

'Chlorophyll-like' compounds as novel biomarkers of stress in corals

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ABSTRACT: The appearance of 'bleached' or pale-coloured corals at numerous locations around the world over the last 3 decades has been linked to various environmental stressors. However, the role and fate of zooxanthellar pigments in response to such stress has received little attention. We provide new insights into the adaptive response of coral zooxanthellae to environmental stress, based upon the analysis of chlorophyll pigments using high performance liquid chromatography. Manipulative field experiments were used to monitor induction and recovery of the reef coral *Goniastrea aspera* from photo-damage. Over a 6 d period the production of up to 6 compounds with very similar light absorbance spectra to chlorophyll *a* (chl *a*) was observed. Higher concentrations of a sub-set of these products were observed in response to elevated solar radiation levels. The fact that the same chl *a*-like compounds were consistently observed under the same experimental conditions means that they may have significant potential as biomarkers of stress in corals and may also provide a means of 'fingerprinting' environmental stress in corals.

KEY WORDS: Coral bleaching · Stress biomarkers · Symbiotic algae · Chlorophyll *a* · HPLC

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INTRODUCTION

Since the early 1980s 'bleached' or pale-coloured corals have been observed at numerous locations around the world (Glynn 1984). The concern expressed over bleaching has resulted in extensive studies of the processes involved. The bleaching of corals has now been linked to various environmental stressors, including elevated and reduced temperature (Hoegh-Guldberg & Smith 1989, Glynn 1993, Coles & Fadalallah 1991, Muscatine et al. 1991), elevated visible and ultraviolet light levels (Hoegh-Guldberg & Smith 1989, Lesser et al. 1990, Kinzie 1993), combined light deprivation and starvation (Yonge & Nicholls 1931), low salinity (Goreau 1964, Fang 1995) and bacterial infection (Kushmaro et al. 1996, Ben-Haim et al. 1999).

It is now evident that corals exhibit a range of pathological and physiological responses to environmental pressures depending on the degree or type of stress to which they are exposed. These range from more obvious and easily quantified responses, such as the loss of

zooxanthellae, to more subtle changes in the host and symbiont, such as increased expressions of heat-shock proteins and antioxidant enzymes (Downs et al. 2000). The ability to measure responses under subtle levels of stress could be exploited to detect changes in biomarkers preceding 'bleaching' events, to provide a valuable tool to both marine scientists and coastal zone managers.

The loss of zooxanthellae (and their pigments) has long been established as a signal of stress in cnidarians (Yonge & Nicholls 1931, Brown & Howard 1985). Nevertheless, despite the focus on coral bleaching research in the last 2 decades (see Hoegh-Guldberg 1999, for review), there has been surprisingly little attention paid to coral algal pigments, either with respect to their involvement in the physiological mechanism of bleaching (Ambarsari et al. 1997) or their potential value as bio-indicators of stress.

Coral pigmentation, in particular chlorophyll *a* (chl *a*) content, is frequently determined by long established spectrophotometric or fluorometric methods

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(e.g. Holm-Hansen et al. 1965, Lorenzen 1967). However, these methods, which have traditionally been used in the characterisation of phytoplankton pigmentation, are prone to inaccuracies associated with the spectral interference of other chlorophylls (e.g. chlorophyll *b*), carotenoids, or from chl *a* degradation products (e.g. chlorophyllides, phaeophytins). The use of high performance liquid chromatography (HPLC) in conjunction with spectrophotometric or fluorescence detectors allows the rapid separation and more accurate quantitation of a wide range of chlorophyll and carotenoid pigments to be achieved (Wright et al. 1991, Ambarsari et al. 1997, Jeffrey et al. 1997). While these techniques are commonly used to provide estimates of phytoplankton abundance and insights into population composition, their application to coral studies has been limited.

In the present work we exploited HPLC with photodiode array detection to provide evidence of a subtle stress response in the symbiotic zooxanthellae of coral. This response would not be discernable through the application of conventional spectrophotometric or fluorometric techniques. We report the identification of 6 chl *a*-like compounds, which may be potential bio-indicators of low-level stress in the zooxanthellae of the coral *Goniastrea aspera*. This response was rapid and recoverable and may be indicative of a novel mechanism involved in the adjustment of corals to environmental stress.

MATERIALS AND METHODS

Study site. Experiments were carried out on colonies of the shallow water coral species *Goniastrea aspera* in Phuket, Thailand (7° 50' N, 98° 25.5' E), during November 2000. Factors responsible for solar bleaching and the temporal progression of bleaching in corals at the site have been described in detail by Brown et al. (1994) and Le Tissier & Brown (1996). Notably, west faces of corals are exposed to higher incident solar radiation than east faces. As such, rotation of coral colonies from east to west is proposed to induce light stress and rotation from west to east to allow recovery from light stress.

Rotation experiments. Coral colonies of approximately 20 cm in diameter were carefully detached from the reef using a hammer and stone-chisel and rotated 180° before being cemented in their new position. Additional colonies were detached from the reef and then replaced in their original position to act as handling controls. All colonies were tagged for future identification. As a result of the manipulation, former east surfaces now faced west and vice versa in rotated colonies. In this discussion, samples from corals rotated

from the west to east sides will be denoted 'W→E' and those rotated from east to west as 'E→W'. Samples from the east and west control or non-manipulated corals will be denoted as 'E_{cont}' and 'W_{cont}', respectively.

Sample collection and preparation. Cores (25 mm diameter) were removed from east and west sides of 5 coral colonies from each of the above conditions using methodology previously described (Brown et al. 1999a). Baseline cores were taken at the beginning of a neap tide sequence at the time of manipulation on 13 November 2000. Experimental cores were collected at 2, 4 and 6 d after the corals were aerielly exposed on the subsequent low spring tide sequence (23, 25 and 27 November).

After collection in the field, the cores were taken, in darkened containers on ice, back to the laboratory where they were frozen at -50°C within 30 min of sampling. Algal pellets were prepared as described by Ambarsari et al. (1997). Briefly, tissue was removed from coral cores by air gunning to produce algal pellets, which were stored under liquid nitrogen prior to transportation to the UK for subsequent studies.

In the laboratory, algal pellets were homogenised in buffer (Tris-HCl, 10 mM) and a sub-sample was stored for the determination of cell numbers. Pigments were extracted from the remaining homogenate using methanol with the aid of ultrasonication. Extracts were clarified using centrifugation (5 min at 1800 × *g*), and the supernatant was drawn off by micro-syringe for analysis by HPLC.

Pigments were analysed by the method of Barlow et al. (1997), modified as described in Brown et al. (2002a). In brief, aliquots of the clarified extract were loaded into the autosampler and maintained at 2°C before being vortex-mixed with 1 M ammonium acetate buffer (1:1, v/v). Buffered extracts were injected (100 µl) onto a Hypersil MOS2 C-8 column (3 µm particle size, 100 × 4.6 mm) maintained at 30°C. Pigments were separated using a binary mobile phase system (min; 70:30 [v/v] methanol:1.0 M ammonium acetate; 100% methanol): (0; 100; 0), (2; 50; 50), (12; 30; 70), (25; 10; 90), (27; 0; 100), (29; 0; 100), (29.1; 0; 100), (35; 0; 100). Flow rate was constant at 0.85 ml min⁻¹.

The analyses were performed using an integrated Thermo-Separations HPLC system comprising: SCM1000 vacuum degasser, P4000 quaternary pump, AS3000 auto-sampler (with sample tray cooling, column over and sample preparation capability) and UV6000LP diode array detector (DAD). System control, data collection and integration were performed using Chromquest software.

Pigment identity was secured through co-elution with authentic pigments obtained from either Sigma Aldrich or from DHI. Confirmation of analyte identity

was achieved through spectral comparison of DAD data to standards using the Chromquest software. Quantification of pigments was carried out at 444 nm.

Algal cell counts from pigment subsamples were obtained using a Neubauer haemocytometer and by following established techniques (Brown et al. 1999a). Pigment concentrations were then calculated as femtomoles cell⁻¹.

Data analysis. Compounds were identified as 'chl *a*-like' by their proximity to chl *a* on the HPLC chromatogram and their spectral similarity to chl *a* determined using the Chromquest software. These compounds were quantified by peak area and denoted by their retention time relative to chl *a* itself.

Statistical comparisons were carried out to compare the concentration of chl *a*-like compounds, chl *a* itself and the total chl *a* pool, i.e. chl *a* plus chl *a*-like compounds (total chl *a*), between control and rotated cores.

RESULTS

Analysis of algal pellets from the coral *Goniastrea aspera* by HPLC with DAD revealed the presence of a range of chlorophyll and carotenoid pigments, principally chlorophyll *c*₂, chl *a*, peridinin, diadinoxanthin, diatoxanthin and β-carotene (Fig. 1a). This pigment signature is characteristic of zooxanthellae and has been reported previously (Kleppel et al. 1989, Ambarsari et al. 1997).

More detailed examination of the chromatograms revealed the presence of a number of additional peaks in the time window around chl *a*. A total of 6 discrete compounds were identified from the samples analysed, all exhibiting characteristic chlorophyll-like absorbance spectra, with blue spectral bands at 418 to 434 nm and red spectral bands at 654 to 662 nm (Figs. 1b & 2). The retention times of these chl *a*-like compounds relative to that of chl *a* were consistent in all samples analysed (Table 1; with 1 exception, which was excluded from further analyses). While chromatographic retention times were highly reproducible, the integrated peak area (and by inference their concentration) showed some variability (Fig. 3).

Data from rotated cores was compared to that from controls in order to study the initial response to the increase (E→W) or decrease (W→E) of light stress (e.g. W→E compared with W_{cont} and with E_{cont}).

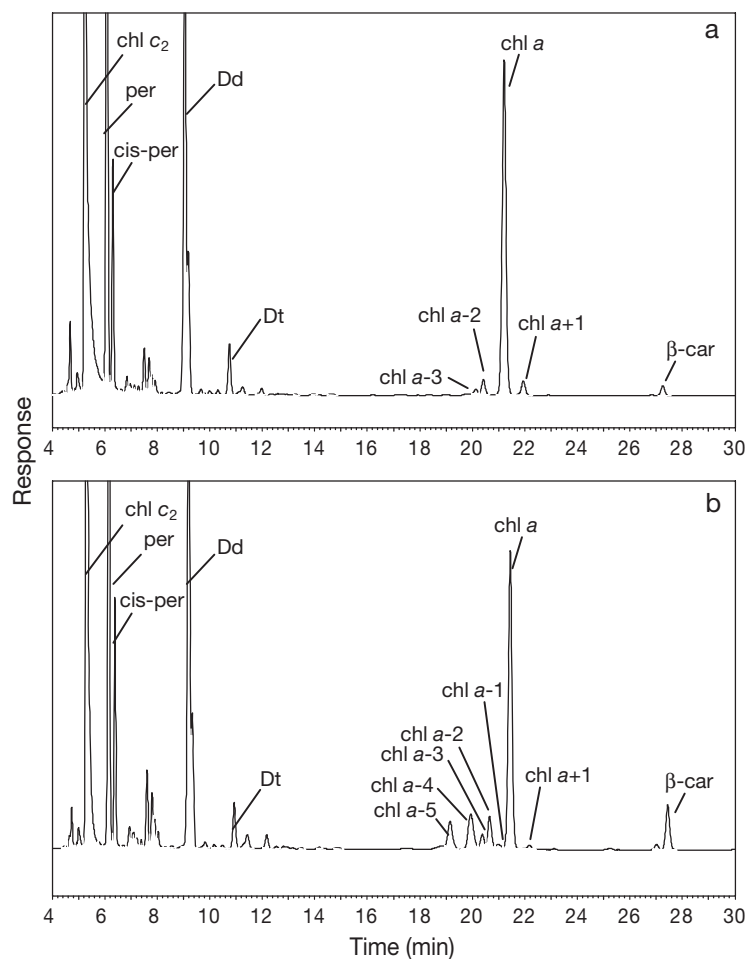


Fig. 1. Representative chromatograms illustrating the peaks corresponding to the chl *a*-like compounds in (a) a control sample showing the chl *a* peak with few additional peaks and (b) a rotated sample (E→W) with a greater number of peaks (chl *a*: chlorophyll *a*; chl *a* ± *x*: chlorophyll *a*-like compounds). The other major pigment peaks denoted are chlorophyll *c*₂ (chl *c*₂), peridinin (per), cis-peridinin (cis-per), diadinoxanthin (Dd), diatoxanthin (Dt) and β-carotene (β-car)

Statistical comparison between experimental conditions showed that there was no significant difference in the concentration of chl *a*-like compounds between E_{cont} and W_{cont} cores throughout the 6 d experiment. There was also no significant difference between W→E cores and W_{cont} cores. However, there was a significant increase in the concentration of chl *a*-like compounds between E→W cores and E_{cont} cores at Day 2, but no significant difference thereafter (Fig. 4a). This indicates an initial increase in chl *a*-like compounds in response to the increased solar radiation stress on the west side of the corals, followed by adaptation by Days 4 and 6.

There was no significant difference in chl *a* concentration between rotated cores and the side of origin (E→W and E_{cont}, W→E and W_{cont}) at Day 2, indicating

Table 1. Retention time (RT) and frequency of occurrence of chl *a*-like compounds relative to chl *a*

	Chl <i>a</i> -5	Chl <i>a</i> -4	Chl <i>a</i> -3	Chl <i>a</i> -2	Chl <i>a</i> -1	Chl <i>a</i>	Chl <i>a</i> +1
Mean RT relative to chl <i>a</i> (min)	-2.26	-1.47	-1.06	-0.78	-0.46	0.00	0.73
SD	0.032	0.024	0.008	0.004	0.007	0.000	0.003
Occurrence (% samples analysed)	72	74	100	100	47	100	100

that chl *a* was conserved upon rotation (Fig. 4b). There was also no significant difference in chl *a* concentration between E_{cont} and W_{cont} cores. As such, the increase in the concentration of total chl *a* in $E \rightarrow W$ cores compared with E_{cont} cores at Day 2 (Fig. 4c) reflected the conservation of chl *a* and the increase in chl *a*-like compounds.

This increase in the concentration of chl *a*-like compounds on rotation $E \rightarrow W$ at Day 2 was associated with an increase in a specific set of compounds (chl *a*-5 to -1) eluting at retention times prior to chl *a* (Fig. 5).

DISCUSSION

Pigment identity

The ubiquity of chl *a* in phytoplankton has long been recognised, and this pigment is routinely used as a proxy of phyto-biomass in oceanographic studies. Traditionally, algorithms based on spectrophotometric and fluorometric measurements have been used to quantify chl *a* in bulk extracts of phytoplankton. However, inaccuracies in such methodologies, arising from the spectral interference of other chlorophyll compounds (e.g. chlorophyll *b*), chlorophyll transformation products (e.g. phaeophorbides, chlorophyllides, phaeophytins) and/or carotenoids has led to the development and application of HPLC-based methodologies allowing more accurate estimates of chl *a* from phytoplankton extracts. The continued development of HPLC methodologies now allows the identification and quantification of >50 individual pigments from phytoplankton extracts (Jeffrey et al. 1997).

Despite the advances in the application of HPLC in studies of oceanic phytoplankton, the study of algal pigmentation in corals has, to a great extent, been neglected. Exceptions include the analysis of chlorophylls *a* and *c* and the carotenoids diadinoxanthin and diatoxanthin (Kleppel et al. 1989, Brown et al. 1999a, 2000a, 2002a). Phaeophytin *a* and pyropheophytin *a* have also been identified as breakdown products of chlorophyll during coral bleaching (Ambarsari et al. 1997).

The present work reported results from the analysis of pigments in a manipulative field experiment designed to study the effect of solar stress on corals (specifically *Goniastrea aspera*). Previous studies at the reef site (Brown et al. 1994, 2000a) have shown that western surfaces of intertidal coral colonies are exposed to higher levels of solar irradiance than eastern surfaces during spring tide exposures. This obser-

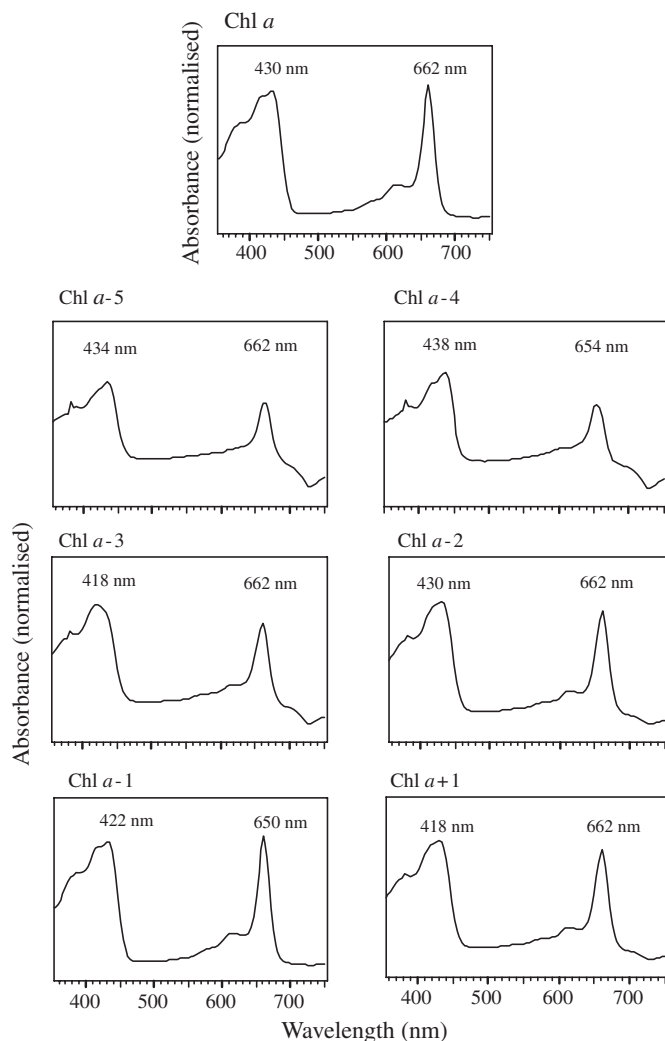


Fig. 2. Light absorbance spectra of chl *a* and the chl *a*-like compounds (chl *a* ± *x*)

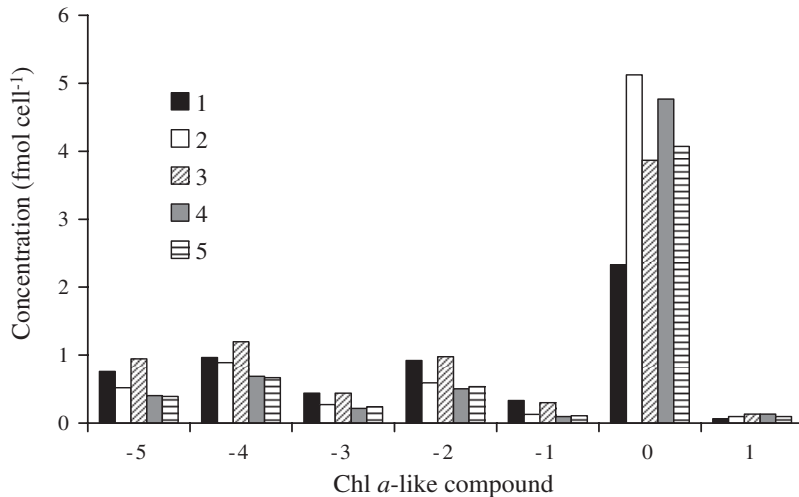


Fig. 3. Concentration of chl *a*-like compounds for 5 replicate samples (1 to 5) relative to chl *a* in the data set of E→W cores at Day 2. Bars represent individual samples

vation was exploited in manipulative field experiments to investigate induction of photo-damage upon rotation of coral cores 180° from east to west and recovery from photo-damage upon rotation from west to east (Brown et al. 2000a).

Pigment analysis of zooxanthellae sampled during the experiment revealed the presence of up to 6 compounds eluting in the region of chl *a*. Transformation products of other key coral pigments including chlorophyll *c* and peridinin were not detected. As seen from Table 1, the occurrence of these chl *a*-like compounds was consistent. From their retention time data and spectral absorbance characteristics (Table 1, Figs. 1 & 2), we ascertained that these compounds do not include commonly proposed chl *a* transformation products, such as chlorophyllide, pyro-pheophorbide, pheophorbide (which would have eluted with shorter retention times than chl *a*), pheophytin, or sterol chlorin esters of pheophorbide (which would have longer retention times).

On the basis of retention time, divinyl chlorophyll *a* is a candidate for one of the chl *a*-like compounds that elute ahead of chl *a*. This pigment is used as a highly specific indicator of the prokaryotic picoplankton, prochlorophytes (Chisholm et al. 1988). Similarly, phytolated chl *c*-like pigments, associated with many prymnesiophyte species (Jeffrey et al. 1997), have been reported to elute in the proximity of chl *a* under reverse-phase HPLC conditions. However, the absorbance characteristics of these compounds eliminate both as potential candidates for the chl *a*-like compounds described here.

It is likely that chl *a* epimers and allomers are included among the compounds identified. The fact that reverse-phase HPLC was used in the analysis and that most of the compounds elute ahead of chl *a* sug-

gests that they are of lower molecular weight and/or more polar than chl *a* itself. The absorbance spectra shown in Fig. 2 also suggest that the porphyrin ring system is intact. Candidate compounds could therefore include chlorophyll-like compounds with oxidatively modified phytol side chains. The oxidation of free phytol is known to produce more polar species, which have been observed in environmental samples (Rontani & Aubert 2005).

At present we are only able to speculate over the likely identity of these chl *a*-like compounds and to eliminate a range of commonly studied chl *a* transformation products. However, only by the utilisation of HPLC was

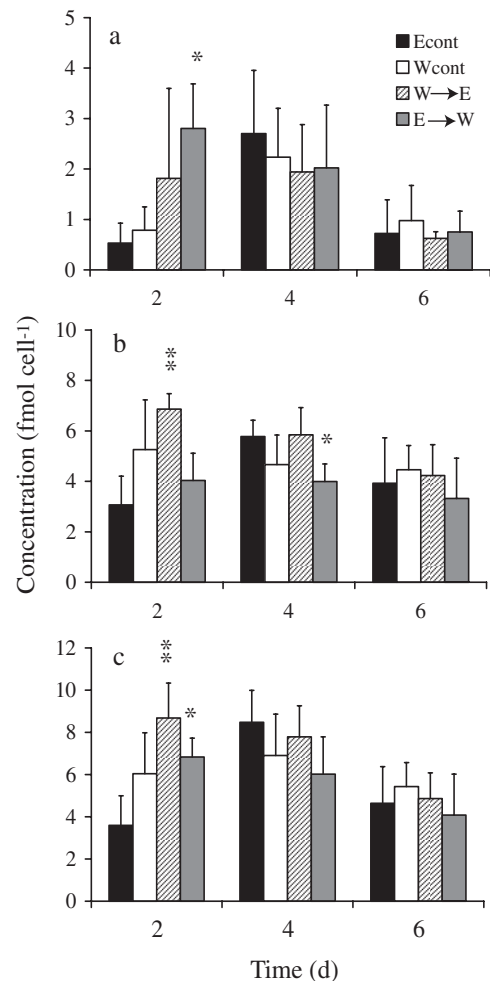


Fig. 4. Concentration of (a) chl *a*-like compounds, (b) chl *a*, and (c) total chl *a* (chl *a* + chl *a*-like compounds) in east and west control (E_{cont} , W_{cont}) and rotated samples ($W \rightarrow E$, $E \rightarrow W$). Bars represent mean values (error bars: +SD); * $p < 0.05$, ** $p < 0.01$, compared with E_{cont} , as determined by paired 2-tailed *t*-test

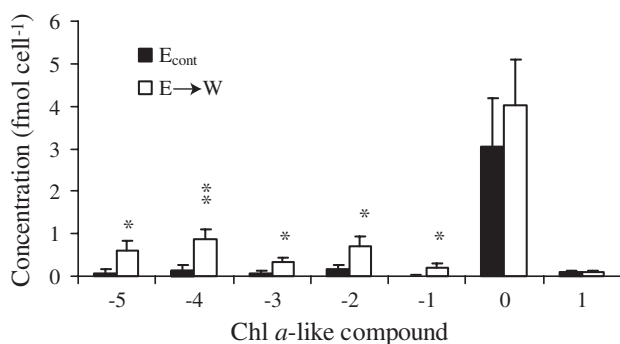


Fig. 5. Concentration of chl *a*-like compounds for $E \rightarrow W$ cores compared with E_{cont} cores at Day 2. Bars represent mean values (error bars: +SD); * $p < 0.05$, ** $p < 0.01$ compared with E_{cont} as determined by paired 2-tailed *t*-test

the presence of these compounds revealed. Had spectrophotometric or fluorometric tools (which could not differentiate between chl *a* and the chl *a*-like compounds) been employed, this subtle expression of pigments would not have been observed. Further studies utilising mass spectrometric detection will be required to elucidate the structures of these chl *a*-like compounds.

Environmental factors

Analysis of the evolution of the chl *a*-like compounds throughout the experiment shows that they are particularly prevalent on Day 2 in samples from coral faces that had been rotated 180° from east to west ($E \rightarrow W$). Although there was variation in the relative concentration of the compounds between sample replicates (resulting in high SDs), there was a significant increase in chl *a*-like compounds in response to increased solar radiation stress on Day 2. There was no significant difference between E_{cont} and W_{cont} , indicating these samples were not under any increased stress. There was no significant change as the cores were moved to a lower irradiance level ($W \rightarrow E$).

By Day 4 there was no significant difference between E_{cont} , W_{cont} , or rotated samples, indicating that the $E \rightarrow W$ samples had adapted to the higher solar radiation conditions. This suggests that the increase in chl *a*-like compounds is associated with a phase in the photo-adaptation process of the coral zooxanthellae.

The increase in total chl *a* in $E \rightarrow W$ samples may be attributed to an increase in chl *a*-like compounds and, specifically, a subset of compounds eluting prior to chl *a*. Notably there was no significant increase in the concentration of chl *a* itself.

Thus, it appears that there was an increase in chl *a*-like compounds in response to increased levels of solar

radiation stress, while the level of chl *a* itself was maintained. If these chl *a*-like compounds represent chl *a* breakdown, it is possible that there was an overall increase in chl *a* production to compensate for any degradation, thereby maintaining chl *a* levels. However, the nature of these products and the mechanism by which they are produced is currently unknown and warrants further investigation.

Previous studies have shown an increase in spectrophotometrically measured chlorophyll during a variety of stress events in both laboratory manipulations and bleaching events in the field (Fitt et al. 1993, Le Tissier & Brown 1996, Jones 1997, Jones & Steven 1997, Ambarsari 1998, Brown et al. 1999b, Brown et al. 2000b, Kerswell & Jones 2003, Saxby et al. 2003, Bhagooli & Yakovleva 2004, Yakovleva & Hidaka 2004). Our results infer that this response, which has been attributed to an increase in chl *a*, may in fact be due to an increase in chl *a*-like compounds. Clearly, the application of HPLC to such studies and the ability to discretely resolve the individual compounds, which may collectively contribute to the spectrophotometric signal, is advantageous.

Mechanism sensitivity

Corals have been shown to be capable of acclimatisation and adaptation to environmental stress (for review see Coles & Brown 2003). Brown et al. (2000b, 2002b) demonstrated that surfaces of corals that were acclimatised to elevated solar radiation subsequently showed improved temperature tolerance as a result of improved photoprotection in the algae and host defences. The time scale over which these defences operate may range from minutes (molecular responses) to days (loss of zooxanthellae) depending on the nature and degree of the stress.

The production of chl *a*-like compounds in response to the rotation occurs relatively rapidly (within 48 h). Conditions at the study location in November are such that solar radiation levels are seasonally low, with corals having been exposed to the highest solar radiation during low tides in April. The rotation experiments are therefore associated with the induction of a low level of stress. As such, the production of the chl *a*-like compounds is a result of a minor stress and the fact that the levels of these compounds return to that of the control samples after 4 d suggests that the mechanism responsible for their formation is rapidly reversed or brought under control through acclimatisation to the stress conditions.

Previous studies (Brown et al. 2000a, 2002a) have demonstrated an increase in the ratio of the xanthophylls diadinoxanthin to diatoxanthin in response to

solar radiation stress. In the present study, this response was not observed (data not shown). From this, it is possible to infer that the expression of chl *a*-like compounds may be a more sensitive response to low-level solar radiation stress than to xanthophyll cycling. However, the xanthophyll ratio is known to respond rapidly (hours) to changing PAR (photosynthetically active radiation) levels (Brown et al. 1999a), while the rate of the chl *a* response is as yet unknown (we report changes measured over a number of days). Therefore, it is possible that, while the change in xanthophyll ratio is a rapid response to the conditions experienced over a period of hours, the chl *a* response is a less rapid response reflecting the conditions experienced over a number of days. To resolve this issue, future, simultaneous study of both chl *a* transformation products and the xanthophylls is required, over a period of several days in response to varying PAR, but must incorporate a sampling frequency of 4 to 6 h.

CONCLUSIONS

Through the application of HPLC with DAD we have identified the occurrence of a number of chl *a*-like compounds that are expressed by zooxanthellae in response to solar radiation manipulation. While the structures of these compounds were not determined, their occurrence is reproducible and consistent. Ongoing studies include the elucidation of mechanisms involved in the generation of these compounds and their structural characterisation.

The occurrence of these compounds was observed under conditions that did not induce a photoprotective response in xanthophyll cycling. They, therefore, have the potential to act as sensitive 'biomarkers' of subtle levels of environmental stress in the coral. Whether the analysis can be used as an early warning of the onset of 'bleaching' will require further verification.

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