format. That is, the goal was to predict taxon-specific chl *a* contributions.

RESULTS

Cyanobacteria

Pigments common to the 3 cyanobacteria studied were chlorophyllide *a* (chlide *a*), ZEA, chl *a*-allomer (chl *a*-allo), chl *a*, chl *a*′, and β-carotene (BETA).

Anacystis nidulans (cf. *Synechococcus* sp.)

This common unicellular cyanobacterium was previously called *A. nidulans* R2 and is likely *Synechococcus* PCC 7942 or *S. elongatus* (PCC6301) in the Expert Protein Analysis System (ExPASy) proteomics server of the Swiss Institute of Bioinformatics (www.expasy.ch). *S. elongatus* is a bloom-forming organism in Florida Bay and its estuarine margins (Phlips et al. 1999) and exhibits changing chl *a*:ZEA ratios with light regimes (Louda 2008).

The only pigment available as a biomarker for this species was ZEA. Chl *a*:ZEA ratios declined from about 3.0:1 in low to about 1.5:1 in moderate to high light, and then dropped slowly to about 1.1:1 at the highest light levels (Fig. 1a). As ZEA was about 1 to 2 orders of magnitude more abundant than BETA, a similar trend was likewise found for chl *a*:total carotenoids. Laza-Martinez et al. (2007) studied *Synechococcus* sp. grown in 100 µmol m^{-2} s^{-1} in a 14L:10D regime and reported a weight ratio of ZEA:chl *a* of 0.836 (thus chl *a*:ZEA = 1.196), corresponding to a molar ratio of chl *a*:ZEA of 0.76:1.

Lyngbya sp.

In addition to the pigments found in *Anacystis nidulans, Lyngbya* sp. also contained myxol (MYXOL), a less polar myxoxanthophyll (MYXO-NP), myxoxanthophyll (MYXO, myxol-2'-glycoside), echinenone (ECHIN), and BETA. MYXO has a sugar moiety (often rhamnose) attached, whereas MYXOL is the non-glycosylated alcohol (Francis et al. 1970, cf. Mohamed et al. 2005).

Chl *a*:ECHIN ratios (Fig. 1b) decreased from about 22:1 in moderate and high light to around 17:1 in intense light. No low light cultures grew or survived for this species. Chl *a*: ECHIN of about 22:1 appears to best represent *Lyngbya* sp. biomass in moderate to high light conditions.

Chl *a*:ZEA ratios (Fig. 1b) decreased from about 17 to 12 to 5:1 with increasing light intensity. Chl *a*:MYXO (Fig. 1c) values went from about 11:1 to 8:1 with increasing light, but trends were poorly correlated.

Scytonema hoffmanii

Pigments in *S. hoffmanii* were as found for *Lyngbya* sp. with the addition of certain sunscreen pigments.

As with the other filamentous cyanobacterium *Lyngbya* sp., the biomarker exhibiting the best stability in its relation to chl *a* was ECHIN (Fig. 1d). The error bars determined for these data encompass a ratio of about 11:1.

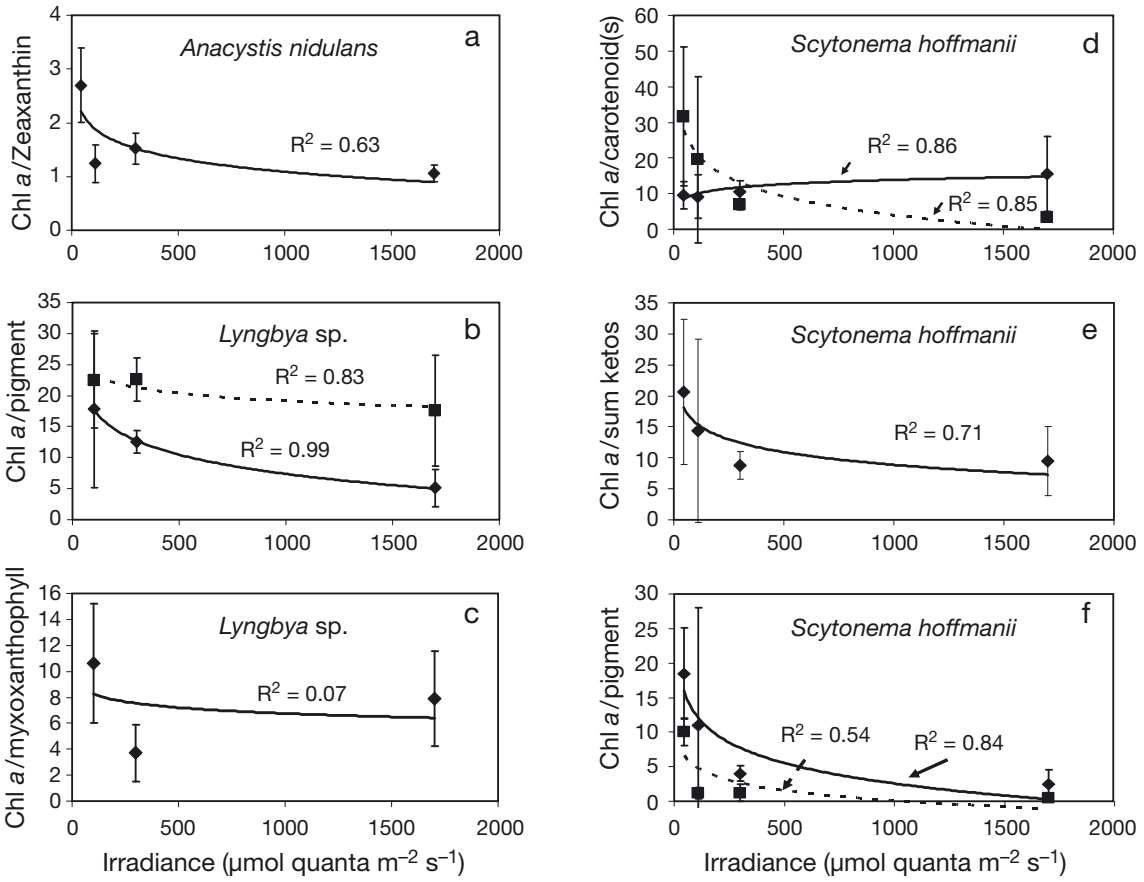

Fig. 1. Pigment ratios versus photic flux density (PFD) for cyanobacteria: (a) *Anacystis nidulans*; chl *a*:zeaxanthin (ZEA). (b,c) *Lyngbya* sp.; (b) chl *a*:ZEA (diamonds, solid line) and chl *a*: echinenone (ECHIN; squares, dotted lined), (c) chl *a*: myxoxanthophyll (MYXO). (d–f) *Scytonema hoffmanii*; (d) chl *a*:ECHIN (diamonds, solid line) and chl *a*:canthaxanthin (CANTH; squares, dashed line), (e) chl *a*:sum ketocarotenoids (= ECHIN + CANTH). (f) chl *a*:MYXO (diamonds, solid line) and chl *a*:scytonemins (squares, dashed line)

Canthaxanthin (CANTH), a diketo-β-carotene, accompanied the mono-keto ECHIN and resulted in chl *a*:CANTH ratios (Fig. 1d) that greatly decreased (~30:1 to 2:1) with light intensity. Plotting chl *a* to the sum of keto-carotenoids (Fig. 1e), it is observed that the trend is dominated by the influence of CANTH.

A highly polar pigment, suggested here to be a visible sunscreen (λ_{max} = 440, 562 nm) of presently unknown structure (UNKN), was present in *Scytonema hoffmanii* grown in high or intense photic flux even in the absence of UV wavelengths. That pigment is still under study but does have a scytonemin (SCYTO)-type nucleus (C. Grant & J. Louda unpublished data). The reduced (SCYTO-red) and oxidized (SCYTO-ox) forms of SCYTO, a dimeric indole-phenol (Garcia-Pichel et al. 1992, Proteau et al. 1993), were present and increased with increasing light levels.

Both the ratios of chl *a*:MYXO (Fig. 1f) and chl *a*:SCYTO (oxidized plus reduced: dashed trace in Fig. 1f) decreased with increasing light intensity, indicating photoprotective roles for each. Under intense light conditions the chl *a*:SCYTO ratios averaged only 0.4:1 to 0.1:1, indicating a 2.5- to 10-fold molar excess, relative to chl *a*, of SCYTO.

Chlorophyta (green algae)

Pigments found in the green algae were chlide *a*, neoxanthin (NEO), VIOLA, ANTH, LUT, ZEA, chl *b*, chl *a*-allo, chl *a*, chl *a'*, pheophytin *a* (PTIN*a*), and BETA. Chlide *a* and PTIN*a* were in extremely low amounts, likely representing minor senescence products (Louda et al. 1998, 2002) and confirming that the cultures were viable with little if any alteration of biomarker pigments (cf. Louda 2008)

Pigment arrays and light-related changes in the desmids *Closterium acerosum* and *Cosmarium turpinii* were identical. Chl *a* to chl *b* values (Fig. 2a) were stable across tested light levels. The ratios were consistent at about 2.6:1 between 100 and 300 µmol quanta m^{-2} s^{-1} with an increase (~3.1:1), representing a rela-

tive decrease in chl *b*, in low light. Chl *a*:LUT ratios (Fig. 2a) dropped with increasing intensity, indicating a photoprotective function for LUT (cf. Demmig-Adams & Adams 2006); R^2 values were 0.6 to 0.7:1 for chl *a*:LUT and 0.8 for chl *a*:chl *b* for both species. Data for chl *a*:LUT from both species often co-plotted such that the triangles (Closterium) and circles (Cosmarium) are indistinguishable in Fig. 2a.

The concentration of chl *a* cell^{-1} (Fig. 2b) decreased more than 4-fold as light increased from 44 to 1700 µmol quanta m^{-2} s^{-1}. Thus, decreasing ratios of chl *a* to other pigments in many instances are due to decreasing chl *a* concentration, while other pigments remain at nearly stable concentrations. For example, in these cases, chl *a* goes from about 400 to 200 fg cell^{-1} as chl *a*:LUT goes from about 10 to 4:1. Additionally, the slight decrease in chl *a*:chl *b* ratios in response to increasing light suggests that the cellular content of both chlorophylls are decreasing, but chl *b* is decreasing faster than chl *a* with increasing light.

ZEA was not detected in the lowest light cultures. At other light levels the LUT:ZEA ratio (not plotted here) was about 12:1 (100–300 µmol quanta m^{-2} s^{-1}) and dropped to about 8:1 under intense light. Chl *a*:ZEA was around 45 to 60:1 in the moderate and high light and about 30 to 35:1 in intense light. Other pigments (neoxanthin, violaxanthin, antheraxanthin) as part of the 'xanthophyll cycle' gave sporadic ratios to each other and to chl *a*. The VIOLA:NEO ratios in low light were about 0.5:1 and then leveled out at about unity at higher light levels.

Chrysophyta, Bacillariophyceae (diatoms)

The diatoms investigated were *Phaeodactylum* sp., tentatively *P. tricornutum*, and *Navicula* sp. Both species contained chlide *a*, chl c_1/c_2 (chl *c* hereafter), pyrochlorophyllide a (pchlide *a*), FUCO, DD, DX, chl *a*-allo, chl *a*, chl *a*′, and BETA.

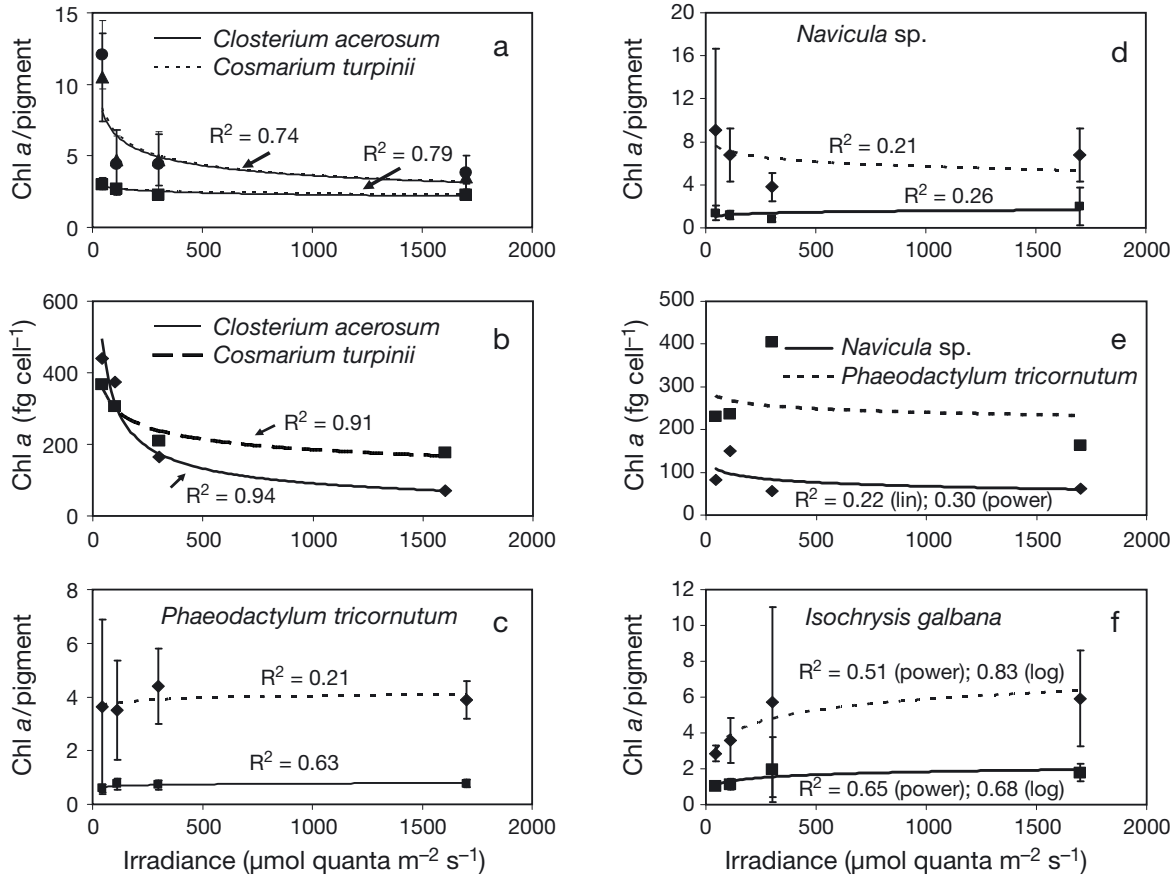

Fig. 2. (a,b) Chlorophyta; *Closterium acerosum* (solid traces) and *Cosmarium turpinii* (dotted traces). (a) Pigment ratios versus photic flux density (PFD) chl *a*:chl *b* (lower, diamonds and squares) and chl *a*:lutein (LUT; upper, triangles and solid circles), (b) chl *a* content cell^{-1} for *C. acerosum* (diamonds, solid trace) and *C. turpinii* (squares, dashed trace). (c–e) Chromophyta, Bacillariophyceae: chl *a*:fucoxanthin (FUCO) (squares) and chl *a*:chl (i.e. chl c_1/c_2) *c* (diamonds) versus light intensity for (c) *Phaeodactylum tricornutum* and (d) *Navicula* sp.; (e) chl *a* cell^{-1} versus light intensity for *P. tricornutum* (squares, dashed trace) and *Navicula* sp. (diamonds, solid trace); (f) Haptophyta, *Isochrysis galbana*; chl *a*:FUCO (squares, solid trace) and chl *a*:chl *c* (diamonds, dashed trace)

Only chl *a*:chl *c* and chl *a*:FUCO gave trends versus light intensity that did not undergo 5-fold to an order of magnitude or greater changes (Fig. 2c,d). Chl *a*:chl *c* was relatively stable at about 4:1 for *Phaeodactylum* sp. but dropped from about 9:1 to 5.5:1 in *Navicula* sp. Additionally, the chl *a*:chl *c* values for both species had very large SDs in low light conditions (Fig. 2c,d). On the other hand, both diatoms gave chl *a*:FUCO ratios about unity (~0.85:1, Fig. 2c; ~1.1:1, Fig. 2d) and did so with extremely low SDs, except for the intense light data from *Navicula* sp.

The contents of chl *a* cell^{-1} (Fig. 2e) decreased from about 250 to 150 fg cell^{-1} in *Phaeodactylum* and from about 100 to 60 fg cell^{-1} in *Navicula*.

Chrysophyta, Prymnesiophyceae: *Isochrysis galbana*

I. galbana contained chlide *a*, chl *c*, FUCO, DD, DIATO, chl *a*-allo, chl *a*, chl *a*′, β-carotene (ALPHA), BETA, and pheophytin-*a* (PHTIN*a*). Phytylated chl *c* (cf. 'T-ISO' in Zapata & Garrido 1997) was quite low in abundance. Given its close elution to chl *a*-allomer on our system, it could serve as an indication of this taxon but would be difficult to use as a routine biomarker. Zapata & Garrido (1997) used a polymeric column and discussed the coelution with chl *a*-allo on monomeric systems, such as used herein. Soret absorption of the phytylated chl *c* is at about 456 nm and is easily seen on the low energy side of the Soret band (~432 nm) of chl *a* as a distinct inflection and could easily be confirmed with the second derivative.

As *Isochrysis galbana* is a non-19′-alkanoyloxyfucoxanthin-containing haptophyte, the most usable marker pigment for this species was FUCO, with the chl *a*:FUCO ratios (Fig. 2f) remaining between 1.1:1 and 2:1 from the low to the higher light experiments, respectively.

Pyrrophyta (dinoflagellates)

Amphidinium carteri contained chl *c*, PERI, dinoxanthin (DINO), DD, DX, chl *a*-allo, chl *a*, chl *a*′, and BETA, as well as 2 glycosylated carotenoids (P-457and P-468). The chl *a*:PERI ratios (Fig. 3a) varied between 0.7:1 and 1.2:1. An increase in this ratio to nearly 2:1 was observed for growth under intense illumination. The chl *a*:chl *c* ratio (Fig. 3a) was 1.9:1 in low to high light and 2.2:1 in intense light.

Ratios of chl *a* to either chl *c* or PERI for cultures grown under intense radiation exhibited very large variability, as indicated by large SDs in Fig. 3a.

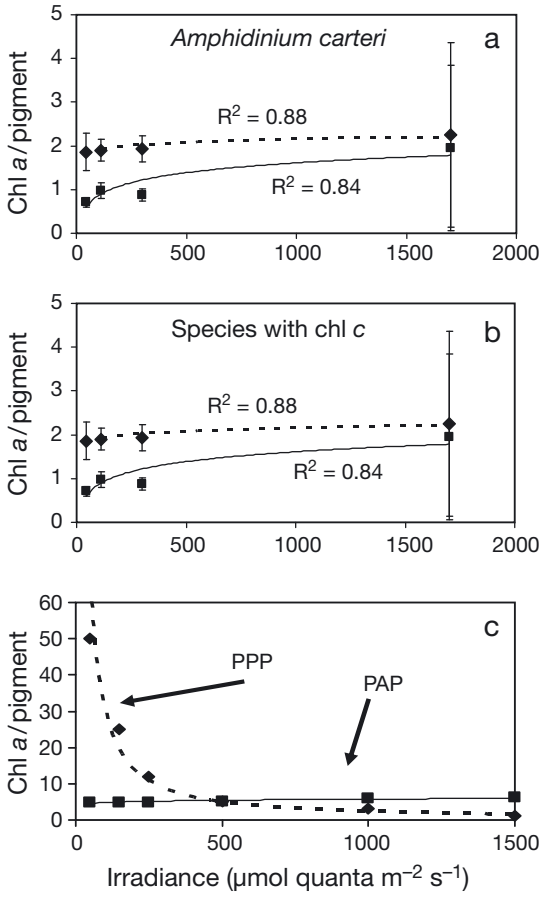

Fig. 3. (a) Dinophyta, *Amphidinium carteri*; chl *a*:peridinin (PERI; squares, solid trace) and chl *a*:chl *c* (diamonds, dashed trace) versus light intensity. (b) chl *a*:diadinoxanthin versus light intensity for all chl *c*-containing species (*Phaeodactylum tricornutum*, *Navicula* sp., *A. carteri*, *Gymnodinium* sp., *Isochrysis galbana*) investigated herein. (c) Generalized (hypothetical) plot of photosynthetic accessory pigments (PAPs) and photoprotective pigments (PPPs) versus light intensity (see Results and Conclusion)

Chromophyte xanthophyll cycle pigments

The ratio of chl *a*:DD (Fig. 3b), or DD+DX (not shown), for these 5 species decreased from low to intense light, often by an order of magnitude (e.g. 20 to 2:1 in diatoms).

DISCUSSION

Cyanobacteria

Marker pigments for cyanobacteria are typically ZEA and/or ECHIN. Ratios of chl *a*:ZEA = 1.1:1 and chl *a*:ECHIN = 11.0:1 have been used for estimating coccoidal or filamentous cyanobacteria, respectively, in Florida Bay (Louda et al. 2000, Louda 2008) and Ever-

glades (Hagerthey et al. 2006) studies. Similar ratios have been previously reported for samples grown without light or nutrient limitations (Wilhelm et al. 1991, Barlow et al. 1995).

The decrease in chl *a*:ZEA ratios for *Anacystis nidulans* (Fig. 1a) from about 2.6:1 to 1.5:1 and then to 1.0:1 with increasing light intensity suggests that ZEA functions to protect the species from photodamage (cf. Paerl et al. 1983, Bidigare et al. 1989). We previously found that *Synechococcus* sp. (possibly *S. elongatus*) had chl *a*:ZEA ratios of 2.5:1 or 1.0:1 in the dark brown humic waters of Whitewater Bay or the clearer waters of Florida Bay proper, respectively (Louda 2008). Even though ZEA acts as a PPP in *A. nidulans*, it is still required as a marker pigment for coccoidal cyanobacteria lacking other carotenoids. ZEA does occur in the chlorophytes as well, but in very reduced concentrations. Aside from chl *a*, the other main pigment in *A. nidulans* is BETA, and since this pigment is nearly omnipresent in oxygenic phototrophs, it is not a suitable marker pigment. The decrease in the ratio of chl *a*:ZEA is explained wholly or partly by decreases in cellular chl *a* contents as previously reported for *A. nidulans* (Allen 1968) and with the largest decreases occurring above 300 µmol photons m^{-2} s^{-1} (cf. Utkilen et al. 1983).

Nichols (1973) found that many freshwater filamentous cyanobacteria have a wide range of carotenoids (e.g. ECHIN, MYXOs, CANTH) not found in picoplanktonic cyanobacteria. In the present study, this observation holds for *Lyngbya* sp. and *Scytonema* sp. As such, we used ECHIN as the marker of choice for filamentous cyanobacteria. Thus, cyanobacteria chl *a* contributions include both back calculations from ratios to ECHIN (filamentous) and ZEA (coccoidal). Even though chl *a*:ECHIN ratios for these species are different, they are relatively stable with changing irradiance, and ECHIN is thus the most likely marker. Microscopic examination of the community would aid in picking the best ratio once the dominant species is identified. For *Lyngbya* sp., the chl *a*:ECHIN ratio is around 20:1, while that for *Scytonema* sp. is around 13:1. Previous work in our laboratory with *Anabaena flos-aquae* and *Microcystis aeruginosa* at different irradiance levels yielded chl *a*:ECHIN ratios of 11:1 (Skoog 2003, Skoog & Louda 2003). The filamentous non-heterocystous genus *Lyngbya* is a worldwide bloom form and *L. majuscule* is of growing concern in tropical/sub-tropical waters (Paul et al. 2005).

The increase in CANTH with increased light intensity, giving dramatically decreasing chl *a*:CANTH (Fig. 1d) and correspondingly decreasing chl *a*:total keto-carotenoids (KETOs: Fig. 1e), points to a photoprotective function for CANTH. On the other hand, the relative stability of chl *a*:ECHIN reveals that ECHIN is linked stoichiometrically to the photosynthetic reaction center as an accessory pigment. As total chl *a* decreases with increased light, the slight increase in chl *a*:ECHIN confirms that ECHIN acts as a PAP rather than strictly as a PPP. This slight increase in the ratio of chl *a* to taxon-specific PAPs is also found in the diatoms and dinoflagellates investigated herein. Perhaps, in higher light conditions, antennae chl *a* may be photon saturated and require less energy input from the accessory pigments.

As the amount of ECHIN decreased only slightly during large increases in the relative amounts of CANTH, it appears that CANTH serves a much stronger photoprotective role than does ECHIN (cf. Lakatos et al. 2001).

Both the ratios of chl *a*:MYXO (Fig. 1f) and the SCYTOs (oxidized plus reduced) decreased dramatically with increasing light intensity, indicating photoprotective roles for each. One report stated that MYXO may not be a PAP but that it is required for thylakoid organization or cell wall function/stability (Mohamed et al. 2005). We think that these functions are not mutually exclusive.

Chlorophyta (green algae)

Closterium acerosum and *Cosmarium turpinii* are green algae that possess the same signature pigments. Chl *b* was the best quantitative marker pigment for these 2 species, and the Chlorophyta in general, with chl *a*:chl *b* ratios at around 2.5:1 for *C. acerosum* and around 2:1 for *C. turpinii,* over the range of light levels investigated. The chl *a*:chl *b* ratio in chl *b*-containing organisms ranges from 2:1 to 3:1 (Halldal 1970, Strain et al. 1971, Meeks 1974), and our data confirm this.

Chrysophyta, Bacillariophyceae (diatoms)

FUCO and chl *c* are the main accessory light-harvesting pigments of diatoms (Stauber & Jeffrey 1988). The diatoms used in this study, *Phaeodactylum* sp. and *Navicula* sp., exhibited the characteristic pigments of diatoms with FUCO as the marker pigment for chl *a* divisional estimation. The molar ratios found during this study were about 0.75:1 for *Phaeodactylum* sp. and 1.1:1 for *Navicula* sp. Chl *a*:FUCO (molar converted) ratios of 2.34:1 (Gieskes et al. 1988), 1.21:1 (Wilhelm et al. 1991), and 1.8 to 2.6:1 (Garibotti et al. 2003) have been reported from studies on North Sea, Pacific, and Antarctic waters.

Both species showed relatively stable ratios of chl *a*:chl *c*, even though they did not compare well with each other. The chl *a*:chl *c* ratios for *Navicula* sp. were

around 6:1 and about 4.1 for *Phaeodactylum* sp. Baumann et al. (1994) suggested a species-specific chl *a*:chl *c* ratio. This would prove to be difficult if a mixed algal sample were being analyzed, and as such, chl *a* divisional estimates would not be calculated correctly. For example, chl c_2 also occurs in dinoflagellates, prymnesiophytes, cryptophytes, and others (see Jeffrey & Vesk 1997, Jeffrey & Wright 2006). For this reason, 'chl *c*' is not considered herein as a marker pigment for determining the chl *a* contributions unless HPLC systems such as C8 (Zapata et al. 2000, 2004) designed for separation of the 'chl *c*' were employed in addition to or in place of a C18 system. Chl c_3, while separated from $-c_1/c_2$ on our system, was not encountered in any species tested during the present study.

Pyrrophyta, Dinophyceae (dinoflagellates)

PERI has long been used as the quantitative marker pigment for 'peridinin-containing' algae belonging to the division Dinophyceae (Johansen et al. 1974, Jeffrey et al. 1975). The chl *a*:PERI ratios varied between 0.8:1 and 1.0:1 for *Amphidinium carteri* grown under low to high light. These ratios are lower than the 1.5:1 conversion factor used by Louda (2008) to estimate the chl *a* contributed from dinoflagellates in studies of Florida Bay. However, the ratio of chl a:PERI (~2.0:1) in intense light (Fig. 3a) easily encompasses the calculations from Florida Bay, an intense light environment. Chl *a*:PERI ratios as high as 2.35:1 (Everitt et al. 1990, Ondrusek et al. 1991) and 3.96:1 (Barlow et al. 1995) have been reported.

The chl *a*:chl *c* ratios for both species studied shows some stability around 2:1 but, as discussed above, since the chl *c* are common light-harvesting pigments across different algal groups, they cannot be standalone division/class estimators in single pigment determinations. They may, however, be helpful in mathematically advanced algorithms such as CHEMTAX (Mackey et al. 1996, Wright & Jeffrey 2005) or BCE (Van den Meersche et al. 2009), especially when HPLC systems (e.g. C8) that completely separate all chl *c* forms are included.

Chrysophyta, Prymnesiophyceae

The main photosynthetic light-harvesting pigments for this division are chl *a*, chl *c*, and FUCO (Montero et al. 2002) plus a wide assortment of 'chl *c*' and FUCO derivatives (Zapata et al. 2004).

For the strain (T-ISO) of *Isochrysis galbana* examined here, we found chl *a*:FUCO values to change slowly as light intensity increased. The chl *a*:FUCO ratios varied between 1:1 and 1.6:1 to just under 2:1. Chl *a*:FUCO ratios of 1.46:1 (Vidussi et al. 2000), 1.18:1 (Descy et al. 2000), 1.69:1 (Barlow et al. 1993), and 3.1 to 3.7 (Zapata et al. 2004) have been reported for studies done on different factors that affect changes in pigment relationships in phytoplankton. Note that we used molar ratios, and the ratios originally reported on a w/w basis were converted to molar ratios for this report. Chl *a*:chl *c* ratios of *I. glabana* increased from about 3:1 to 6:1 with increasing light intensity. The *I. galbana* cultured in our study would fall in the 'Type-3 Haptophyte' array as defined by Zapata et al. (2004). Their 2 strains, grown at 60 to 70 µmol quanta m^{-2} s^{-1} (12L:12D) gave chl *a*:FUCO of 3.70:1 and 3.11, as converted to molar ratios.

A different sample of 'T-ISO', provided by the Harbor Branch Oceanographic Institution (Ft. Pierce, FL, USA), contained phytylated chl *c* (Louda unpubl. 2009). However, that sample was not cultured during the present study. Phytylated chl *c* (cf. Zapata et al. 2004) would be useful in better defining mixed natural cultures containing *Isochrysis galbana*.

Without the wide variety of chl *c* and FUCO derivatives given for numerous haptophytes (Zapata et al. 2004), the pigment array of the *Isochrysis galbana* strain studied here would place this isolate with diatoms and other chrysophytes.

CONCLUSIONS

Throughout this study, we found that the ratio of chl *a* to various other lipophilic pigments either decreased rapidly with increasing light intensity or remained relatively stable with sporadic slight increases. It is therefore concluded that those pigments which maintain some level of stability in their relationship (stoichiometry) to chl *a* are primary PAPs. On the other hand, pigments which exhibit relative significant increases in relation to chl *a*, thus giving decreasing chl *a*:pigment ratios with increasing photic flux, are concluded as being mainly PPPs. That is not to say that a pigment acting 'mainly' as a PPP cannot also be involved in energy transfer during photosynthesis (cf. Demmig-Adams & Adams 2006 and references therein). As noted, chl *a* content per cell decreases with increasing photic flux, and this phenomenon partially or wholly explains the decreasing chl *a*:PPP ratios observed.

Fig. 3c shows the generalized trends for PAP- and PPP-type pigments. The ratio values of chl *a* to either PPP or PAP are hypothetical but reflect the ranges reported herein. Thus, PAP-type pigments include ECHIN (cyanobacteria), chl *b* (chlorophytes), FUCO (diatoms, other chrysophytes), and PERI (PERI-containing dinoflagellates). PPP-type pigments include

ZEA, CANTHA, MYXO, and SCYTO in cyanobacteria; LUT and all 'xanthophyll-cycle' pigments (ZEA, VIOLA, ANTH, NEO) in chlorophytes; and DD + DX in chrysophytes and dinoflagellates.

When using any of the PPP-type pigments for community estimation, consideration of the average light field (photo flux density, PFD) is an absolute requirement. Given slight differences in the ratio of chl *a*:PAP pigments, light field measurement should also be factored into all determinations. When using chl *a* as a proxy e.g. for biomass and organic carbon, the average light field data are also a much needed metric, as the amount of chl *a* per cell decreases with increasing light level.

In many cases, the majority of the decreases in chl *a*: marker (viz. PPP) pigment ratios is due to decreasing amounts of chl *a* with increasing PFD. Chl *a*:PAP ratios change very little with light.

The measure of PAR during sampling and monitoring can provide ancillary data with which to improve pigment-based chemotaxonomic estimates of microalgal community structure and dynamics.

Acknowledgements. This work has been supported in part by past contracts to J.W.L. from the Division of Marine Fisheries, National Oceanographic and Atmospheric Administration (NOAA), and the South Florida Water Management District (SFWMD). The assistance of J. Morrison of the Living Materials section of Carolina Biological Supply during the early stages of culture material selection is noted. We thank P. Mongkhonsri for expert assistance in routine laboratory functions and maintenance, especially HPLC operations. This study was performed during the pursuit of the MS and PhD degrees by C.S.G.

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Editorial responsibility: Warwick Vincent, Sante-Foy, Canada

Submitted: June 4, 2010; Accepted: October 1, 2010
Proofs received from author(s): November 29, 2010