SHORT COMMUNICATION

Biochemical comparison of bleaching and non-bleaching *Montipora digitata* (Order Scleractinia) in the Philippines

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Populations of bleaching (B) and non-bleaching (NB) *Montipora digitata* (Dana, 1846, Order Scleractinia) inhabit similar zones, with similar temperature ranges, in the reef of Bolinao in the northwestern Philippines. Colonies of the former (B) have been observed to bleach during periods of elevated sea surface temperatures and calm waters, and have declined in abundance over recent years. Biochemical analyses revealed significantly higher amounts of chlorophyll-a in the NB colonies compared to the B. The kinds and quantities of mycosporine-like amino acids were not significantly different between the two, except for palythine which was higher in the NB corals, and palythene which was more abundant in the B. The greater quantities of chlorophyll-a in the tissue of NB corals indicate higher abundances of symbiotic zooxanthellae, and the likely importance of building up nutritional reserves in order to overcome environmental stress. An additional consideration concerns the possible role of palythine in bleaching resistance.

KEYWORDS
climate change, sea surface warming, bleaching resistance, mycosporine-like amino acids, *Montipora digitata*

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INTRODUCTION

The adverse impacts of climate change, including factors such as rising sea water temperatures, and associated effects of ultraviolet radiation (Hoegh-Guldberg and Bruno 2010, Pandolfi et al. 2011, Hughes et al. 2012) have been associated with coral bleaching (mostly of the Order Scleractinia). Coral bleaching is the loss of pigment in the symbiotic algae (zooxanthellae) inhabiting the coral tissues, or actual expulsion of the zooxanthellae themselves (Brown 1997). In the Philippines, bleaching has been observed in several experiments involving the species *Montipora digitata* (Levy et al. 2010, Shaish et al. 2010a,b, Gomez et al. 2011), a major component of Indo-Pacific reef communities (Veron 2000).

Transplants of *M. digitata* were obtained by breaking off fragments from natural colonies and were established in a reef flat north of the island of Santiago in Bolinao, northwestern Philippines (described in Levy et al. 2010, Shaish et al. 2010b and Gomez et al. 2011, Fig. 1). Levy et al. (2010) collected their specimens from a shallow portion of the Malilnep channel, north of Santiago Island, and experimented with different designs of coral "nurseries" made of rope. Shaish et al. (2010b) gathered their fragments from the Lucero area west of Santiago Island and reared them in nurseries constructed with plastic mesh enclosed by PVC frames before transplanting them onto the natural reef after several months. Gomez et al. (2011) sourced their material from relatively extensive populations on the shallower portions of the reef flat also at Lucero and directly attached their transplants to previously cleared surfaces of dead microatolls using underwater epoxy.

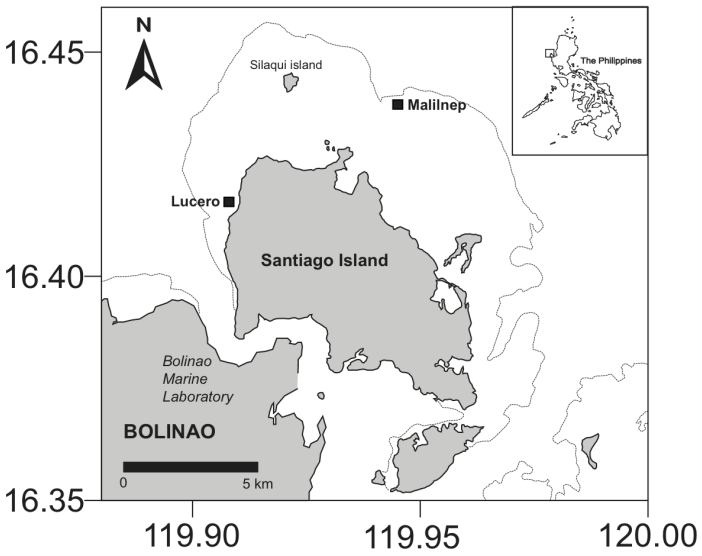

Figure 1. Map of study sites.

The majority of the transplants used by Levy et al. (2010) and Shaish et al. (2010b) were observed to bleach during the warming episode of June 2007 (Shaish et al. 2010a), a factor that is believed to have contributed to significant mortality thereafter. The warming was attributed to a combination of elevated seawater temperature, unusually low tide and high levels of irradiance due to clear skies. The colonies used by Gomez et al. (2011) did not bleach over the duration of their experiment which also included the warm summer months. The causes of mortality, as documented by the authors over approximately an annual cycle, included competition with neighboring organisms such as sponges and algae, and predation by invertebrates such as the gastropod *Drupella*.

Coral bleaching is mainly attributed to increases in seawater temperature and/or exposure to ultraviolet (UV) radiation (Brown 1997, Banaszak and Lesser 2009). As previously described by Banaszak and Trench (1995), responses of host-symbiont systems to UV radiation vary to a great extent and are influenced by other factors such as phenotypic plasticity and the production of protective proteins. For example, corals accumulate water-soluble UV-absorbing compounds (mycosporine-like amino acids or MAAs) (Dunlap and Shick 1998, Lesser 2000, Shick and Dunlap 2002, Shick 2004) that have been characterized specifically as oxocarbonyl- or imino-MAAs and which absorb optimally in the wavelength region of 310-360 nm (Bandaranayake 1998). Part of the function of MAAs in biosynthetic systems is to prevent damage of intracellular UV-sensitive components without interfering with the action of photosynthetically active radiation (PAR) (Banaszak and Trench 1995), mainly absorbing UV radiation and dissipating it as heat (Shick et al. 2000). MAAs are also believed to exhibit antioxidant properties (Dunlap and Shick 1998).

The pathway of MAA biosynthesis was previously reported to be confined to algae, bacteria and fungi (Shick and Dunlap 2002). MAAs in corals were believed to be produced by the symbiotic zooxanthellae inhabiting the coral tissue, and consequently translocated and modified in the host (Dunlap and Chalker 1986). Recent findings indicate that the coral itself is also capable of producing MAAs (Shinzato et al. 2011).

This study reports the biochemical characterization of *M. digitata* colonies collected from populations that have been observed to have higher (B) and lower (NB) bleaching prevalence, respectively, in the reef complex of Bolinao. Specifically, the objective is to determine if there are significant differences between the B and NB corals in terms of levels of chlorophyll-a, protein and mycosporine-like amino acids. It appears that the NB corals are able to resist the detrimental effects of increasing seawater temperature and UV levels, at least within the current range of conditions prevailing in their local habitats.

MATERIALS AND METHODS

Coral collection

Colonies of bleaching *M. digitata* had become scarce by the time of the present study (October 2009), and could be found only in the area around Malilnep, while the NB colonies were obtained from the Lucero reef flat (Fig. 1).

Fragments of *M. digitata* from 5 B colonies and 5 NB colonies were collected between 1030 and 1130 h in October 2009 from a depth of 1 to 1.5 m from Malilnep and Lucero, respectively (Fig. 1). Three fragments approximately 5 cm in length were carefully broken off by hand from the top of each coral colony. The independent experimental unit was the coral colony, yielding 5 replicates each of B and NB.

The fragments were frozen in liquid nitrogen immediately after collection until ready for processing in the laboratory a few days later. Samples were lyophilized and homogenized into fine powder for use in the analyses. Levels of chlorophyll (chl)-a and protein were expressed on the basis of total weight (g) of each sample, consisting of an entire coral fragment. This representation was considered more accurate than using surface area as a basis, because estimation of the latter is subject to error due to irregularity of the shape of coral branches (Ferrier-Pages et al. 2007).

Chloropyhyll-a determination

A portion of the sample powder was suspended in 90% acetone at 4°C with agitation overnight. Solutions were centrifuged at 3000 rpm and the absorbance of the supernatant was read at 665 and 645 nm. Computation of chl-a concentration followed the formula of Lichtenthaler (1987).

Protein determination

An aliquot of the sample powder was digested for 2 hrs with 1 N NaOH, following a modified method of Lowry et al. (1951). After centrifugation at 3000 rpm, the supernatant was extracted and the absorbance was read at 660 nm against a blank. Known concentrations of bovine serum albumin were used for standard curve calibration.

Mycosporine-like amino acid (MAA) determination

MAA content analysis was carried out by extraction of the powdered sample in aqueous methanol for 24 h at 25°C with agitation. Samples were centrifuged at 3000 rpm and the bulk of the organic solvent was removed by evaporation *in vacuo*. Extracts were kept at -20°C prior to chromatographic analysis. For the HPLC separation of MAAs, the dried extracts were reconstituted with 25% aqueous methanol and passed through 0.2 µm pore-sized syringe filters. HPLC resolution was carried out using a Shimadzu LC-10AD Liquid Chromatograph with a photodiode detector.

In terms of method optimization using isocratic solvent systems, preliminary trials showed that employing a reversed-phase octadecyl silane (C18) column with a guard precolumn using the same material rendered the best separation of peaks compared to the systems with C8 columns. Elution with 0.2% HOAc in 0.5% MeOH as mobile phase in isocratic mode at 0.7 ml/min flow rate produced peaks with relatively high peak purity. Due to the unavailability of commercial standard MAA compounds for quantification, peak selection and quantification were based on published relative retention times (RRT) detected at wavelengths of maximum absorption (λ). Peak selection and approximation of MAA concentration using published molar extinction coefficients (ε, M cm^{-1}) satisfied the criteria of high purity (>0.99985) for both the upper and lower parts of the peaks after integration and baseline correction. RRTs should also fall within ± 0.5 min of the published values. Table 1 presents corresponding literature values for the quantitative parameters (absorption maximum, molar extinction coefficient, relative retention time [RRT]) of mycosporine-like amino acids (MAAs) that were extracted in this experiment. Quantities of MAAs were expressed as mmol mg^{-1} protein.

Statistical analyses

Nested analysis of variance (ANOVA, Zar 1984), using treatment x colony as factors, was employed to determine significant differences between B and NB *M. digitata* in terms of chl-a and MAA content. Note that colonies were independent from each other. Data were transformed to satisfy assumptions of normality and homoscedasticity, as follows: fourth-root transformation for chl-a and square-root transformation for MAAs.

A multivariate test, one-way analysis of similarities (ANOSIM) (Clarke 1993, Clarke and Gorley 2001), was used to test for possible significant differences among the treatments (different colonies; B versus NB) in terms of types and quantities of MAAs. A test of similarities in percentages (SIMPER, Clarke 1993) determined which types of MAAs contributed to significant differences or similarities among groups.

RESULTS

Levels of chl-a and MAAs in bleaching and non-bleaching *M. digitata* are presented in Figures 2 and 3. Amounts of chl-a (Fig. 2) ranged between 0.0063 $\pm$ 0.00078 and 0.0067 $\pm$ 0.00054 (SD) mg g^{-1} sample for the B corals, and between 0.0052 $\pm$ 0.0012 and 0.0088 $\pm$ 0.00064 mg g^{-1} in the NB corals. Levels of chl-a were significantly higher in NB corals (Table 2A), with significant differences among colonies contributing to a significant treatment x colony interaction term.

There were no statistically significant differences in the total amounts of MAAs per unit weight of protein (expressed as mmol mg^{-1} protein) between fragments of the B and NB colonies, although there was significant treatment x colony interaction (Table 2B, Fig. 3). An analysis of Similarity Percentages

Table 1. Literature values for the quantitative parameters (absorption maximum, molar extinction coefficient, relative retention time [RRT]) of mycosporine-like amino acids (MAAs).

MAA	Absorption Maxima (nm)	Molar Extinction Coefficient (M cm^{-1})	Reference	Relative Retention Time (RRT, min)	Reference
Palythine	320	36200	Dunlap et al. 1986	6.1	Volkmann and Gorbushina 2006
Palythinol	332	43500	Dunlap et al. 1986	9.9	Volkmann and Gorbushina 2006
Shinorine	334	44700	Shick and Dunlap 2002	5.5	Volkmann and Gorbushina 2006
Porphyra-334	334	42300	Shick and Dunlap 2002	8.4	Volkmann and Gorbushina 2006
Palythene	360	50000	Dunlap et al. 1989	13.2	Whitehead and Vernet 2000
Mycosporine-glutaminol	310	25000	Moliné et al. 2011	9.5	Volkmann and Gorbushina 2006
Mycosporine-glutaminol-glucoside	310	25000	Moliné et al. 2011	10.5	Volkmann and Gorbushina 2006

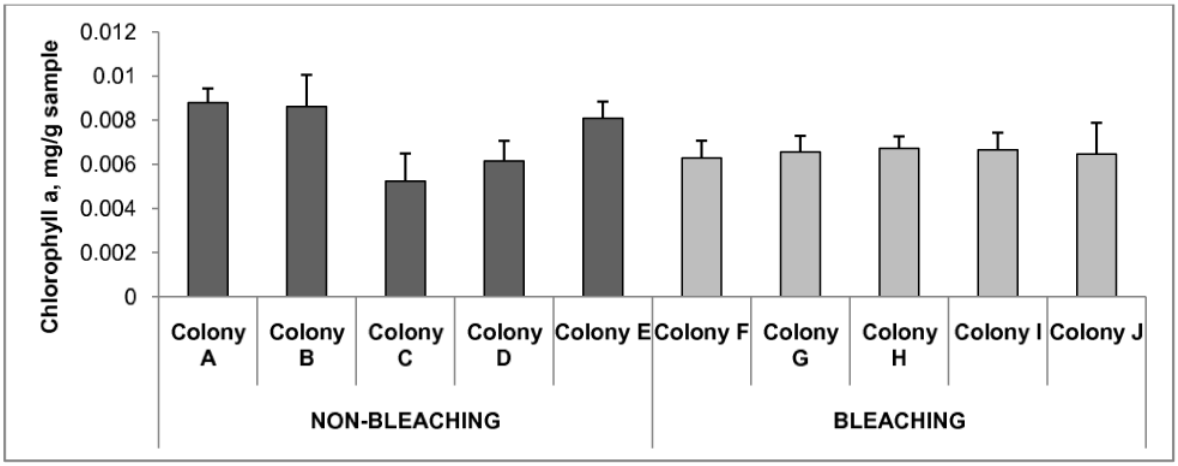

Figure 2. Comparison of chlorophyll-a (mg g^{-1}sample) in fragments of bleaching and non-bleaching corals. A fragment group originates from an independent colony, of either a bleaching or non-bleaching variant.

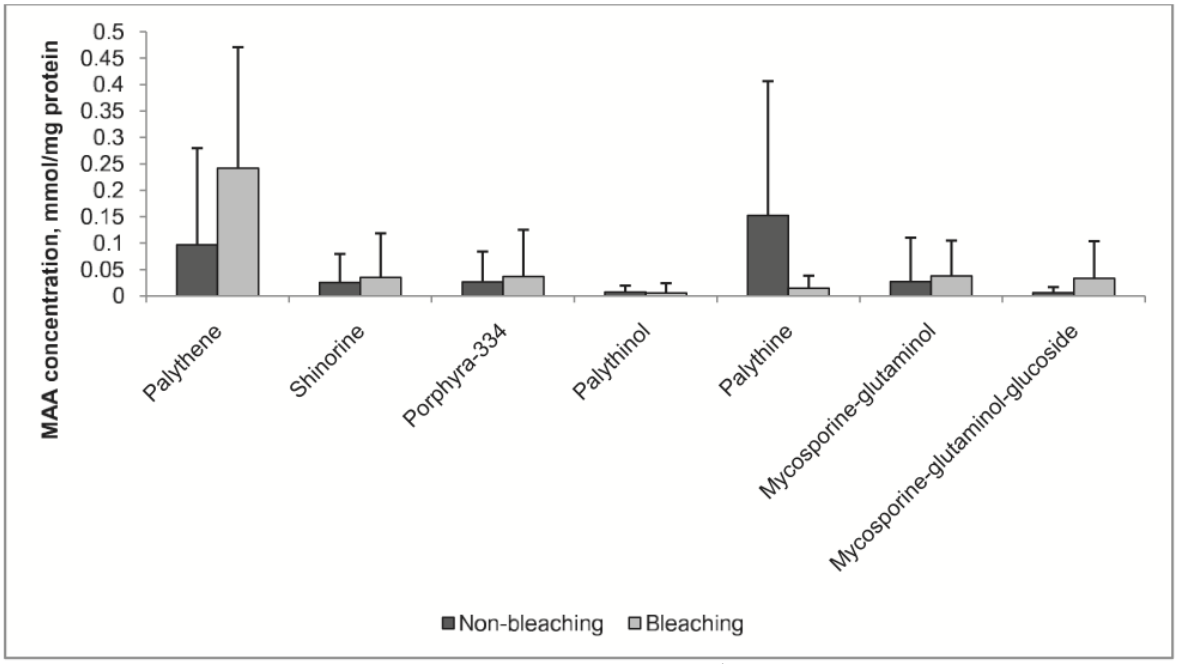

Figure 3. Comparison of the mean MAA concentrations (mmol mg^{-1} protein) found in fragments of bleaching and non-bleaching corals.

Table 2. Results of nested Analysis of Variance (ANOVA) comparing levels of chlorophyll-a (mg g^{-1} sample), and total MAA (mmol mg^{-1} protein) in fragments from bleaching (B) and non-bleaching (NB) corals.

A. Chl-a (mg g^{-1} sample) - fourth root transformed

	Df	Sum Sq	Mean Sq	F value	*P* (>F)
Treatment	1	5.31E-06	5.31E-06	5.704	**0.0269**
Treatment:Colony	8	3.08E-05	3.85E-06	4.132	**0.00474**
Residuals	20	1.86E-05	9.31E-07		

B. Total MAA (mmol mg^{-1} protein) - square root transformed

	Df	Sum Sq	Mean Sq	F value	*P* (>F)
Treatment	1	0.1304	0.13037	2.217	0.1521
Treatment:Colony	8	1.4196	0.17744	3.017	**0.0215**
Residuals	20	1.1762	0.05881		

PLATE. *Montipora digitata* in Bolinao, Pangasinan, Philippines. Non-bleaching (left) and bleaching (right) variants during a non-bleaching episode (photo by A. Punongbayan).

(SIMPER, Clarke 1993) revealed that the B and NB corals differed only with respect to the MAA compounds, palythene (contributing 42.3% to the between-treatment differences) and palythine (contributing 19.7%). Palythene occurred in higher amounts in the B corals, while palythine was more abundant in the NB.

DISCUSSION

The bleaching *M. digitata* contained significantly lower levels of chl-a per unit weight of sample than the non-bleaching colonies. The levels of MAAs per mg of protein were not significantly different between B and NB corals. A somewhat different result was obtained by Edmunds et al. (2003) on *Montastrea franksi* in Florida, where colonies under varying degrees of bleaching still exhibited similar levels of protein, MAAs and other parameters included under "tissue composition." In contrast, Yakovleva and Hidaka (2009) found differences in the composition and quantity of MAAs between different color morphs of *Galaxea fascicularis* in Okinawa, Japan.

All colonies were collected within the same time of the day on the same date in October 2009 which was a non-bleaching year. Conditions were not stressful for the corals at the time of collection. Nevertheless, colonies from Malilnep that are known to bleach had declined significantly in abundance by the time of sampling.

Unpublished records of water temperature (R. Villanueva, the Marine Science Institute), obtained using an *in situ* sensor (model HOBO Water Temp Pro v2, Onset Corp.) from March 2011 to December 2013, indicate almost identical ranges for both Lucero and Malilnep. Values lay between 27°C (cool months) and 32° (warm months). It is reasonable to assume that these temperature trends reflect what has prevailed over the recent history of the two sites, including when populations of both B and NB corals were thriving and served as source material for the various experiments described here (Levy et al. 2010, Shaish et al. 2010a,b, Gomez et al. 2011). Hence, the differences in biochemical composition of the B and NB corals could not be ascribed to different temperature histories.

Based on appearance, both types of corals belong to the same species (see Plate) though genetic analyses were not performed to confirm this.

Since levels of MAAs per mg of protein did not differ significantly between the B and NB colonies, what appears to be the main factor distinguishing them is their metabolic status at the time of collection as indicated by the total amount of chl-a in their tissues (significantly higher in the NB corals). The results of this study point to the possible importance of the nutritional status of the coral and its associated symbiotic algae in contributing to its resilience to environmental stress.

An additional possible explanation lies in the kinds of MAAs found in the B and NB colonies. The calculated concentrations of the suite of MAAs observed in both bleaching and non-bleaching *M. digitata* showed no significant statistical variation except for palythene (higher in B) and palythine (more abundant in NB). In a controlled experiment involving increasing UV exposure in the Caribbean coral *Porites furcata*, levels of

palythine were found to have tripled after 128 days (Torres-Perez and Armstrong 2012). Its maximum wavelength of absorption, 320 nm, suggests optimal absorption in the region of UV-B radiation (290-320 nm).

For future investigations, genetic characterization of coral species that show different susceptibilities to bleaching would be useful to determine, among others, the enzymes that are up-regulated upon exposure to stress conditions. Genotypic profiling of the symbiotic zooxanthellae should accompany that of the host corals.

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CONFLICTS OF INTEREST

None.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

HTY conceptualized the study and research design, and led the writing of the manuscript. DMLE analyzed the samples and contributed portions of the manuscript and literature review. MNEM supervised the methodological approach, contributed to the design of the study and provided ideas for the manuscript. CSB helped collect the coral samples, performed the statistical analyses and contributed to the review of literature. EDG was overall research program leader, solicited financial support for the study and contributed ideas for the research.

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