Coral Reefs (2006) 25: 99–109
DOI 10.1007/s00338-005-0071-8

REPORT

Eric J. Hochberg · Amy M. Apprill
Marlin J. Atkinson · Robert R. Bidigare

Bio-optical modeling of photosynthetic pigments in corals

Received: 21 June 2005 / Accepted: 17 October 2005 / Published online: 20 December 2005
© Springer-Verlag 2005

Abstract The spectral reflectance of coral is inherently related to the amounts of photosynthetic pigments present in the zooxanthellae. There are no studies, however, showing that the suite of major photosynthetic pigments can be predicted from optical reflectance spectra. In this study, we measured cm-scale *in vivo* and in situ spectral reflectance for several colonies of the massive corals *Porites lobata* and *Porites lutea*, two colonies of the branching coral *Porites compressa*, and one colony of the encrusting coral *Montipora flabellata* in Kaneohe Bay, Oahu, Hawaii. For each reflectance spectrum, we collected a tissue sample and utilized high-performance liquid chromatography to quantify six major photosynthetic pigments, located in the zooxanthellae. We used multivariate multiple regression analysis with cross-validation to build and test an empirical linear model for predicting pigment concentrations from optical reflectance spectra. The model accurately predicted concentrations of chlorophyll *a*, chlorophyll c_2, peridinin, diadinoxanthin, diatoxanthin and β-carotene, with correlation coefficients of 0.997, 0.941, 0.995, 0.996, 0.980 and 0.984, respectively. The relationship between predicted and actual concentrations was 1:1 for each pigment, except chlorophyll c_2. This simple empirical model demonstrates the potential for routine, rapid, non-invasive monitoring of coral-zooxanthellae status, and ultimately for remote sensing of reef biogeochemical processes.

Communicated by Biological Editor H.R. Lasker

E. J. Hochberg (✉) · M. J. Atkinson
School of Ocean and Earth Science and Technology,
Hawaii Institute of Marine Biology,
University of Hawaii, 1346, Kaneohe, HI 96744, USA
E-mail: hochberg@hawaii.edu
Tel.: +1-808-9569108
Fax: +1-808-9569225

A. M. Apprill · R. R. Bidigare
Department of Oceanography, School of Ocean and
Earth Science and Technology, University of Hawaii,
1000 Pope Road, Honolulu, HI 96822, USA

Keywords Corals · Pigments · Reflectance · High-performance liquid chromatography · Bio-optical modeling

Introduction

Reef-building corals contain within their tissues photoautotrophic dinoflagellates called zooxanthellae. In this symbiosis, the coral animal provides protection, a relatively stable light environment, and inorganic nutrients to the zooxanthellae, which in return supply photosynthate and other products to the coral-host (Muscatine 1967, 1990). Zooxanthellae are responsible for high rates of gross primary production commonly observed in coral-rich communities, and zooxanthellae photosynthesis has been shown to enhance coral calcification (Gladfelter 1985). Thus, the status of a hermatypic coral is fundamentally linked to the photosynthetic activity of its zooxanthellae population.

Zooxanthellae have the major photosynthetic pigments chlorophyll *a*, chlorophyll c_2, peridinin, diadinoxanthin, diatoxanthin, and β-carotene (Jeffrey and Haxo 1968; Brown et al. 1999b). Peridinin is a diagnostic pigment for (i.e., unique to) dinoflagellates (Kirk 1994), and high concentrations of this light-harvesting pigment give zooxanthellae their characteristic golden-brown color [relative to the predominantly green color of chlorophyll (Jeffrey and Haxo 1968)]. Chlorophylls *a* and c_2, along with peridinin, are complexed to protein and serve as major light-harvesting components. β-carotene residing with chlorophyll *a* in Core Complex I functions in photosystem I (Kirk 1994); unattached β-carotene may also serve a photoprotective role (Falkowski and Laroche 1991; Kirk 1994). Another photoprotective mechanism is the xanthophyll cycle, where, under high light conditions, diadinoxanthin is converted to diatoxanthin, with excess light dissipated as heat (Brown et al. 1999a). The coral host tissue often contains absorbing compounds; those that fluoresce typically produce bright green

colorations in the coral, while those that do not fluoresce lead to pink or blue colorations (Kawaguti 1944; Dove et al. 2001; Hochberg et al. 2004). These host pigments are collectively referred to as green fluorescent proteins (GFPs), and they have been postulated to serve in both photoprotective and photoenhancing roles (Kawaguti 1944; Salih et al. 2000; Dove et al. 2001; Mazel et al. 2003).

Researchers have long recognized the importance of photosynthetic pigments to coral ecology and biogeochemistry. The history of research in this area includes basic characterization of pigment suites (Jeffrey and Haxo 1968; Chalker and Dunlap 1981; Gil-Turnes and Corredor 1981), as well as investigation into environmental controls on pigment concentrations and on coral metabolic parameters (Dustan 1979; Falkowski and Dubinsky 1981; Brown et al. 1999a; Fitt et al. 2000; Lesser et al. 2000). In the wake of several well-publicized mass-bleaching events, recent studies have focused heavily on coral and zooxanthellae physiological responses to stressors, primarily high light and high water temperature (Kleppel et al. 1989; Porter et al. 1989; Szmant and Gassman 1990; Fang et al. 1995; Fitt and Warner 1995; Edmunds et al. 2003). Recent evidence indicates wide genetic variability among zooxanthellae, in some instances correlated to environmental conditions (Rowan and Knowlton 1995). It therefore seems possible that zooxanthellae pigment concentrations are similarly variable.

For the most part, only general environmental trends have been described for photosynthetic pigments in corals. Chlorophyll *a* tends to exhibit an inverse relationship with light intensity, showing increases in both cellular chlorophyll *a* concentration and chlorophyll *a* per unit coral colony surface area with decreases in prevailing irradiance (e.g., with increasing depth; Falkowski et al. 1990). Increases in per-cell pigment levels correspond with decreases in zooxanthellae density. In shallow water, chlorophyll *a* is also higher in zooxanthellae living in shaded microenvironments, but zooxanthellae densities remain similar to those of unshaded environments. Most attention has been paid to coral pigment concentrations under stress conditions (Kleppel et al. 1989; Fang et al. 1995; Brown et al. 1999a, 2002; Kuffner 2002). Under high light and temperature, chlorophyll *a*, chlorophyll c_2 and peridinin concentrations decrease, while concentrations of diatoxanthin, β-carotene, and chlorophyll degradation products increase. During low salinity or thermal stress events, coral pigment concentrations often decrease through expulsion of zooxanthellae and/or reduction of pigment per zooxanthella, leaving the coral visibly pale or even white (i.e., coral "bleaching"). These dramatic decreases in pigment concentrations adversely affect coral physiological status, potentially leading to mortality.

Despite their recognized importance, there has been little effort to describe natural ranges of pigments in natural, undisturbed, "healthy" coral populations. By far, researchers have directed their efforts at quantifying chlorophyll *a* (Falkowski and Dubinsky 1981; Wyman et al. 1987; Porter et al. 1989; Szmant and Gassman 1990; Fitt et al. 2000, 1993). Those few studies that include measurements of accessory pigments have only examined a small number of individual coral colonies and/or species (Jeffrey and Haxo 1968; Gil-Turnes and Corredor 1981; Fang et al. 1995; Myers et al. 1999; Lesser et al. 2000; Stambler and Dubinsky 2005). The most common approaches to quantifying coral pigments include spectrophotometry (Kawaguti 1944; Dustan 1979; Falkowski and Dubinsky 1981; Wyman et al. 1987; Porter et al. 1989; Szmant and Gassman 1990; Fitt et al. 1993, 2000) and chromatography (Jeffrey and Haxo 1968; Gil-Turnes and Corredor 1981; Kleppel et al. 1989; Fang et al. 1995; Brown et al. 1999a; Lesser et al. 2000). Both are invasive techniques that are laborious and can be expensive. A rapid, non-invasive and inexpensive technique could facilitate more extensive investigations into this aspect of coral ecology, as well as routine monitoring of these pigments, which are ideal parameters for evaluating coral bleaching status (Fitt et al. 2001).

Recognizing that pigments determine optical absorption properties of corals, a few recent studies have explored the possibility of measuring light reflected from coral surfaces to predict pigment or zooxanthellae concentrations. Berkelmans and Willis (1999) found limited success correlating zooxanthellae densities with the subjective coral color categories "normal," "moderate bleaching" and "heavy bleaching." Kizner et al. (1995) explored the correlation between chlorophyll *a* concentrations and spectrographic measurements of photographic slides. Myers et al. (1999) found a linear relationship between peridinin concentration and reflected light integrated over the region 510–610 nm. Joyce and Phinn (2003) found a statistically significant but highly variable linear relationship in 80 *Porites* sp. samples between chlorophyll *a* concentration and reflectance at 675 nm. Finally, Stambler and Dubinsky (2005) placed small coral colonies into an integrating sphere and found a power-law relationship between chlorophyll-*a*-specific, spectral averaged absorption and chlorophyll *a* concentration.

While the aforementioned studies have found relationships between coral pigments and reflected (or absorbed) light, all of the resulting models are bivariate: optical data are condensed into a single variable, discarding spectral information. The various photosynthetic pigments, however, each exhibit *spectral* absorption, and their combined absorptions are responsible for the characteristic shape of coral spectral reflectance (Myers et al. 1999; Hochberg et al. 2003). The purpose of this study is to begin development of a spectral reflectance-based bio-optical model for coral pigment concentrations.

Methods

Study Area

We haphazardly sampled individual colonies of the massive corals *Porites lobata* and *Porites lutea* (formerly *Porites evermanni*), the branching coral *Porites compressa*, and the encrusting coral *Montipora flabellata* at three sites in Kaneohe Bay, Oahu, Hawaii in January, April and August 2004. The sites included the fore reef (21°29′30″N, 157°47′30″W; ~20 m depth), back reef (21°28′40″N, 157°49′20″W; 2–4 m depth), and a patch reef (21°27″N, 157°47′W; < 1 m depth). Colonies from the patch reef site were transported to an outdoor water table at the Hawaii Institute of Marine Biology (21°26′N, 157°47′12″W), where they were sampled *in vivo* after a week-long exposure to different lighting regimes (shade and no-shade; not pertinent to this manuscript). Colonies at the fore reef and back reef sites were sampled *in situ*. In all, we acquired 37 paired samples of pigments and coral reflectance, 22 from *P. lobata*, 12 from *P. lutea*, two from *P. compressa*, and one from *M. flabellata*.

Spectral measurements

We measured and processed spectral reflectance R (implicitly a function of wavelength) for visible wavelengths (400–700 nm) following methods described in Hochberg and Atkinson (2000). The sampling unit consisted of a 2 m long fiber optic cable (400 μm diameter) attached to an Ocean Optics USB2000 portable spectrometer (wavelength range 330–850 nm, with ~0.3 nm sample interval and ~1.3 nm optical resolution, wavelengths calibrated to Ocean Optics HG-1 Hg–Ar lamp), which in turn was operated by a palmtop computer. The spectrometer and computer were in a waterproof housing, which enabled the spectrometer to be fully diver-operated. The fiber optic cable connected to the spectrometer through the housing wall via a vacuum feedthrough (Ocean Optics). The fiber optic cable tip collected light over a solid angle of ~0.1 sr, which at a distance of 10 cm projected to a circular area of 10 cm^2 (diameter ~3.5 cm). For each single measurement of R, a diver pointed the collecting tip of the fiber optic cable at the target on the coral and triggered acquisition (and storage on the palmtop) of the spectrum by pressing a button on the housing. Immediately thereafter, the diver pointed the collecting tip at a Spectralon diffuse reflectance target (same depth as the target point on the coral) and triggered the storage of its spectrum. In this manner, both spectra could be acquired within 1–2 s. Because the spectrometer was a 12-bit system with limited dynamic response, we used a 10% reflectance Spectralon so that measured light intensity from the coral and the Spectralon were of the same order (coral R averages near 10%: Hochberg et al. 2004), thus maximizing the measurable coral signal. To ensure a constant ambient light field between the two measurements, the Spectralon was placed immediately adjacent to the target point on the coral, and the diver's position was held constant for the 1–2 s required for the measurements. If light flashes due to wave focusing were obvious at the time of sampling, we shaded both the coral and Spectralon from direct light so that they were illuminated only by the ambient diffuse light field. Spectra were acquired in units of digital counts.

We corrected all spectra for baseline electrical signal, then calculated R as the ratio of digital counts measured over the coral to the digital counts measured over the Spectralon, corrected to 100% reflectance, for each pair of measurements. We linearly interpolated R to 1 nm intervals over the wavelength range 400–700 nm, then filtered the result using the Savitsky–Golay method (Savitsky and Golay 1964; Steiner et al. 1972). For each point from which pigment samples would be taken (R was measured prior to tissue sampling), we measured 10–20 replicate R's, and these were averaged for combined analysis with the pigment data. Thus, R's used for development of the bio-optical model were both time-averaged (20–60 s) and area-averaged (~10 cm^2).

Pigment measurements

Immediately after each spectral reflectance sample, two 6 mm diameter tissue samples were removed using a cork borer from a spot within the 10 cm^2 area of the reflectance measurement. The samples were wrapped in aluminum foil and placed on ice in the dark for no more than 3 h before transferring to a −50°C freezer for 2 weeks, followed by liquid nitrogen immersion for the 2 months prior to analysis. We used a jet of water to remove tissue from one of the cores in each sample so that the skeleton could be examined for species identification. The second core in each sample was ground using a mortar and pestle, then extracted in 3 ml of HPLC-grade acetone with 100 μl of the internal standard canthaxanthin at 4°C. After 12 h, the extract was pipetted off, and the sample was resuspended in 3 ml of HPLC-grade acetone for 12 additional hours at 4°C. The extracts were then combined, vortexed, and centrifuged for 5 min. Samples of 1 ml extract and 300 μl HPLC-grade water were injected onto a Varian 9012 HPLC system equipped with a Varian 9300 autosampler, a Timberline column heater (26°C), and a Spherisorb 5 μm ODS2 analytical (4.6×250 mm) column with a corresponding guard cartridge with instrument parameters following Bidigare et al. (2005). Pigments were detected using ThermoSeparations Products UV 2000 ($\lambda = 360$ nm) and FL2000 detectors. The solvent system for HPLC pigment analysis consisted of solvent A (methanol:0.5 M ammonium acetate, 80/20, v/v), solvent B (acetonitrile:water, 87.5/12.5, v/v), and solvent C (ethyl acetate) with a flow rate of 1 ml min^{-1}. Solvents A and B contained 0.01% 2,6-di-ter-butyl-*p*-cresol (0.01% BHT, w/v; Sigma-Aldrich). The linear gradient

used for pigment analysis included: 0.0' (90% A, 10% B), 1.00' (100% B), 11.00' (78% B, 22% C), 27.50' (10% B, 90% C), 29.00' (100% B), 30.00' (100% B), 31.00' (95% A, 5% B), 37.00' (95% A, 5% B), 38.00' (90% A, 10% B). Individual peaks were determined by an absorption UV/VIS diode array detector. Peak identity was determined by comparing retention times with standards (chlorophyll *c*, peridinin, diadinoxanthin, diatoxanthin, chlorophyll *a*, and β-carotene) and extracts prepared from an *Amphidinium* sp. culture. Pigment concentrations were determined through conservative interpretation of chromatogram peak areas using the WOW software package (Spectra-Physics); concentrations were based on the linear relationship between the weight of the respective standard injected and the peak area (Bidigare et al. 2005).

Statistical Analysis

We employed multivariate multiple regression with cross-validation to determine the ability of spectral data to predict pigment concentration. Our predictor variables were coral R at different wavelengths, and the response variables were concentrations of the pigments chlorophyll *a*, chlorophyll c_2, peridinin, diadinoxanthin, diatoxanthin and β-carotene. The least squares regression coefficients $\hat{\mathbf{B}}$ were calculated through matrix algebra by, $\hat{\mathbf{B}} = (\mathbf{X}'\mathbf{X})^{-1}\mathbf{X}'\mathbf{Y}$ where $\mathbf{Y}$ was the matrix of pigment data, and $\mathbf{X}$ was the matrix of R data (including a column of ones to allow for a constant term in the model). In the cross-validation technique, all but one of the spectrum-pigment pairs were used to compute regression coefficients. The coefficients were then applied to the single withheld spectrum, thus providing an unbiased prediction of pigment concentrations for that spectrum. The process was repeated for each of the 37 spectrum-pigment pairs.

The multivariate multiple regression technique required that the number of samples be greater than the number of variables (so that $\mathbf{X}'\mathbf{X}$ would be non-singular). Each of the cross-validation steps considered only 36 spectrum-pigment pairs for computation of regression coefficients. The full-resolution spectral data consisted of 301 wavelengths in the range 400–700 nm: the number of variables was far greater than the number of samples. Therefore, it was necessary to reduce the number of wavelengths considered in the analysis. Because the ultimate application of the bio-optical model is to estimate pigment concentrations through remote sensing, we applied the relative spectral response of visible wavebands from AVIRIS (Airborne Visible/InfraRed Imaging Spectrometer, Jet Propulsion Laboratory) to the R data, reducing the number of wavebands to 31. Table 1 lists waveband centers and bandwidths used to bin the R data.

We evaluated model accuracy by comparing pigment concentrations predicted from R with actual concentrations as measured by HPLC, assuming that HPLC provided the best possible estimates of true pigment values. For each of the six pigments, we performed a model I linear regression between predicted and actual values, computing correlation coefficients and assessing significance of the regression (analysis of variance F-test). To determine whether linear relationships were also 1:1 relationships, we computed 95% confidence intervals for regression parameters (slope b_1 and intercept b_0, as in $y = b_1x + b_0$), thus testing the null hypotheses that regression slopes were equal to one (H_0: $b_1 = 1$) and that regression intercepts were equal to zero (H_0: $b_0 = 0$). We computed prediction error (in μg cm^{-2}) as [actual concentration—predicted concentration] and relative prediction error (in %) as

$$\frac{\text{actual concentration} - \text{predicted concentration}}{\text{actual concentration}} \times 100.$$

Finally, we investigated whether concentration errors and relative errors varied with pigment levels, testing the null hypothesis that the slope of error versus concentration was equal to zero. It is important to note that the linear regressions in the error analysis were designed to evaluate the relationships between predicted and actual pigment concentrations. These regressions should not be confused with the multivariate multiple regression used to build the bio-optical model.

Table 1 Waveband centers and widths used for binning full-resolution R to AVIRIS wavebands

Band number	Band center (nm)	Band width FWHM (nm)
1	403.8	11.2
2	413.4	11.1
3	423.1	11.0
4	432.8	10.8
5	442.4	10.7
6	452.1	10.6
7	461.8	10.5
8	471.4	10.4
9	481.1	10.3
10	490.8	10.2
11	500.4	10.1
12	510.1	10.0
13	519.8	10.0
14	529.4	9.9
15	539.1	9.8
16	548.8	9.8
17	558.5	9.8
18	568.1	9.7
19	577.8	9.7
20	587.5	9.7
21	597.2	9.6
22	606.8	9.6
23	616.5	9.6
24	626.2	9.6
25	635.9	9.6
26	645.6	9.7
27	655.0	10.9
28	664.9	9.6
29	674.4	9.5
30	684.0	9.5
31	693.5	9.5

FWHM, full width at half-maximum

Results

All 22 *P. lobata* and both *P. compressa* colonies exhibited typical triple-peaked brown-mode *R* (after Hochberg et al. 2004), with positive reflectance features near 575, 605 and 650 nm (Fig. 1, top). Of the *P. lutea* colonies, eight showed the same brown-mode *R*; one of these had an enhanced 575 nm feature. Three *P. lutea* colonies had blue-mode *R* (apparent absorption feature near 580 nm), and a single moderately bleached *P. lutea* colony showed neither brown- nor blue-mode *R*, with very high reflectance between 600 and 650 nm and only a slight shoulder near 575 nm. The lone *M. flabellata* colony exhibited blue-mode *R*. Though decreased in spectral resolution from 1 to ~10 nm, the AVIRIS-equivalent *R*'s all showed the same spectral shapes as the full-resolution *R*'s (Fig. 1, bottom). Pigment concentrations varied by one to two orders of magnitude, depending on the pigment (Fig. 2).

The multivariate multiple regression model (coefficients listed in Table 2) performed very well, with correlation coefficients between actual and cross-validation-predicted pigment concentrations ranging from 0.941 for chlorophyll c_2 to 0.997 for chlorophyll *a* (Table 3). Model I regressions of these correlations were significant at the 99% level for all pigments (Table 3, Fig. 2). Significance tests on these regression parameters showed that no intercepts were significantly different from zero and that only chlorophyll c_2 had a slope significantly different from one (Table 3, Fig. 2). The high correlation coefficients and significances of the regressions both indicated strong, tight linear relationships between predicted and actual pigment concentrations. That the regression slopes and intercepts were near one and zero, respectively, indicated that the relationships were 1:1.

Maximum absolute values of prediction errors were 2.25, 0.99, 1.88, 0.49, 0.23 and 0.30 μg cm^{-2} for chlorophyll *a*, chlorophyll c_2, peridinin, diadinoxanthin, diatoxanthin and β-carotene, respectively. These maximum errors occurred near the middle of each pigment's concentration range (Fig. 3). Mean concentration errors were on the order of 10^{-14}–10^{-12} μg cm^{-2}, with standard deviations on the order of 0.1–0.8 μg cm^{-2} (Table 4). Based on relative prediction errors, chlorophyll *a* was the most accurately predicted pigment, with

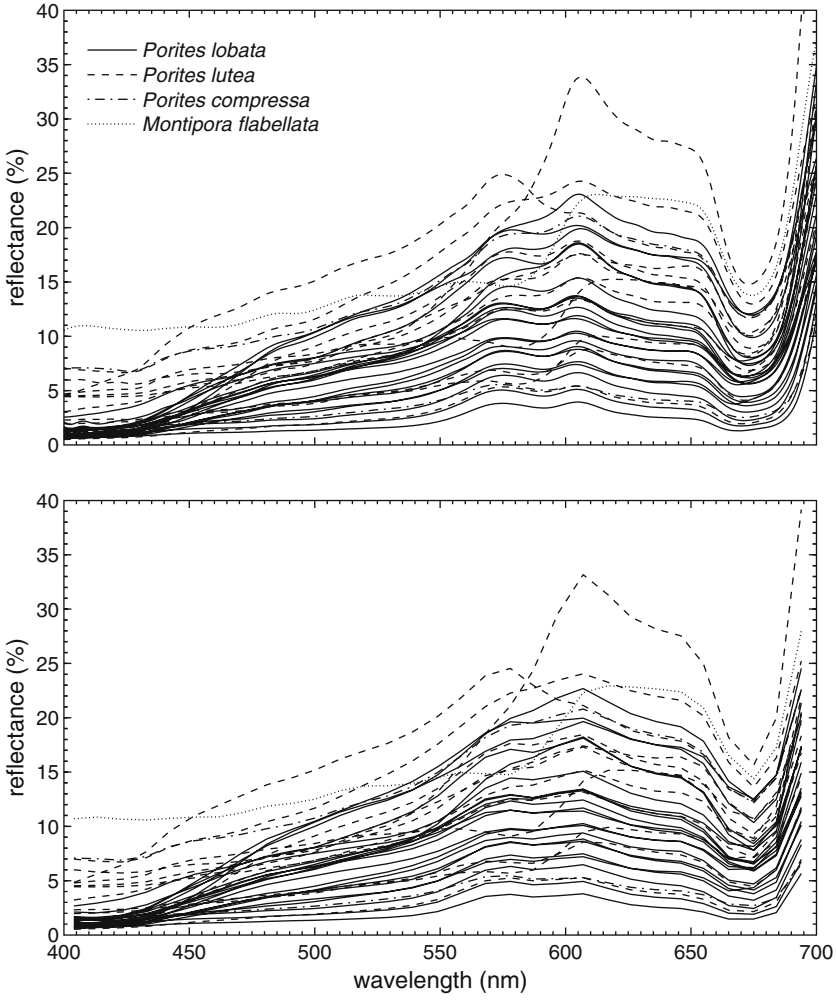

Fig. 1 Spectral reflectance *R* of *P. lobata* (*n* = 22), *P. lutea* (*n* = 12), *P. compressa* (*n* = 2), and *M. flabellata* (*n* = 1) in Kaneohe Bay, Oahu, HI. *Top*: full-resolution spectra. *Bottom*: spectra binned to match AVIRIS wavebands specified in Table 1

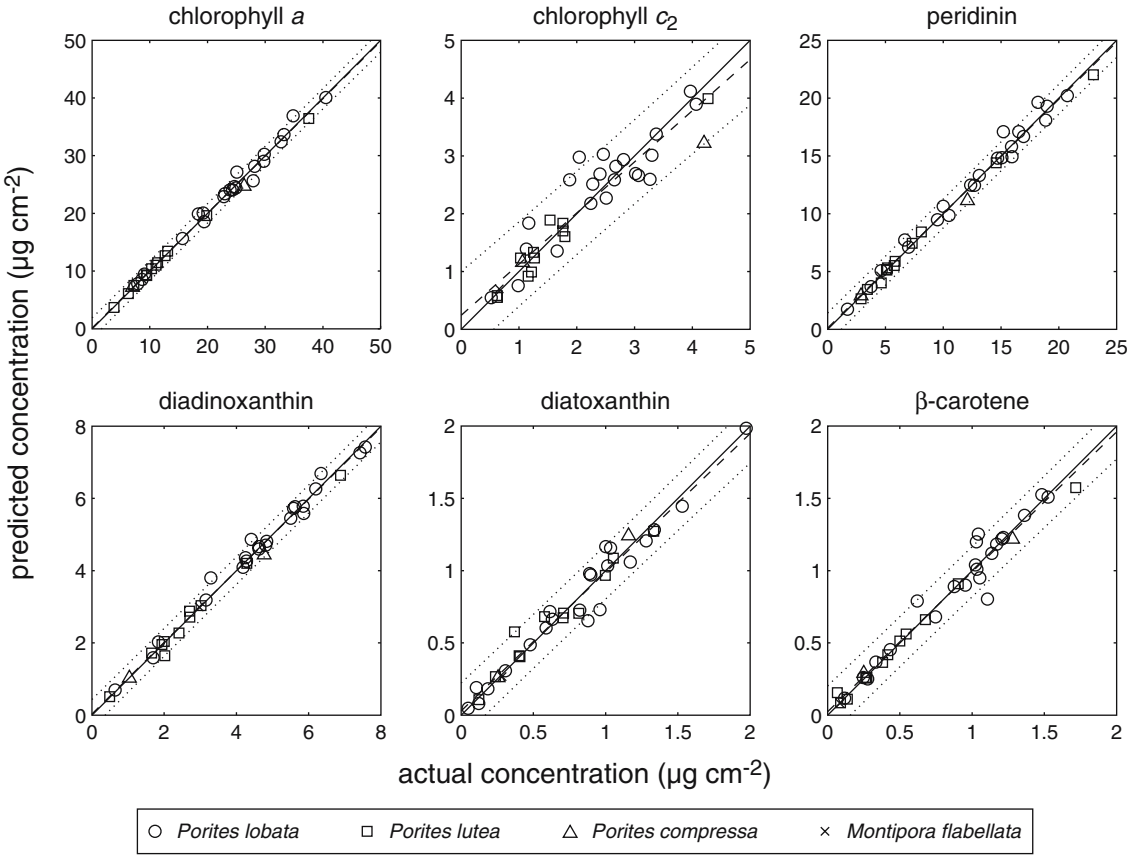

Fig. 2 Predicted versus actual pigment concentrations. Predictions are results of multivariate multiple regression with cross validation to avoid bias. *Solid lines* show 1:1 relationship. *Dashed lines* show model I regression of predicted against actual concentrations; *dotted lines* show regression 95% confidence intervals. Regression statistics in Table 3

all 37 predictions falling within ±10% of actual values (Fig. 4). The least accurately predicted pigment was chlorophyll c_2, with 18 of 37 predictions falling within ±10% of actual concentrations. For all six pigments, at least 33 of 37 (i.e., ~90%) predictions fell within ±25% of actual concentrations. Mean relative errors ranged from 0.1 to 3.3%, with standard deviations ranging from 3.4 to 23.5% (Table 4). The largest relative errors occurred at low concentrations in diadinoxanthin, diatoxanthin and β-carotene (Fig. 4). The spectra corresponding to these errors were all typical brown-mode R belonging to either *P. lobata* or *P. lutea*. Finally, prediction errors did not vary with concentration: slopes of prediction error against actual concentration were not significantly different from zero for all pigments, both for concentration and relative errors. The lone exception was concentration errors in chlorophyll c_2, which had a slight but significantly positive trend.

Discussion

R as reported here is a passive measurement that represents a convolution of the light flux reflected (i.e., not absorbed) by the coral, as well as the light flux fluoresced (i.e., absorbed and immediately re-emitted at longer wavelengths). Since fluorescence intensity varies with incident irradiance, regressions computed using R may be sensitive to fluctuating light conditions. Chlorophyll fluorescence near 685 nm by zooxanthellae is well documented and is the basis for PAM and FRR fluorometric methods of assessing photosystem II status (Beer et al. 1998; Lombardi et al. 2000; Gorbunov et al. 2001). This type of fluorescence is certainly present in all of the R's in our data set. However, regression coefficients around the chlorophyll fluorescence band are very small relative to other regions of the spectrum (Table 2), indicating that these wavelengths are not necessarily important for predicting photosynthetic pigment concentrations and therefore that the regression is not sensitive to this type of fluorescence. Many corals also contain host pigments that fluoresce, predominantly at green wavelengths (Kawaguti 1944; Dove et al. 2001; Salih et al. 2000; Mazel et al. 2003). Homologous coral-host pigments do not fluoresce, but absorb between 560 and 590 nm, resulting in blue-mode R (Dove et al. 1995; Hochberg et al. 2004). The current data set contains four individuals of the latter non-fluorescing variety. Because pigment concentrations were accurately predicted for blue-mode individuals, we reason that the regression is

Table 2 Bio-optical model coefficients for predicting photosynthetic pigment concentrations (μg cm^{-2}) in corals from *R*

Waveband	chl *a*	chl c_2	per	diadin	diato	*β*-car
C	38.81	4.94	18.37	7.94	1.56	1.73
1	−20451.7	−4069.98	−10575.7	−2667.35	−1089.51	−1402.46
2	28644.31	6436.25	12806.08	2907.88	2233	2460.35
3	6324.11	−1540.02	7700	3148.58	−2622.04	−1275.39
4	−23115.33	−4027.67	−17583.25	−6576.43	1191.5	−71.41
5	28871.31	10552.16	23378.73	8105.62	2925.86	1773.71
6	−58015.45	−15027.54	−36599.4	−10852.4	−5034.63	−3650.57
7	77946.09	11625.06	39717.12	11226.98	2836.77	3823.61
8	−68758.6	−7944.31	−33941.33	−10877.13	1800.98	−1933.31
9	48892.3	10261.11	24787.69	11492.96	−6962.72	−568.12
10	−47093.19	−12062.53	−22192.01	−12165.21	9114.19	1233.96
11	77867.89	12310.46	44968.16	16215.74	−6058.49	626.95
12	−122023.3	−10302.18	−75496.05	−23213.58	1265.75	−3164.24
13	96255.49	2004.46	62224	17894.24	−177.28	2242.09
14	−16381.61	4411.54	−14493.91	−1751.7	2134.06	1057.68
15	−11650.21	−6458.24	−8594.19	−4279.34	−1059.38	−1116.13
16	8083.66	4847.01	6547.47	2393.65	−1049.7	−259.28
17	−5846.48	−519.18	−3745.4	−1076.42	1272.12	619.63
18	6854.03	296.12	5068.61	793.12	−1420.65	−502.74
19	−26988.29	−3707.18	−14389.04	−3710.05	987.39	−609.34
20	48412.86	6464.84	24375.53	7253.69	−234.31	1716.15
21	−49082.71	−6513.37	−23865.23	−7859.27	227.64	−1560.48
22	38117.26	5438.68	16272.44	6352.13	−201.25	1105.31
23	−20835.04	−4550.54	−5908.74	−2698.06	−599.51	−1182.67
24	6738.13	2848.34	1696.2	−496.61	765.72	1138.75
25	−11766.84	−1655.74	−8488.15	−964.64	108.74	−848.34
26	36026.17	4409.52	20629.85	6443.23	−694.83	1269.93
27	−39958.5	−5865.36	−21200.05	−8030.05	974.07	−1059.36
28	16236.62	3484.83	9336.65	4309.79	−900.43	−29.08
29	−7406.73	−1976.84	−6163.55	−2773.18	10.67	79.77
30	4795.39	735.5	4440.66	1605	250.57	78.43
31	−390.02	39.92	−589.88	−174.67	−11.87	9.4

Values were determined by multivariate multiple regression using the full data set of 37 pigment-*R* pairs. Wavebands are as listed in Table 1. *C* constant term. chl *a* chlorophyll *a*. chl c_2 chlorophyll c_2. per peridinin. diadin diadinoxanthin. diato diatoxanthin. *β*-car *β*-carotene

Table 3 Statistics for model I regressions of predicted against actual pigment concentrations

Pigment	*r*	*F*	*p*	H_0: $b_1 = 1$	H_0: $b_0 = 0$
Chlorophyll *A*	0.997	5417.8	0	ns	ns
Chlorophyll C_2	0.941	270.3	0	a	ns
Peridinin	0.995	3365.7	0	ns	ns
Diadinoxanthin	0.996	4114.1	0	ns	ns
Diatoxanthin	0.980	833.1	0	ns	ns
β-carotene	0.984	1070.8	0	ns	ns

Regression *r*-, *F*- and *p*-values indicate strong linear relationship between actual and predicted pigment concentrations. Slope and intercept hypothesis tests indicate that the relationship is 1:1 for all pigments except chlorophyll c_2, which has a slope slightly less than one. $n = 37$ ($df = 35$) for all regressions. H_0 null hypothesis. b_1 slope of regression line. b_0 intercept of regression line. *ns* not significant at 95% confidence limit. [a]significant at 95% confidence limit

also able to account for the 560–590 nm absorption. Unfortunately, our sample does not include individuals exhibiting green fluorescence. Thus, it is not possible to determine whether such fluorescence will negatively affect the regression.

For the purposes of our model, we have taken the HPLC results to represent "true" pigment concentrations, but this method does have its own associated errors, potentially leading to errors in the model. First, machine errors in the HPLC device can influence elution output. Second, some corals were less amenable than others to sampling by the cork borer, resulting in small tissue tears. In these instances, samples could have been either slightly larger or smaller than if the borer had perfectly cut the coral tissue. This would have resulted in over, or underestimates, respectively, for areal pigment concentrations. Finally, we delimited chromatogram peaks conservatively, including only peak areas definitively belonging to the pigment in question. This conservatism could result in underestimates of pigment

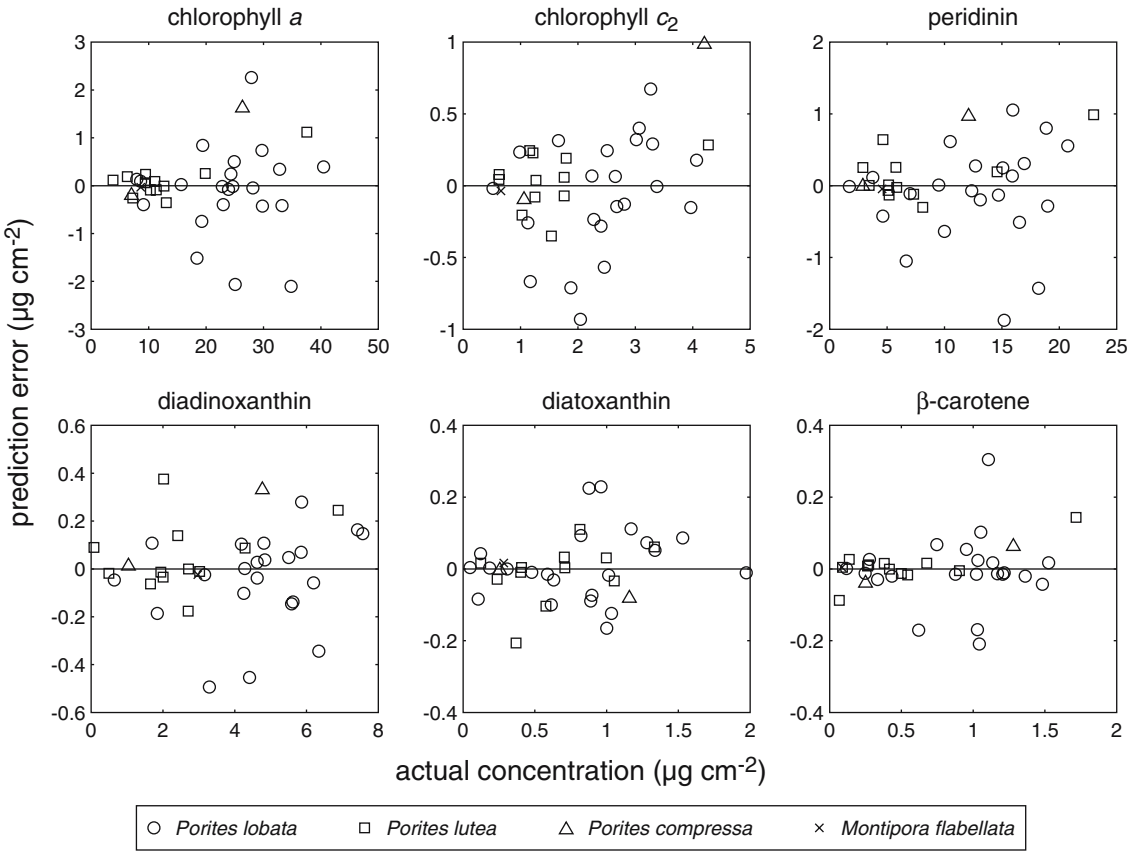

Fig. 3 Concentration prediction errors. There are no trends of error with concentration (slopes not significantly different from zero), except for chlorophyll c_2, which shows a slight positive trend. *Solid lines* are 0 μg cm^{-2} error level

Table 4 Means and standard deviations for concentration and relative errors of pigment predictions

Pigment	Concentration error (μg cm^{-2})		Relative error (%)	
	Mean	SD	Mean	SD
Chlorophyll *A*	2×10^{-12}	0.81	−0.1	3.4
Chlorophyll C_2	7×10^{-13}	0.37	−1.9	18.7
Peridinin	1×10^{-13}	0.60	−0.1	5.6
Diadinoxanthin	-3×10^{-13}	0.18	2.8	20.0
Diatoxanthin	6×10^{-14}	0.09	−2.1	19.6
β-carotene	2×10^{-13}	0.08	−3.3	23.5

SD standard deviation

concentrations. However, these are common issues faced in the quantification of pigments in general, and by following standardized quality assurance protocols, we have ensured that our HPLC data are as accurate as the method allows (Bidigare et al. 2005). The fact that our model fits the data so well indicates that HPLC errors are small relative to actual pigment concentrations, and we may regard the HPLC results as accurate. More to the point, the bio-optical model very closely reproduces pigment concentrations as derived from HPLC, which is the state-of-the-art technique for such measurements.

A more likely source of error in our data is inexact co-sampling of pigments and *R*. Because different researchers were responsible for optical acquisitions and pigment coring, in some instances, the two measurements could have been displaced from each other by several centimeters. Zooxanthellae living in different microenvironments adapt to prevailing shade or high light conditions by altering their pigment concentrations (Falkowski et al. 1990). Spatially offset *R* and pigment samples may introduce errors into the regression model, leading to inaccurate predictions, especially for pigments with inherently low concentrations. We suggest that this is the source of the few large relative prediction errors (~20%) at low concentrations in diadinoxanthin, diatoxanthin and *β*-carotene. Conversely, the pigments with the highest concentrations, chlorophyll *a* and peridinin, appear insensitive to this source of error, at

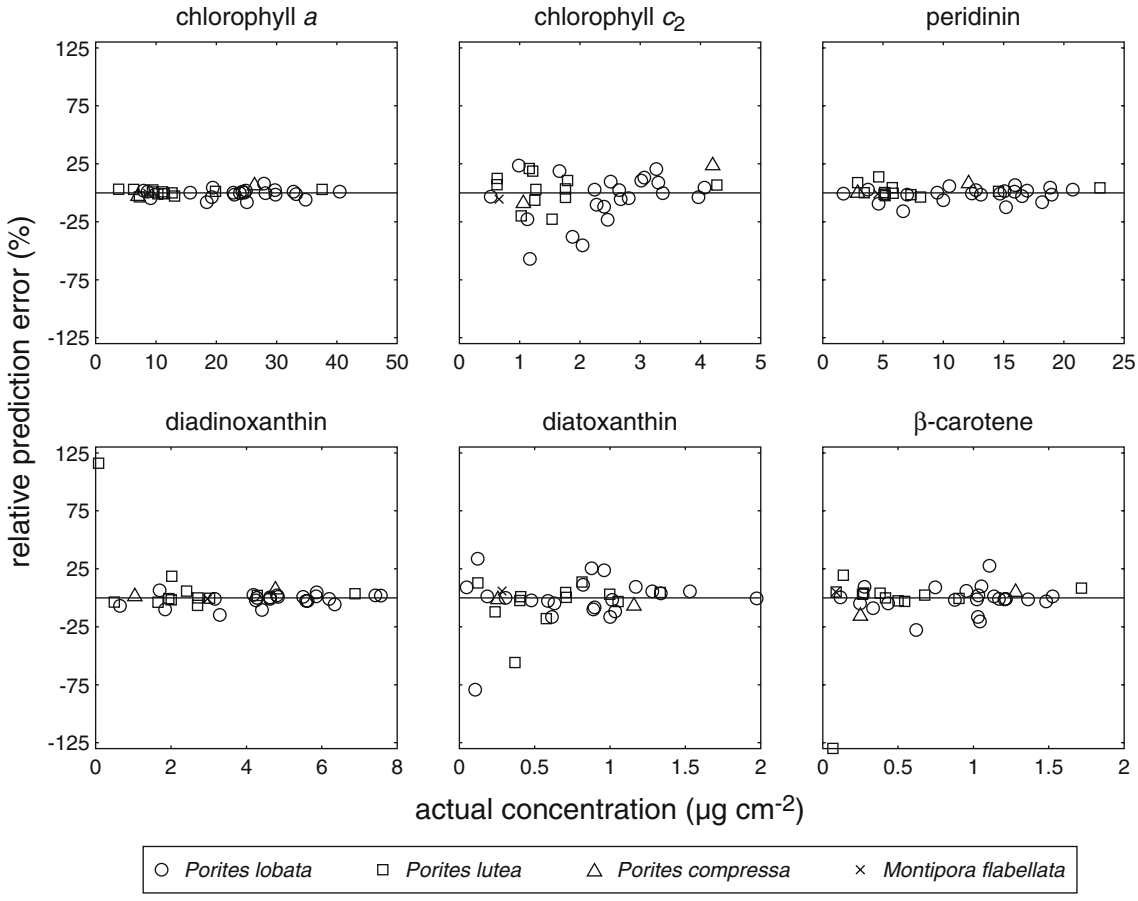

Fig. 4 Relative prediction errors. Most predictions lie within 25% of actual pigment concentrations, and relative magnitude of error does not vary with concentration (slopes not significantly different from zero). *Solid lines* are 0% error level

least in the present study where spatial offsets were at most 5 cm. It is also useful to note that small absolute errors at low pigment concentrations result in large relative errors. Thus, the combination of analytical and sampling errors more strongly affects predictions for lower-concentration accessory pigments. We expect these errors to decrease as more points are added to the data set, which will allow for more robust computation of regression coefficients, as well as for inclusion of more wavebands in the model (as sample size increases, more predictor variables can be added).

Stambler and Dubinsky (2005) used an integrating sphere to measure absorption properties for 143 coral colonies and colony fragments. They found an inverse power relationship between chlorophyll *a* concentration and spectral-averaged chlorophyll-*a*-specific absorption (reported $r^2 = 0.57$). Joyce and Phinn (2003) computed linear correlations between chlorophyll *a* concentration and *R* at individual visible and near-infrared wavelengths for 80 *Porites* sp. corals, determining that the regions 460–510 and 630–700 nm were most sensitive to variations in chlorophyll content. These authors also found a statistically significant negative linear relationship between *R* at 675 nm and chlorophyll *a* concentration, though the correlation coefficient was low (reported $r = 0.31$). Taking average values from four species of Caribbean corals, Myers et al. (1999) found a negative linear relationship between *R* integrated across the region 510–610 nm and peridinin concentration (reported $r^2 = 0.797$). In contrast to these earlier studies, which reduced spectral information to univariate parameters, our multivariate multiple regression of pigment concentration against *R* at 31 AVIRIS wavebands yielded squared multiple correlations R^2 (after Rencher 1995) of 0.99 for both chlorophyll *a* and peridinin. This result highlights the spectral nature of photosynthetic pigment absorption and demonstrates the utility of spectral-based models.

Concentrations for all pigments in this study range over at least an order of magnitude; diadinoxanthin spans two orders of magnitude (Fig. 2). Each pigment varies continuously across its concentration range. Conversely, visual assessments of the corals listed 25 colonies as "normally pigmented," ten as "pale," and two as "bleached." These assessments are both qualitative and subjective, and they inadequately describe the status of the coral-zooxanthellae symbiosis. In fact, there are few studies describing natural variability in coral pigment concentrations, or even describing what a "healthy" level of pigmentation might be (see review in

Fitt et al. 2001). Bio-optical models enable reliable, repeatable and quantitative measurements of pigment concentrations. The availability of this type of measurement sets the stage for far more detailed characterization of coral photosynthetic status under variable environmental conditions, including low salinity, thermal stress and nutrient loading events.

Our error analysis indicates that passive optical measurements of spectral reflectance provide very accurate predictions, nearly 1:1 relationships with actual values, of coral photosynthetic pigments across two orders of magnitude in concentration, and for four coral species belonging to two genera. These results are not surprising. The relative concentrations of pigments both in the zooxanthellae and coral host ultimately determine the shape and magnitude of observed spectral reflectance. In this study, all specimens exhibit basic brown- or blue-mode R without significant fluorescence features. Thus, reflectance is dominated by zooxanthellae, and therefore photosynthetic, pigments. The current data set is limited, representing only 37 individuals from four species, all from a single locale (albeit three different reef geomorphologic zones). The very accurate pigment predictions for the lone *M. flabellata* colony are encouraging, because the predictions were based on a model built only from *Porites* data, but further sampling is required to determine whether this technique can be extended to other coral taxa and growth forms, e.g. branching. Bleaching studies can help determine whether relative proportions of pigments change as corals become more fully bleached, as well as the extent to which photosynthetic pigment degradation products contribute to R. Provided accurate quantification of surface area, it may also be possible to apply bio-optical models to other reef autotrophs such as fleshy macroalgae, encrusting coralline algae and algal turfs. Such further development of the model requires laborious collection of pigment-spectrum pairs for each application, especially for irregularly shaped subjects, where quantifying surface area is problematic. However, the approach is conceptually straightforward and may be deployable in the near-term for coral monitoring, at the least.

Considerably more developmental research is necessary to enable routine application of bio-optical models in remote sensing of "coral reef color." Andréfouët et al. (2003) utilized airborne spectral imagery to identify pigments in microbial mats in atoll rims, and Andréfouët et al. (2004) demonstrated that R for reef macroalgae is scalable from the organism- to community-scale, again using airborne spectral imagery. However, these two studies were independent of significant water column radiative transfer effects, as the microbial mats were subaerial, and the macroalgae inhabited shallow (≤ 2 m) reef flats. Also, the imagery utilized in both studies had very high spatial resolution ($\sim$1 m ground sample distance). It remains to be seen whether R and pigment concentrations scale similarly from the 10 cm^2 scale of this study to the 1–10 m^2 scale of coral patches and other reef communities. Conversely, it may not be necessary to scale data if measurements of R and pigments can be made at remote sensing scales. Provided that pigments and R do correlate at remote sensing scales, another hurdle is effective retrieval of sea floor R from remote sensing data (i.e., radiative transfer inversion). This is an area of active research, and there have been several recent encouraging developments (Lee et al. 2001; Louchard et al. 2003; Mobley et al. 2005). The final step would be to link imaging technology with a bio-optical model, identifying those wavebands both (1) for which R is retrievable (including signal-to-noise issues) from remote sensing data, and (2) that provide accurate predictions of pigment concentrations. All of these steps are conceptually straightforward, but require significant research effort. Thus, remote sensing measurement of reef pigments is feasible, though it is currently a long-term prospect.

HPLC represents the state-of-the-art for pigment characterization, and thus can be the standard against which other techniques are compared. While HPLC analysis is both intrusive to the coral and laborious, our simple bio-optical model non-intrusively and rapidly provides accurate, quantitative predictions on pigment concentrations. There is clear potential for simple and low-cost (the entire system costs less than US$5,000) spectral measurements to provide for routine *in situ* monitoring of coral-zooxanthellae status, and eventually for remote sensing of coral reef color.

Acknowledegments We would like to thank Stephanie Christensen for invaluable technical support with the HPLC analysis. We would also like to thank M. Grant Susner and Chris Conger for assistance with field sampling. This work was funded by NASA award NNG04GO65G to E. J. Hochberg, NOAA award NA07-OA0571 to M. J. Atkinson, and PADI Project AWARE Foundation award to A. M. Apprill. This is SOEST contribution 6681 and HIMB contribution 1214.

References

Andréfouët S, Payri C, Hochberg EJ, Che LM, Atkinson MJ (2003) Airborne hyperspectral detection of microbial mat pigmentation in Rangiroa atoll (French Polynesia). Limnol Oceanogr 48:426–430

Andréfouët S, Payri C, Hochberg EJ, Hu C, Atkinson MJ, Muller-Karger FE (2004) Use of in situ and airborne reflectance for scaling-up spectral discrimination of coral reef macroalgae from species to communities. Mar Ecol Prog Ser 283:161–177

Beer S, Ilan M, Eshel A, Weil A, Brickner I (1998) Use of pulse amplitude modulated (PAM) fluorometry for in situ measurements of photosynthesis in two Red Sea faviid corals. Mar Biol 131:607–612

Berkelmans R, Willis BL (1999) Seasonal and local spatial patterns in the upper thermal limits of corals on the inshore Central Great Barrier Reef. Coral Reefs 18:219–228

Bidigare RR, Van Heukelem L, Trees CC (2005) Analysis of algal pigments by high-performance liquid chromatography. In: Andersen RA (ed) Algal culturing techniques. Elsevier Academic Press, Burlington, MA, pp 327–346

Brown BE, Ambarsari I, Warner ME, Fitt WK, Dunne RP, Gibb SW, Cummings DG (1999a) Diurnal changes in photochemical efficiency and xanthophyll concentrations in shallow water reef corals: evidence for photoinhibition and photoprotection. Coral Reefs 18:99–105

Brown BE, Dunne RP, Ambarsari I, Le Tissier MDA, Satapoomin U (1999b) Seasonal fluctuations in environmental factors and variations in symbiotic algae and chlorophyll pigments in four Indo-Pacific coral species. Mar Ecol Prog Ser 191:53–69

Brown BE, Dunne RP, Goodson MS, Douglas AE (2002) Experience shapes the susceptibility of a reef coral to bleaching. Coral Reefs 21:119–126

Chalker BE, Dunlap WC (1981) Extraction and quantitation of endosymbiotic algal pigments from reef-building corals. Proc fourth Int Coral Reef Symp 2:45–50

Dove SG, Takabayashi M, Hoegh-Guldberg O (1995) Isolation and partial characterization of the pink and blue pigments of Pocilloporid and Acroporid corals. Biol Bull 189:288–297

Dove SG, Hoegh-Guldberg O, Ranganathan S (2001) Major colour patterns of reef-building corals are due to a family of GFP-like proteins. Coral Reefs 19:197–204

Dustan P (1979) Distribution of zooxanthellae and photosynthetic chloroplast pigments of the reef-building coral *Montastrea annularis* Ellis and Solander in relation to depth on a West Indian coral reef. B Mar Sci 29:79–95

Edmunds PJ, Gates RD, Gleason DF (2003) The tissue composition of *Montastraea franksi* during a natural bleaching event in the Florida Keys. Coral Reefs 22:54–62

Falkowski PG, Dubinsky Z (1981) Light-shade adaptation of *Stylophora pistillata*, a hermatypic coral from the Gulf of Eilat. Nature 289:172–174

Falkowski PG, Jokiel PL, Kinzie RA (1990) Irradiance and corals. In: Dubinsky Z (ed) Ecosystems of the world 25: coral reefs. Elsevier, Amsterdam, pp 89–107

Falkowski PG, Laroche J (1991) Acclimation to spectral irradiance in algae. J Phycol 27:8–14

Fang L-S, Liao C-W, Liu M-C (1995) Pigment composition in different-colored scleractinian corals before and during the bleaching process. Zool Stud 34:10–17

Fitt WK, Spero HJ, Halas J, White MW, Porter JW (1993) Recovery of the coral Montastrea annularis in the Florida Keys after the 1987 Caribbean "bleaching event". Coral Reefs 12:57–64

Fitt WK, Warner ME (1995) Bleaching patterns of four species of Caribbean reef corals. Biol Bull 189:298–307

Fitt WK, McFarland FK, Warner ME, Chilcoat GC (2000) Seasonal patterns of tissue biomass and densities of symbiotic dinoflagellates in reef corals and relation to coral bleaching. Limnol Oceanogr 45:677–685

Fitt WK, Brown BE, Warner ME, Dunne RP (2001) Coral bleaching: interpretation of thermal tolerance limits and thermal thresholds in tropical corals. Coral Reefs 20:51–65

Gil-Turnes S, Corredor J (1981) Studies of the photosynthetic pigments of zooxanthellae in Caribbean hermatypic corals. Proc Fourth Int Coral Reef Symp 2:51–54

Gladfelter EH (1985) Metabolism, calcification and carbon production II: organism-level studies. Proc Fifth Int Coral Reef Congr 4:527–542

Gorbunov MY, Kolber ZS, Lesser MP, Falkowski PG (2001) Photosynthesis and photoprotection in symbiotic corals. Limnol Oceanogr 46:75–85

Hochberg EJ, Atkinson MJ (2000) Spectral discrimination of coral reef benthic communities. Coral Reefs 19:164–171

Hochberg EJ, Atkinson MJ, Andréfouët S (2003) Spectral reflectance of coral reef bottom-types worldwide and implications for coral reef remote sensing. Remote Sens Environ 85:159–173

Hochberg EJ, Atkinson MJ, Apprill A, Andréfouët S (2004) Spectral reflectance of coral. Coral Reefs 23:84–95

Jeffrey S, Haxo F (1968) Photosynthetic pigments of symbiotic dinoflagellates (zooxanthellae) from corals and clams. Biol Bull 135:149–165

Joyce KE, Phinn SR (2003) Hyperspectral analysis of chlorophyll content and photosynthetic capacity of coral reef substrates. Limnol Oceanogr 48:489–496

Kawaguti S (1944) On the physiology of reef corals VI. Study on the pigments. Palao Trop Biol Stn Stud 2:617–674

Kirk JTO (1994) Light and photosynthesis in aquatic environments. Cambridge University Press, Cambridge, 509p

Kizner Z, Achituv Y, Dubinsky Z, Thieberger Y (1995) A novel, nondestructive bioassay for assessing areal chlorophyll a in hermatypic cnidarians. Limnol Oceanogr 40:1166–1173

Kleppel G, Dodge R, Reese C (1989) Changes in pigmentation associated with the bleaching of stony corals. Limnol Oceanogr 34:1331–1335

Kuffner IB (2002) Effects of ultraviolet radiation and water motion on the reef coral, Porites compressa Dana: a transplantation experiment. J Exp Mar Biol Ecol 270:147–169

Lee Z, Carder KL, Chen RF, Peacock TG (2001) Properties of the water column and bottom derived from Airborne Visible Infrared Imaging Spectrometer (AVIRIS) data. J Geophys Res Oceans 106:11639–11651

Lesser MP, Mazel C, Phinney D, Yentsch CS (2000) Light absorption and utilization by colonies of the congeneric hermatypic corals *Montastraea faveolata* and *Montastraea cavernosa*. Limnol Oceanogr 45:76–86

Lombardi MR, Lesser MP, Gorbunov MY (2000) Fast repetition rate (FRR) fluorometry: variability of chlorophyll a fluorescence yields in colonies of the corals, Montastraea faveolata (w.) and Diploria labyrinthiformes (h.) recovering from bleaching. J Exp Mar Biol Ecol 252:75–84

Louchard EM, Reid RP, Stephens FC, Davis CO, Leathers RA, Downes TV (2003) Optical remote sensing of benthic habitats and bathymetry in coastal environments at Lee Stocking Island, Bahamas: a comparative spectral classification approach. Limnol Oceanogr 48:511–521

Mazel CH, Lesser MP, Gorbunov MY, Barry TM, Farrel JH, Wyman KD, Falkowski PG (2003) Green-fluorescent proteins in Caribbean corals. Limnol Oceanogr 48:402–411

Mobley CD, Sundman LK, Davis CO, Bowles JH, Downes TV, Leathers RA, Montes MJ, Bissett WP, Kohler DDR, Reid RP, Louchard EM, Gleason A (2005) Interpretation of hyperspectral remote-sensing imagery by spectrum matching and look-up tables. Appl Optics 44:3576–3592

Muscatine L (1967) Glycerol excretion by symbiotic algae from corals and *Tridacna* and its control by the host. Science 156:516–519

Muscatine L (1990) The role of symbiotic algae in carbon and energy flux in reef corals. In: Dubinsky Z (ed) Ecosystems of the world 25: coral reefs. Elsevier, Amsterdam, pp 75–87

Myers MR, Hardy JT, Mazel CH, Dustan P (1999) Optical spectra and pigmentation of Caribbean reef corals and macroalgae. Coral Reefs 18:179–186

Porter JW, Fitt WK, Spero HJ, Rogers CS, White MW (1989) Bleaching in reef corals: physiological and stable isotopic responses. Proc Natl Acad Sci USA 86:9342–9346

Rencher AC (1995) Methods of multivariate analysis. John Wiley and Sons, NY, 627p

Rowan R, Knowlton N (1995) Intraspecific diversity and ecological zonation in coral-algal symbiosis. Proc Natl Acad Sci USA 92:2850–2853

Salih A, Larkum A, Cox G, Kühl M, Hoegh-Guldberg O (2000) Fluorescent pigments in corals are photoprotective. Nature 408:850–853

Savitsky A, Golay MJE (1964) Smoothing and differentiation of data by simplified least squares procedures. Anal Chem 36:1627–1639

Stambler N, Dubinsky Z (2005) Corals as light collectors: an integrating sphere approach. Coral Reefs 24:1–9

Steiner J, Termonia Y, Deltour J (1972) Comments on smoothing and differentiation of data by simplified least square procedure. Anal Chem 44:1906–1909

Szmant AM, Gassman NJ (1990) The effects of prolonged "bleaching" on the tissue biomass and reproduction of the reef coral *Montastrea annularis*. Coral Reefs 8:217–224

Wyman K, Dubinsky Z, Porter J, Falkowski P (1987) Light absorption and utilization among hermatypic corals: a study in Jamaica, West Indies. Mar Biol 96:283–292