Plant, Cell and Environment (2006) **29**, 2133–2142 doi: 10.1111/j.1365-3040.2006.01587.x

The impact of coral bleaching on the pigment profile of the symbiotic alga, *Symbiodinium*

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ABSTRACT

Bleaching of corals by loss of symbiotic dinoflagellate algae and/or photosynthetic pigments is commonly triggered by elevated temperatures coupled with high irradiance, and is a first-order threat to coral reef communities. In this study, a high-resolution high-performance liquid chromatography method integrated with mass spectrometry was applied to obtain the first definitive identification of chlorophyll and carotenoid pigments of three clades of symbiotic dinoflagellate algae (*Symbiodinium*) in corals, and their response to experimentally elevated temperature and irradiance. The carotenoids peridinin, dinoxanthin, diadinoxanthin (Dn), diatoxanthin (Dt) and β-carotene were detected, together with chlorophylls *a* and c_2, and phaeophytin *a*, in all three algal clades in unstressed corals. On exposure to elevated temperature and irradiance, three coral species (*Montastrea franksi* and *Favia fragum* with clade B algae, and *Montastrea cavernosa* with clade C) bleached by loss of 50–80% of their algal cells, with no significant impact to chlorophyll *a* or c_2, or peridinin in retained algal cells. One species (*Agaricia* sp. with clade C) showed no significant reduction in algal cells at elevated temperature and irradiance, but lost substantial amounts of chlorophyll *a* and carotenoid pigments, presumably through photo-oxidative processes. Two coral species (*Porites astreoides* and *Porites porites* both bearing clade A algae) did not bleach. The impact of elevated temperature and irradiance on the levels of the photoprotective xanthophylls (Dn + Dt) and β-carotene varied among the corals, both in pool size and xanthophyll cycling, and was not correlated to coral bleaching resistance.

Key-words: symbiosis; xanthophyll cycling; zooxanthellae.

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INTRODUCTION

The colouration of many reef-building corals is derived from the photosynthetic pigments of their endosymbiotic, dinoflagellate algae of the genus *Symbiodinium*. The pigment complement of dinoflagellates is distinct from terrestrial plants and chlorophyte algae in containing chlorophylls c_1 and/or c_2, and the major xanthophylls peridinin or fucoxanthin (or fucoxanthin derivatives) (Kirk 1994). Dinoflagellates including *Symbiodinium* also contain the xanthophylls diadinoxanthin (Dn) and diatoxanthin (Dt) (Ambarsari *et al*. 1997), which are functionally equivalent to the photoprotective xanthophyll cycle pigments violaxanthin, antheraxanthin and zeaxanthin in terrestrial plants (Demmig-Adams & Adams 1996).

Recently, *Symbiodinium* pigments have been the subject of keen interest in relation to their significance in 'coral bleaching', a phenomenon defined as the loss of colour of corals, caused by the elimination of symbiotic algal cells or degradation of algal pigments. Bleaching is triggered by a range of environmental stressors, including temperature extremes and high irradiance, and is deleterious to corals (reviewed in Brown 1997). Bleached corals display depressed growth rates, increased susceptibility to pathogens and mechanical damage, and reduced survivorship (Szmant & Gassman 1990; Meesters & Bak 1993; Harvell *et al*. 1999). The increased frequency and severity of episodes of mass coral bleaching in recent years have been attributed to elevated temperature and irradiance associated with global climate change, and is recognized as a major threat to coral reef ecosystems (Stone *et al*. 1999; Wilkinson 1999; Hughes *et al*. 2003).

The primary mechanism underlying temperature/irradiance triggered bleaching is thought to involve photoinhibition of *Symbiodinium* photosystems, perhaps arising from depressed levels of the D1 protein in photosystem II (PSII) (Warner, Fitt & Schmidt 1999) or impairment of the Calvin cycle (Jones *et al*. 1998), leading to reduced rates of electron transport. The resultant excess of excitation energy in the photosynthetic antennae activates singlet excited states of chlorophyll *a* to triplet excited states, causing the production of reactive oxygen species (ROS), including singlet oxygen ($^1O_2$) and superoxide radicals (O_2^-) in bleaching

corals (Lesser *et al*. 1990; Lesser 1996, 1997; Tchernov *et al*. 2004). Elevated levels of ROS cause widespread damage to membrane lipids, cellular proteins and chlorophyll *a* (Asada & Takahashi 1987; Matille *et al*. 1996) and are widely believed to trigger the breakdown of algal photosynthetic pigments and host-mediated elimination of algal cells (Franklin *et al*. 2004; Tchernov *et al*. 2004).

Impacts on *Symbiodinium* photosynthetic pigments during bleaching are frequently measured exclusively as alterations in algal chlorophyll (Szmant & Gassman 1990; Fitt *et al*. 1993; Rowan *et al*. 1997) and comparatively little information is available on the impacts of bleaching on the total *Symbiodinium* pigment profile, especially the xanthophylls. Exceptionally Ambarsari *et al*. (1997) and Dove *et al*. (2006) showed significant fluctuations in the algal carotenoids peridinin and Dn in bleached corals together with both chlorophylls *a* and c_2. Ambarsari *et al*. (1997) and Brown *et al*. (1999) obtained evidence of a functional xanthophyll cycle in *Symbiodinium*, thought to operate as part of non-photochemical quenching (NPQ), also observed in other marine algae (Demers *et al*. 1991), and analogous to the photoprotective xanthophylls cycle in terrestrial plants. Under high light, Dn is de-epoxidated to Dt, which protects PSII from excess excitation and the consequent oxidative damage; and Dt is transformed back to Dn in darkness (Olaizola & Yamamoto 1994; Olaizola *et al*. 1994). Xanthophyll pigments may also have photoprotective benefits beyond NPQ, including stabilization of the chloroplast thylakoid under high irradiance (Havaux *et al*. 1996; Tardy & Havaux 1997; Havaux & Niyogi 1999) and quenching of ROS (Havaux & Niyogi 1999; Havaux *et al*. 2000).

Although the photoprotective function of the Dn/Dt cycle is established in *Symbiodinium*, the response of these pigments under conditions that trigger coral bleaching is less understood. Studies on bleaching using pulse modulated amplitude (PAM) fluorimetry indicate that mechanisms of NPQ including xanthophyll cycling may have profound impacts on the tolerance of corals to thermal-irradiance stress (Warner, Fitt & Schmidt 1996; Rowan 2004). Furthermore, high temperature/light may have significant impacts on the abundance of other carotenoids such as β-carotene, which is known to be a particularly efficient antioxidant when in close proximity to sites of ROS production (Burton 1990; Young & Britton 1993).

High-performance liquid chromatography (HPLC) studies on dinoflagellate pigments including those in *Symbiodinium* have largely relied on photodiode array (PDA) detection of electronic absorption spectra for the identification of pigments and their transformation products (e.g. Ambarsari *et al*. 1997; Barlow, Cummings & Gibb 1997; Dove *et al*. 2006). This method is limited by the various pigments with similar or indistinguishable UV-Vis spectra (Borrego *et al*. 1998), and liquid chromatography-mass spectrometry (LC-MS) is increasingly used as a complement or replacement to PDA detection in pigment analysis (Goericke, Shankle & Repeta 1999; Airs, Atkinson & Keely 2001). Furthermore, previous analyses of *Symbiodinium* pigments have used binary mobile phase methodologies with short run times (Ambarsari *et al*. 1997; Brown *et al*. 1999, 2002a). Development of a quaternary mobile phase system by Airs *et al*. (2001) provides greater resolution of complex pigment extracts and can be applied directly to LC-MS to produce definitive information on pigment composition.

The purpose of this study was to profile the pigments in *Symbiodinium* using the improved HPLC-PDA and LC-MS methodologies, and to investigate the impact of elevated temperature/irradiance on the pigment composition under experimental conditions. As a first approach to assess the pigment variation within the genus, members of three clades of *Symbiodinium*, distinguished by their SSU rRNA gene sequence, in a total of six coral species were studied.

MATERIALS AND METHODS

Experimental design

The experimental material comprised samples of six coral species of boulder or plate-like morphology collected at 4–6 m depth from patch reefs in Bermuda's northern lagoon: *Porites porites* and *Porites astreoides*, which bear *Symbiodinium* of clade A, *Montastrea franksi* and *Favia fragum* with clade B, and *Montastrea cavernosa* and *Agaricia* sp. with clade C (Savage *et al*. 2002a). Fragments of approximately 5 cm^2 of each species were removed from parent colonies using a hammer and chisel. The coral samples of each species were assigned randomly to four groups. The first group was assayed immediately, and the other three groups were allowed to acclimate for 14 d in aquaria shaded from direct solar irradiance (ca 14:10 h light:dark, with midday irradiance of 800 μmol m^{-2} s^{-1} PAR above the aquaria). The aquaria were supplied with running, unfiltered seawater at a flow rate of 1.25 L min^{-1} and ambient sea temperature of 26–28 °C. The acclimation period was well in excess of the time required for algal photoacclimation to the new light environment (Harland & Davies 1994; Savage, Trapido-Rosenthal & Douglas 2002b). After acclimation, one group was harvested immediately, and the other two groups transferred to either control aquaria or treatment aquaria. Conditions in control aquaria matched acclimation aquaria, but the treatment aquaria were kept at 32 °C and exposed to direct solar irradiance with clear, almost cloudless skies and irradiance peaking at 2000 μmol m^{-2} s^{-1} PAR above the aquaria at midday. Corals were harvested at midday after 48 h exposure to control and treatment conditions. Individual corals were wrapped in aluminium foil before removal from the aquaria and then immediately transferred to a –80 °C freezer. The frozen coral samples were thawed, airbrushed in zooxanthella isolation buffer (ZIB; comprising 0.4 M NaCl, 10 mM Na_2EDTA and 20 mM Tris pH 7.5), washed three times by centrifugation (500 *g*, 15 min, 4 °C) and then divided into three aliquots, which were recentrifuged. The first aliquot was stored for pigment

analysis (refrozen at –80 °C); the second was transferred to DMSO buffer comprising 20% DMSO, 0.25 M Na_2EDTA, saturated NaCl pH 7.5 for molecular typing of the *Symbiodinium*; and the third aliquot was stored in 2% formalin in ZIB for determination of cell density using a haemocytometer. The surface area of the airbrushed coral skeleton was determined by the tinfoil method of Marsh (1970). The experimental sequence was repeated four times during June and July 2003. The results were pooled and treated as one data set, following statistical analysis that confirmed non-significant differences between experiments.

Identification and quantification of *Symbiodinium* pigments

Pigments were extracted from frozen *Symbiodinium* isolates by sonication in 100% acetone (AR grade), following the demonstration by Airs *et al*. (2001) and Walker (2004) that this procedure minimizes pigment degradation, including the oxidative degradation of chlorophyll *a* that can occur with methanol as a solvent. Extracts were filtered through solvent-extracted cotton wool plugs, dried by rotary evaporation, redissolved in acetone and methylated with freshly prepared diazomethane (Black 1983) to stabilize the sample and improve chromatographic separation (Airs *et al*. 2001). The extracts were dried under a stream of nitrogen and transported dry at 4 °C to the UK for analysis. Preliminary pigment identifications were made by HPLC with PDA detection and confirmed by LC-MS. Once the identifications were secure, the pigments were quantified by HPLC against standards. HPLC procedures were adapted from Airs *et al*. (2001) and were as follows.

Symbiodinium pigments were separated by reverse phase HPLC, using a Hewlett Packard HPLC system, comprising a 1100 autosampler, a 1050 vacuum degasser, a 1050 quaternary pump and a 1040A Photo-Diode Array (Hewlett Packard, Bracknell, UK). The HPLC column (5 μm Luna C18 column with a C18 ODS Octadecyl guard column, Phenomenex Ltd, Macclesfield, UK) was maintained at 30 °C and eluted with a quaternary mobile phase gradient (0.5 mL min^{-1}) set to the following programme [percentages of ammonium acetate (0.01 M), methanol, acetonitrile and ethyl acetate given respectively]: 0–10 min: hold 5, 80, 15 and 0%; 10–50 min: gradient to 1, 33, 15 and 51%; 50–55 min: gradient to 0, 1, 1 and 98%; 55–60 min: hold 0, 1, 1 and 98%; 60–65 min: gradient 5, 80, 15 and 0%; 65–75 min: hold 5, 80, 15 and 0%.

Absorbance chromatograms were extracted at 450 nm for all carotenoids and chlorophyll c_2, 430 nm for chlorophyll *a* and 410 nm for phaeopigments. The area of each peak was modelled by a Gaussian curve, using SPSS Peakfit Software (SPSS Inc, Chicago, IL, USA). Pigments were quantified with respect to known concentrations of chlorophyll *a* and phaeophytin *a* (standards provided by the Analytical Chemistry Group, University of York, UK) and surrogate carotenoid standards (Sigma Chemical Company Ltd, Poole, UK) via relative response factors, run every third sample throughout the analysis.

Pigment samples were analysed by LC-MS using a Finnigan system, comprising a Thermo-Separations AS3000 autosampler, P4000 gradient pump, UV2000 UV/Vis detector and a MAT LCQ ion trap mass spectrometer (Finnigan, San Jose, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source operated in the positive ion mode. Pigments were separated in the reversed phase mode using a 7 × 250 mm Apex ODS11 5 μm column (Jones Chromatography Ltd, Pontypridd, UK) and HPLC conditions described earlier. MS parameters were: capillary temperature 150 °C, APCI vapourizer temperature 450 °C, discharge current 5 μA, sheath gas flow 40 (arbitrary units), default isolation width 5 Th, collision energy 25%. The ionization of chlorophylls was enhanced by the post-column addition of formic acid (Airs & Keely 2000).

Molecular typing of *Symbiodinium*

DNA was extracted using QIAgen DNA-easy plant mini kits (Qiagen, Valencia, CA, USA)according to the manufacturer's instructions. The clade of each *Symbiodinium* isolate was determined by PCR-RFLP analysis of the SSU rRNA gene with 'host excluding primers' ss3z and ss5z (Rowan & Powers 1991) and digested with the restriction enzymes *Taq*I and *Dpn*II, following the procedure of Bythell *et al*. (1997).

Statistical analysis

Data were analysed with SPSS v.12 software (SPSS Inc.) using *t*-tests, one-way or two-way analysis of variance (ANOVA) or multivariate analysis of variance (MANOVA), with Tukey's *post hoc* test to identify significant differences between treatments in ANOVA tests. Where indicated in the text, the data were logarithmically transformed to obtain normal distributions with homogenous variances.

RESULTS

The *Symbiodinium* pigments

Symbiodinium pigment profiles were dominated by chlorophyll *a* and peridinin, both of which comprised isomers in the HPLC eluant appearing as two or more peaks in HPLC chromatograms (Fig. 1a & Table 1). All *Symbiodinium* extracts also contained the pigments Dn, Dt, β-carotene and phaeophytin *a* (Fig. 1a & Table 1). All samples additionally contained the minor xanthophyll dinoxanthin and the alteration product of Dn, diadinochrome, neither of which has been reported previously in HPLC studies of *Symbiodinium* (Kleppel, Dodge & Reese 1989; Ambarsari *et al*. 1997; Dove *et al*. 2006). LC-MS data (mass-to-charge ratios of protonated ions and fragment ions) were particularly important in distinguishing between Dn, Dt and dinoxanthin which all shared similar UV-Vis spectrum absorption bands. There were no qualitative differences in the pigment profiles of *Symbiodinium* of different clades or coral species.

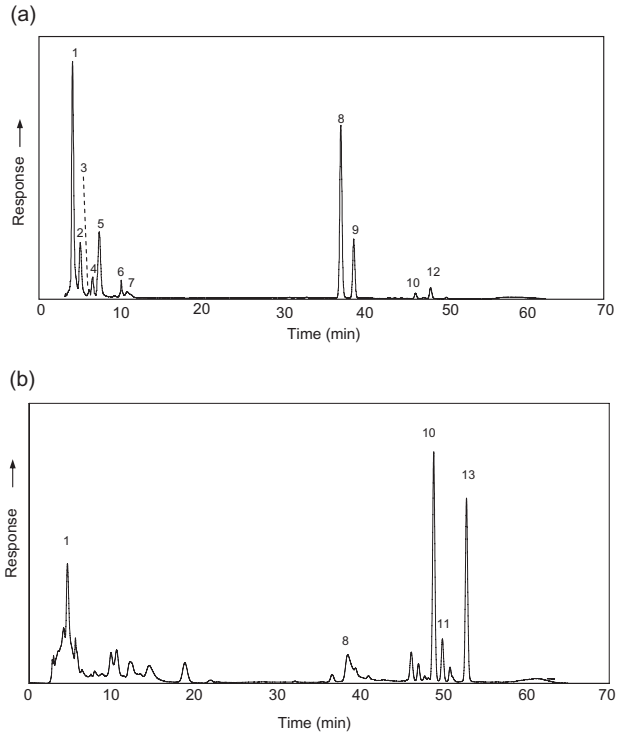

Figure 1. High-performance liquid chromatography-photodiode array max-plot chromatograms of pigments extracted from *Symbiodinium*. Peaks 1 to 13 are identified in Table 1. (a) *Agaricia* sp. colony under control conditions (ambient temperature and shade). (b) *Agaricia* sp. colony treated with elevated temperature and irradiance.

Samples of *Agaricia* sp. that had been subjected to the elevated temperature/irradiance treatment yielded small chlorophyll *a* peaks and large peaks of the chlorophyll *a* breakdown products phaeophytin *a* and pyrophaeophytin *a* as well as numerous peaks provisionally identified as alteration products of chlorophylls and carotenoids (Fig. 1b and Table 1).

The data set for the post-acclimation corals was used to assess the quantitative variation in concentration of pigments normalized to chlorophyll *a*. The variation across the six coral species was statistically significant (MANOVA Wilk's Lambda: species; $F_{25,102} = 2.614$, $P < 0.001$). One-way ANOVA of individual carotenoids revealed that this variation could be attributed principally to peridinin (species; $F_{5,30} = 7.893$, $P < 0.001$), which attained a significantly higher concentration in the algae of *Agaricia* sp. than in the other coral species, and not to other pigments, including the Dn + Dt pool (species; $F_{5,30} = 0.238$, $P > 0.05$) and β-carotene (species; $F_{5,30} = 1.728$, $P > 0.05$).

Impact of elevated temperature and irradiance on *Symbiodnium* cell density

Symbiodinum cell density was adopted as the primary index of coral bleaching in response to elevated temperature and irradiance. Over the 14 d acclimation period, no significant change in algal density was obtained: (two-way ANOVA: species $F_{5,46} = 38.970$, $P < 0.001$; treatment $F_{1,46} = 0.134$, $P > 0.05$; interaction $F_{5,46} = 4.22$, $P > 0.05$). For the post-acclimation corals, the number of algal cells per

Table 1. Identification data for *Symbiodinium* pigments obtained by high-performance liquid chromatography-photodiode array and liquid chromatography-mass spectrometry

Peak number in Fig. 1	Identification	Retention time T_R (min)	UV-Vis spectrum absorption bands	Relative molecular mass (RMM)[a]	*m/z* of protonated molecules $[M + H]^+$	*m/z* of fragment ions
1	Peridinin	4	474	630	631	613, 553, 535
2	*cis* peridinin	5	330, 467	630	631	613, 553, 535
3	Dinoxanthin	7	417, 441, 469	642	643	625, 565
4	Chlorophyll c_2	8	456	609	601[d]	
5	Diadinoxanthin	9	427, 445, 476	582	583	565, 547
6	Diatoxanthin	11	427, 452, 480	566	567	549, 531
7	Diadinochrome	12	441, 469	582	583	565, 547
8	Chlorophyll *a*	37	433, 664	893.5	871[c]	593, 533
9	Chlorophyll *a*[b]	38	431, 664	893.5	871[c]	593, 533
10	Phaeophytin *a*	46	410, 665	871	871[c]	593, 533
11	Phaeophytin *a*[b]	47	410, 665	871	871[c]	593, 533
12	β-carotene	48	454, 480	536	537	
13	Pyrophaeophytin *a*	49	411, 667	813	813	535.3

[a]From Jeffrey, Mantoura & Wright (1997).
[b]Peaks 9 and 11 are stereoisomers (epimers) of the preceding pigments.
[c]The *m/z* of the protonated molecules and fragment ions of chlorophyll *a*, phaeophytin *a* and their stereoisomers (peaks 8 to 11, Fig. 1) are identical. This arises by the post-column addition of formic acid to the sample to enhance ionization of chlorophyll *a* for mass spectrometry (Airs & Keely 2000). The procedure effectively converts chlorophyll *a* to phaeophytin *a* by the replacement of Mg^{2+} by two protons. Chlorophyll *a* and phaeophytin *a* are easily distinguished by their UV-Vis absorption bands however, which when considered with their mass spectra, provides a sound basis for their identification.
[d]The *m/z* of the protonated molecules of chlorophyll c_2 arises by the addition of 14 Da by methylation with diazomethane and the replacement of Mg^{2+} by two protons by post-column addition of formic acid.

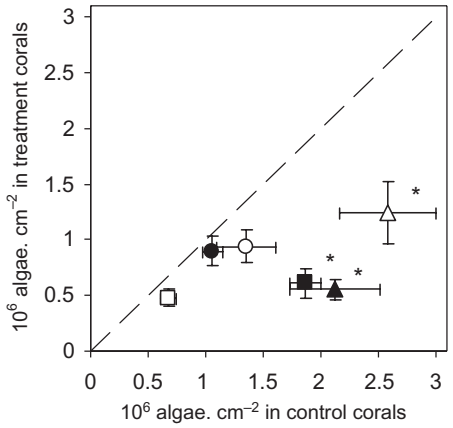

Figure 2. *Symbiodinium* cell density in corals subjected to treatment (elevated temperature and sunlight) and control (ambient temperature and shade) conditions. *Symbiodinium* clade A: *Porites porites* (●) and *Porites astreoides* (○). Clade B: *Montastrea franksi* (▲) and *Favia fragum* (△). Clade C: *Montastrea cavernosa* (■) and *Agaricia* sp. (□). Error bars ± SE. Data were log transformed to conform to normality and homogeneity of variance before analysis with two-way analysis of variance. Coral species $F_{5,58} = 6.449$, $P < 0.001$; treatment $F_{1,58} = 48.809$, $P < 0.001$; interaction $F_{5,58} = 2.619$, $P < 0.05$. Asterisk (*) indicates coral species with significant differences between control and treatment values, as determined by Tukey's *post hoc* analysis.

unit colony surface area varied significantly among the coral species (one-way ANOVA, species; $F_{5,30} = 21.145$, $P < 0.001$) but without consistent differences among the three algal clades. When treated with elevated temperature and irradiance, the algal density in three species, *F. fragum* (B), *M. franksi* (B) and *M. cavernosa* (C), was significantly depressed, relative to the control corals (Fig. 2), but no significant differences were obtained for the clade A corals, *P. porites* and *P. astreoides*, and for *Agaricia* sp. (clade C).

Impact of elevated temperature and irradiance on chlorophyll *a* and major accessory pigments

The chlorophyll *a* content per algal cell varied significantly with coral species in post-acclimation colonies (one-way ANOVA, species; $F_{5,32} = 5.186$, $P < 0.001$), and *post hoc* tests revealed that the values for *Agaricia* sp. were significantly higher than the other species tested. No significant change in chlorophyll *a* values occurred in the 14 d acclimation period (two-way ANOVA: species $F_{5,48} = 2.566$, $0.01 < P < 0.05$; treatment $F_{1,48} = 0.001$, $P > 0.05$; interaction $F_{5,48} = 0.498$, $P > 0.05$). Elevated temperature and irradiance caused no significant change in chlorophyll *a*, peridinin or chlorophyll c_2 content per algal cell in five corals species, including those that lost significant numbers of algae. *Agaricia* sp. experienced a significant reduction in pigmentation, however, occurring specifically in chlorophyll *a*, peridinin and chlorophyll c_2 (Fig. 3). Reductions in chlorophyll *a* in treated *Agaricia* sp. were accompanied by

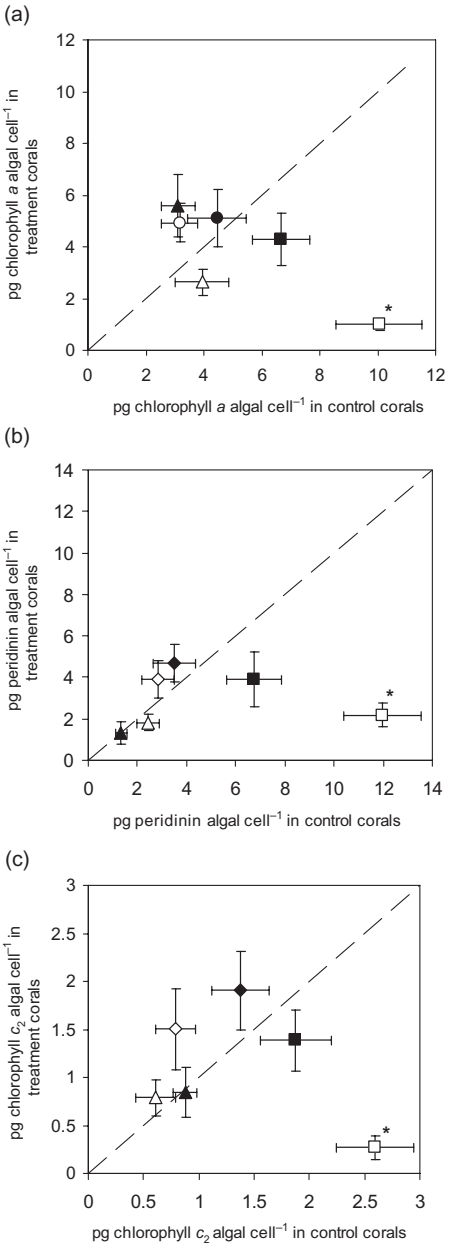

Figure 3. Abundance of major light harvesting and accessory pigments of *Symbiodinium* in corals subjected to treatment (elevated temperature and sunlight) and control (ambient temperature and shade) conditions. *Symbiodinium* clade A: *Porites porites* (●) and *Porites astreoides* (○). Clade B: *Montastrea franksi* (▲) and *Favia fragum* (△). Clade C: *Montastrea cavernosa* (■) and *Agaricia* sp. (□). Error bars ± SE. Data were log transformed to conform to normality and homogeneity of variance. Asterisk (*) indicates coral species with significant differences between control and treatment values, as determined by Tukey's *post hoc* analysis. (a) Chlorophyll *a* per algal cell. Two-way analysis of variance (ANOVA); coral species $F_{5,68} = 0.988$, $P > 0.05$; treatment $F_{1,68} = 4.743$, $P < 0.05$; interaction $F_{5,68} = 10.243$, $P < 0.001$. (b) Peridinin per algal cell. Two-way ANOVA; coral species $F_{5,68} = 8.451$, $P < 0.01$; treatment $F_{1,68} = 4.114$, $P < 0.05$; interaction $F_{5,68} = 7.303$, $P < 0.01$. (c) Chlorophyll c_2 per algal cell. Two-way ANOVA; coral species $F_{5,68} = 1.182$, $P > 0.05$; treatment $F_{1,68} = 1.166$, $P > 0.05$; interaction $F_{5,68} = 2.985$, $P < 0.05$.

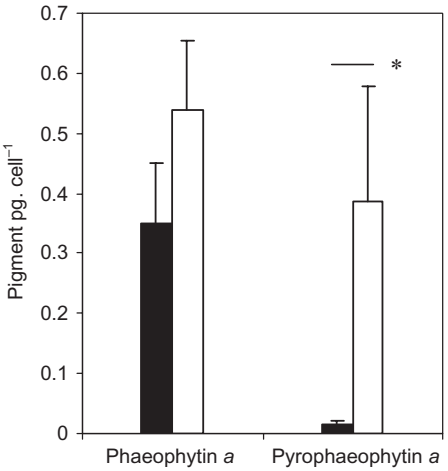

Figure 4. Chlorophyll transformation products, phaeophytin *a* and pyrophaeophytin *a* in algae from control (black bars) and treatment (clear bars) *Agaricia* sp. *t*-tests. Phaeophytin *a*: $t_{14} = 0.889$, $P > 0.05$. Pyrophaeophytin *a*: t_{14} (equal variances not assumed) = 0.336, $P < 0.05$. Error bars ± SE. Asterisk (*) indicates significant difference.

an increase in the chlorophyll transformation product pyrophaeophytin *a*, but not phaeophytin *a* (Fig. 4).

Impact of elevated temperature and irradiance on photoprotective *Symbiodinium* pigments

The Dn + Dt pool size per cell was significantly greater in algae from elevated temperature/irradiance treated colonies than control colonies of three species: *P. porites*, *P. astreoides* and *M. franksi*; and significantly reduced for *Agaricia* sp. (Fig. 5a). When the data were normalized to chlorophyll *a* content, a significant difference between treatment and control colonies was obtained only for *P. porites* (Fig. 5b). (Data for *Agaricia* sp. were not included in this analysis because the algal cells in the treatment corals had been bleached of > 90% of their chlorophyll *a*).

Xanthophyll cycling (increases in Dt relative to the total Dn + Dt pool size) was significantly elevated in treatment colonies relative to controls for all species except *P. porites* (Fig. 5c). This was despite the fact that the algal cells displayed a significantly increased Dn + Dt pool size relative to chlorophyll *a* only in *P. porites*. The increase in xanthophyll cycling in *Agaricia* sp. could be attributed to reduction in Dn content in treatment colonies relative to the controls ($t_{13} = 6.738$, $P < 0.001$) and not to an increase in Dt ($t_{13} = 1.990$, $P > 0.05$).

The β-carotene levels per cell were increased in the treatment corals relative to controls in *P. astreoides* and *M. franksi* (two-way ANOVA; species $F_{4,48} = 2.615$, $P < 0.05$; treatment $F_{1,48} = 0.526$; $P > 0.05$; interaction $F_{4,48} = 4.136$, $0.01 > P > 0.001$ and least significant difference *post hoc* analysis), but the remaining corals showed no significant increase. When normalized to chlorophyll *a*, changes in β-carotene content were not significant for any species (two-way ANOVA; species $F_{4,48} = 1.542$, $P > 0.05$; treatment

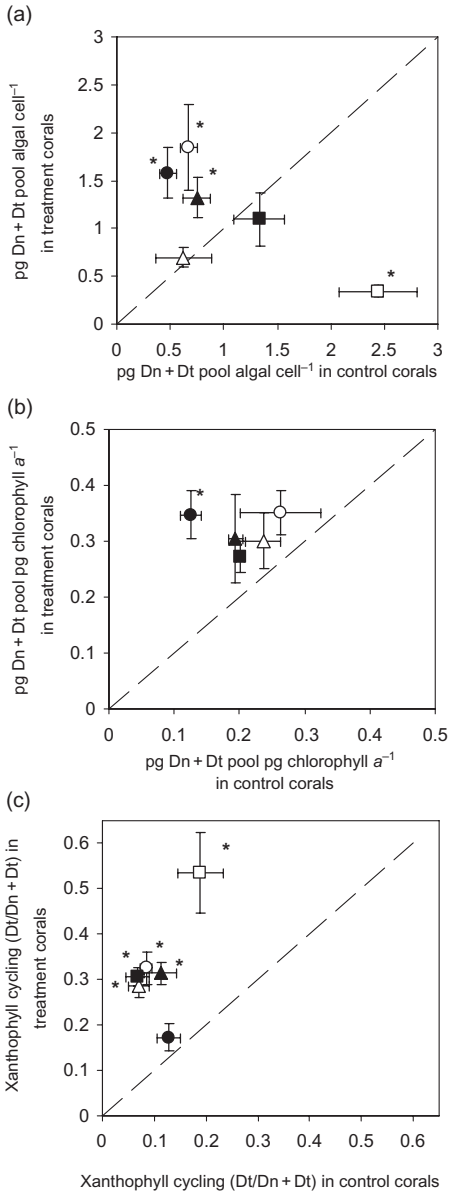

Figure 5. Diadinoxanthin (Dn) and diatoxanthin (Dt) in corals subjected to treatment (elevated temperature and sunlight) and control (ambient temperature and shade) conditions. *Symbiodinium* clade A: *Porites porites* (●) and *Porites astreoides* (○). *Symbiodinium* B: *Montastrea franksi* (▲) and *Favia fragum* (△). *Symbiodinium* C: *Montastrea cavernosa* (■) and *Agaricia* sp. (□). Error bars ± SE. Data were transformed to conform to normality and homogeneity of variance. Asterisk (*) indicates coral species with significant differences between control and treatment values, as determined by Tukey's *post hoc* analysis. (a) Xanthophyll pool (Dn + Dt) size per cell. Two-way analysis of variance (ANOVA) after logarithmic transformation: coral species $F_{5,66} = 1.158$, $P > 0.05$; treatment $F_{1,66} = 0.446$, $P > 0.05$; interaction $F_{5,66} = 16.784$, $P < 0.001$. (b) Xanthophyll pool (Dn + Dt) size per pg chlorophyll *a*. Two-way ANOVA after square-root transformation: coral species $F_{4,54} = 0.859$, $P > 0.05$; treatment $F_{1,54} = 18.062$, $P < 0.001$; interaction $F_{4,54} = 1.305$, $P > 0.05$. (c) Xanthophyll cycling (Dt / [Dn + Dt]). Two-way ANOVA after arcsin-square root transformation: coral species $F_{5,66} = 5.782$, $P < 0.001$; treatment $F_{1,66} = 77.570$, $P = 0.001$; Interaction $F_{5,66} = 2.576$, $P < 0.05$.

$F_{1,48} = 0.580$; $P > 0.05$; interaction $F_{4,48} = 1.362$, $P > 0.05$) (*Agaricia* sp. excluded from the analysis).

Clade analysis of *Symbiodinium*

PCR-RFLP analysis resolved the *Symbiodinium* from all coral colonies to the clade as identified previously by Savage *et al*. (2002a): A in *P. porites* and *P. astreoides*, B in *M. franksi* and *F. fragum*, and C in *M. cavernosa* and *Agaricia* sp.

DISCUSSION

Coral bleaching and pigment damage

'Bleaching' in the botanical sense usually refers to the destruction of photosynthetic pigments by photo-oxidative processes that can occur in both higher plants and algae (Brown, Houghton & Hendry 1991; Asada 1996; Velitchkova & Picorel 2004; Ledford & Niyogi 2005). Coral biologists have adopted the term to also refer to a significant reduction in algal density that effectively leads to a loss of algal pigment in the whole symbiosis. In the current study, three coral species bleached by loss of significant numbers of symbiotic algae: *M. franksi* and *F. fragum* both bearing clade B and *M. cavernosa* with clade C, with no significant loss of chlorophylls, carotenoids, nor increases in chlorophyll *a* transformation products, for example, pyrophaeophytin *a*, detected in the remaining algal cells of these corals. These findings support the conclusion of numerous field and laboratory studies that detected no significant loss of chlorophyll per algal cell in bleached coral using the spectrophotometric methods of Jeffrey & Humphrey (1975) on unseparated pigment extracts (Fitt *et al*. 1993; Le Tissier & Brown 1996; Jones 1997). However, these spectrophotometric analyses could not rule out the confounding influence of chlorophyll *a* transformation products on their chlorophyll *a* estimations. The HPLC approach in the current study provides definitive evidence that this potential problem is not significant; at least for the systems examined here, the chlorophyll *a* in algal cells retained by bleached coral can be intact. It is interesting however that these findings are in contrast to recent reports of an increased presence of chlorophyll oxidation products in thermally stressed corals that did not undergo significant declines in algal density (Dove *et al*. 2006). In the current study, it is unknown whether none of the algal cells experienced appreciable pigment degradation or the algal cells with damaged pigments were eliminated selectively. This unresolved issue presents an obvious course for future research.

Extensive pigment damage was observed in one coral species in the current investigation, and was similar to that observed in a HPLC analysis of *Symbiodinium* in the coral *Goniastrea aspera* (Ambarsari *et al*. 1997). *Agaricia* sp. bearing clade C algae did not lose significant numbers of algae, but displayed dramatic reduction of chlorophyll *a*, peridinin and chlorophyll c_2, together with an increase in pyrophaeophytin *a*, but with no significant increase in the other chlorophyll transformation product phaeophytin *a*. Pyrophaeophytin *a* is transformed from chlorophyll *a* via phaeophytin *a* without the destruction of the chlorophyll macrocycle. The increased presence of pyrophaeophytin *a* accounted for less than 5% of the original amount of chlorophyll *a*, leaving most of the degraded chlorophyll unaccounted for. Most of the chlorophyll *a* is likely to have been eliminated by photo-oxidative reactions involving opening of the chlorophyll macrocycle by attack of singlet oxygen and leading to colourless molecules, including linear tetrapyrroles and maleimides (Carpenter, Elser & Elser 1986; Grice *et al*. 1996). These molecules would not be detected using the analytical approach adopted here.

Agaricia sp. tends to occupy shaded habitats on the reef and a lack of a significant change in chlorophyll *a* following collection may indicate a limited photoacclimatory ability in this species. A shade-adapted pigment profile could plausibly have made *Agaricia* sp. particularly vulnerable to pigment-bleaching by elevated temperature and irradiance. As a consequence, the light levels used in this study may have been appreciably greater than those routinely experienced by *Agaricia* sp. in the field and as a result, may not be relevant to field bleaching of this species. Despite this uncertainty about the ecological relevance of the bleaching response of *Agaricia* sp., the data provide evidence that the degradation of algal pigments is not necessarily an important prerequisite for algal expulsion.

The corals *P. porites* and *P. astreoides* bearing clade A did not undergo bleaching by loss of algae or pigment. The chief significance of this result is that it is consistent with several field surveys and field transplant experiments (Rowan & Knowlton 1995; Rowan *et al*. 1997; Baker 2001; LaJeunesse 2002) that have led to the description of clade A as bleaching-resistant (Rowan 1998; Knowlton & Rohwer 2003). An important caveat to this interpretation of our data, however, is that both corals are *Porites* species, and bleaching resistance could equally be a trait of the genus *Porites* as of *Symbiodinium* clade A.

Coral bleaching and photoprotective pigments

Interconversion of the pigments Dn and Dt is part of a well-documented process of NPQ believed to be both central to photoprotection in *Symbiodinium* of the coral *G. aspera* (Brown *et al*. 1999) and vital to the prevention of ROS production in corals (Brown *et al*. 2002b). The pigment β-carotene (detected in all pigment extracts in the current study) is also an important antioxidant in marine algae (Ben-Amotz, Shaish & Avron 1989). Contrary to the expectation that these pigments may play a protective role against bleaching, no clear link between coral bleaching resistance and the abundance of Dn + Dt or β-carotene in *Symbiodinium* was obtained in this study. In particular, the elevated temperature/irradiance treatment caused a significant increase in xanthophyll pool size in symbiotic algae in just three of the six species tested, although the increases were only significant in *P. porites* (bleaching-resistant) when normalized to chlorophyll *a*. Importantly, algae in the

other bleaching resistant coral *P. astreoides* did not display increased Dn + Dt pool sizes or β-carotene.

Elevated temperature/irradiance caused increased levels of Dt relative to the Dn + Dt pool in all corals except *P. porites*. Thus, photoprotective xanthophyll cycling was activated in the bleaching resistant *P. astreoides* and also in the corals that bleached through loss of algal density. Xanthophyll cycling, appeared to be highest in algae of *Agaricia* sp., which experienced severe pigment loss (see earlier discussion) but this result is misleading because it was mediated by the preferential destruction of Dn and not an increase in Dt.

Xanthophyll cycling is likely to be one of several mechanisms that underpin the environmental tolerance of a coral–algal symbiosis. Symbiotic dinoflagellates, unlike free-living marine algae, exist in coral tissue equipped with additional photoprotective and thermoprotective mechanisms, for example, fluorescent proteins (Dove 2004) and heat shock proteins (Black, Voellmy & Szmant 1995), which may complement or even surpass photoprotective mechanisms linked to the algal pigment profile and thus help maintain the integrity of the symbiosis (Brown *et al*. 2002a).

In conclusion, the analysis by HPLC-PDA and LC-MS provides definitive data on the pigment profile of *Symbiodinium* of clades A, B and C and how the impacts of bleaching on pigment profiles may vary among coral–algal associations. The data demonstrate that corals may bleach by pigment-bleaching, as also observed in the field by Ambarsari *et al*. (1997), and by a reduction in algal density without a significant impact on the chlorophyll *a* in remaining algal cells. The abundance of Dt and Dn may fluctuate in corals experiencing thermal/irradiance stress without correlation to bleaching resistance.

ACKNOWLEDGMENTS

We thank the Natural Environment Research Council (NERC) and the Engineering and Physical Sciences Research Council (EPSRC) for financial support, Nicola Francis and Sandra Zielke for technical support, and Dr Owen Atkin, Professor Barbara Brown and Dr Ross Jones for comments that improved this manuscript. This is contribution 1677 from the Bermuda Biological Station for Research, Inc.

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Received 5 April 2006; received in revised form 18 July 2006; accepted for publication 20 July 2006

