

# The continuity and intensity of ultraviolet irradiation affect the kinetics of biosynthesis, accumulation, and conversion of mycosporine-like amino acids (MAAs) in the coral *Stylophora pistillata*

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## Abstract

Colonies of *Stylophora pistillata* unexposed to ultraviolet radiation (UVR) in laboratory aquaria contained minimal amounts of mycosporine-like amino acids (MAAs). The concentration of MAAs increased rapidly during exposure to broadband UVR, then stabilized between 14 and 28 d of exposure. The four MAAs known to be produced by diverse zooxanthellae (*Symbiodinium* spp.) in culture were the first to increase in coral colonies, followed by six additional MAAs. Decreases in these four primary MAAs between days 14 and 28 were balanced by stoichiometric increases in the six secondary MAAs, suggesting a precursor–product relationship. Discontinuing UV exposure caused rapid declines in the concentrations of primary MAAs, while secondary MAAs continued to increase, likely a manifestation of different rates of turnover of the putative enzymes involved in the de novo biosynthesis of primary MAAs and their conversion to secondary MAAs. The continued production of certain MAAs after UV exposure ceased indicates that UV is not required catalytically but is a signal that induces the enzymes of the biosynthetic pathway. Biochemically inhibiting the biosynthesis of primary MAAs after a pool of these MAAs had accumulated did not affect their conversion to secondary MAAs. Doubling the UV irradiance increased the rate of accumulation, first, of primary MAAs and, later, of secondary MAAs. Under normal UV irradiance, the eventual decline in the accumulation rate of primary MAAs was quantitatively explained by their conversion to secondary MAAs. Under doubled UV irradiance, where MAA concentrations were higher, the deceleration of accumulation of primary MAAs was twice as great as the acceleration in secondary MAAs, suggesting a regulation of the de novo biosynthesis of MAAs, perhaps by their own progressive, concentration-dependent attenuation of UVR.

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Mycosporine-like amino acids (MAAs), natural marine sunscreens that efficiently absorb and dissipate solar ultraviolet radiation (UVR), are ubiquitous among reef-building corals, where their concentrations are related primarily to irradiances of natural and artificial UVR (reviewed by Shick et al. 1996; Dunlap and Shick 1998; Gleason 2001). In the geographically widespread, zooxanthellate scleractinian *Stylophora pistillata* (Esper 1797), UVB (280–320 nm) radiation is the particular stimulus for accumulating structurally diverse MAAs (Shick et al. 1999). As products of a branch of the shikimate pathway, MAAs probably originate in the zooxanthellae, although nonsymbiotic metazoans also contain MAAs, which they obtain from their food (reviewed by Shick and Dunlap 2002).

As the number of structurally identified MAAs has increased, so have attempts to elucidate their biosynthetic interrelationships (Hirata et al. 1979; Carreto et al. 1990; Franklin et al. 1999; Shick et al. 1999; Conde et al. 2003; Portwich and Garcia-Pichel 2003). This interest has extended to structural modifications of phototrophically produced MAAs by heterotrophic bacteria and metazoan consumers (Shick et al. 1992; Dunlap et al. 1998; Dunlap and Shick 1998; Whitehead et al. 2001).

Therefore, an accounting of the complement of MAAs in a zooxanthellate coral must consider not only those emanating from the algal endosymbionts (but see Shick et al. 2002) and those absorbed from the diet, but also from the host's processing any of the foregoing. Withholding food to eliminate dietary MAAs (as done by Shick et al. 1999) simplifies the analysis, but starving *S. pistillata* decreases its zooxanthella population (Shick et al. 1999; Titlyanov et al. 2001).

and protein content (Ferrier-Pagès et al. 2003), so this complicates quantification of the accumulation of MAAs because the capacity (zooxanthella density) for producing MAAs changes during the experiment, as does the divisor (protein) to which MAA concentration is usually normalized. Shick et al. (unpubl. data) have shown that dietary input of MAAs maximally accounts for <5% of the total MAA pool accumulated by *S. pistillata* exposed to UVR under experimental conditions. At the same time, feeding sustains the population of zooxanthellae that contribute the vast majority of the MAAs and maintains the coral's protein content. Thus, feeding a predominantly phototrophic coral a diet of known MAA content does not unduly complicate the analysis.

A further issue is that zooxanthellae in culture often produce a more limited suite of MAAs than that found in the intact symbioses from which they were isolated. The most MAAs produced by a given culture of zooxanthellae is three; collectively, all cultures of *Symbiodinium* spp. that have been examined produce a total of four MAAs—mycosporine-glycine, shinorine, and porphyra-334 (Banaszak et al. 2000), plus mycosporine-2 glycine (J. M. Shick and C. Ferrier-Pagès unpubl. data). Mycosporine-glycine is structurally the simplest MAA, and the other three can be derived from it by single amino acid substitutions (Shick and Dunlap 2002; Portwich and Garcia-Pichel 2003). Therefore, I refer to the first four as primary or *Symbiodinium* MAAs. A culture of zooxanthellae isolated from *S. pistillata* originating in the Red Sea produces primarily shinorine (Banaszak et al. 2000) plus traces of mycosporine-glycine (J. M. Shick and C. Ferrier-Pagès unpubl. data), whereas the coral colony contains 10 MAAs (Shick et al. 1999). This suggests that the host (and, conceivably, associated bacteria; Rohwer et al. 2002) modifies the primary MAA(s) provided by the zooxanthellae to yield an array of secondary MAAs (Shick et al. 1999; reviewed by Shick and Dunlap 2002).

As suggested by Banaszak et al. (2000), this discrepancy between the cultured zooxanthellae and the holobiont in their complements of MAAs also might arise if an undefined stimulus from the host causes the zooxanthellae in hospite to alter qualitatively the MAAs that they produce. This seems not to be the case in *S. pistillata* from the northern Red Sea, based on an experiment in which adding a homogenate of host tissue to cultures of zooxanthellae did not qualitatively affect the MAAs in the latter (J. M. Shick et al. unpubl.) and the fact that zooxanthellae freshly isolated from the host contain, at most, trace amounts of only primary MAAs.

Nevertheless, a given symbiosis often contains a more genotypically diverse assemblage of zooxanthellae than is found when zooxanthellae isolated from it are placed in culture (Santos et al. 2001). The culture of zooxanthellae from *S. pistillata* that we used (provided by T. C. LaJeunesse), although isolated from a specimen collected in the same area of the Red Sea, did not originate from the particular coral colonies that we studied, which have not yielded culturable zooxanthellae. The cultured zooxanthellae (Banaszak et al. 2000), and those in our principal experimental colony (T. C. LaJeunesse pers. comm.), are of ribotype A1, but they are not necessarily genotypically identical (LaJeunesse 2001). Thus, the possibility exists that the zooxanthellae in hospite in our corals produce MAAs in addition to shinorine and

mycosporine-glycine, albeit probably not more than the four aforementioned *Symbiodinium* MAAs. Therefore, for the purposes of this paper on *S. pistillata*, I consider these as the four possible primary MAAs produced by the zooxanthellae, and I consider any other MAAs as secondary MAAs derived from them, probably by processing in the host (Dunlap and Shick 1998).

*S. pistillata*, with its rich diversity of MAAs, is particularly suited to a kinetic and stoichiometric analysis of the accumulation and conversion of MAAs because colonies propagated asexually in the absence of UVR contain only traces of individual MAAs, which increase differentially after a UV stimulus is applied (Shick et al. 1999). The present study investigates the kinetics of their accumulation (and their depletion following the cessation of exposure to UVR or when the shikimate pathway is subsequently inhibited), with the goals of elucidating their likely biosynthetic interrelationships and the mechanisms of regulating these processes, including the quantitative response to UV irradiance (dose rate).

## Materials and methods

*Maintenance of corals*—Prior to the experiments, asexually propagated colonies of the zooxanthellate scleractinian *Stylophora pistillata*, originally from the Gulf of Aqaba, Jordan, were maintained in 300-liter aquarium mesocosms in the Centre Scientifique de Monaco's laboratories in the Musée Océanographique. Conditions were as described previously (Shick et al. 1999), except that incident photosynthetically active radiation (PAR) from metal halide lamps was slightly lower, 200–225  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . Unweighted irradiances of UVA and UVB incident on the water's surface, measured using an IL1400A radiometer with SEL033 UVA (peak sensitivity at 360 nm) and SEL240B UVB (peak sensitivity at 300 nm) sensors (International Light), were at background levels, 1.5  $\text{W m}^{-2}$  and  $\leq 1 \text{ mW m}^{-2}$ , respectively. Standard cylindrical pieces (nubbins) approximately 1.5–3.0 cm in length and 0.5–1.0 cm in diameter were cut from several parent colonies, suspended on nylon monofilaments, and allowed to regenerate in the maintenance aquarium until the cut surfaces were again covered with tissue. Corals were fed weekly with freshly hatched nauplii of *Artemia* (batch Utah B2005). Temperature and salinity were 27°C and 38 practical salinity units (psu) in the maintenance aquarium and in all experiments.

Preliminary experiments on three morphologically distinct colonies indicated that, following 2–4 weeks of exposure to PAR plus broad-spectrum UVR (290–400 nm), two of them contained the 10 identifiable MAAs reported previously (Shick et al. 1999), whereas the third contained only seven of these MAAs. Also, the three colonies accumulated MAAs at different rates, and the third exhibited a different ratio among its individual MAAs. Accordingly, pieces of these qualitatively different colonies could not be mixed in a given experiment studying the complementary changes in individual MAAs, so each experiment used nubbins cut from only one colony.

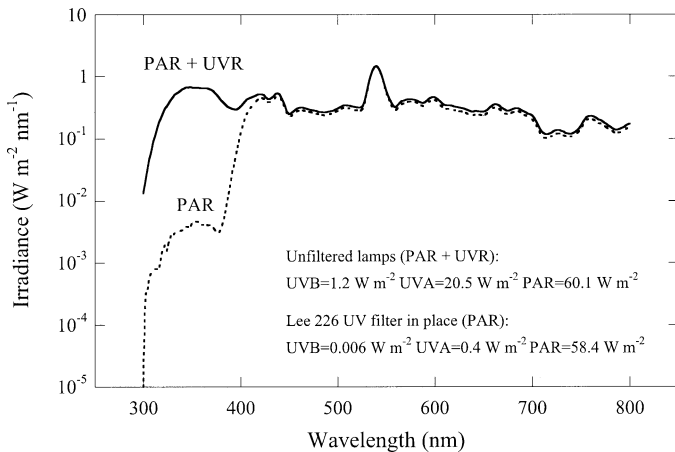


Fig. 1. Combined spectral irradiance, measured at the water's surface in the experimental aquarium, provided by Osram Powerstar 400 W HQI-T metal halide (in shielded housings) and Q-Panel 40 W UVA-340 fluorescent lamps. Inset gives integrated values for UVB (300–320 nm), UVA (320–400 nm), and PAR (400–700 nm), with and without attenuation by a Lee 226 polycarbonate filter.

**Experimental aquarium**—The same 50-liter aquarium used by Shick et al. (1999) was employed in most of the present experiments. It was constructed to allow all UV-exposed and unexposed corals to be held in the same tank, in which the spectral irradiance could be independently controlled above its two longitudinal halves, between which pumps circulated the seawater, thus avoiding any statistical tank effect. PAR was provided by two shielded 400 W Osram Powerstar HQI-T metal halide lamps mounted  $\approx 90$  cm above the aquarium and attenuated by a neutral-density screen, and UVR emanated from a UVA-340 fluorescent lamp (Q-Panel Lab Products) placed above each half of the aquarium, 4 cm from the surface of the water. The combined spectral irradiance from these lamps (Fig. 1) was measured at the water's surface using a LiCor 1800UW scanning spectroradiometer. Nubbins were suspended 1–1.5 cm below the surface of the water. Certain colonies were shielded from UVR (Fig. 1) by a UV-opaque (50% transmission at 400 nm) Lee 226 polycarbonate cutoff filter (Lee Filters). All experiments employed a 12 : 12 light : dark photoperiod, with the UV lamps being illuminated only for the middle 8 h of the 12 h light phase.

The LiCor scanning spectroradiometer was available only briefly, so the IL radiometer and UV sensors were calibrated against the LiCor. Subsequent UV measurements in the experimental aquarium were measured using the IL sensors and adjusted using the calibration. Because of differences in the sensors' spectral sensitivities, the adjusted experimental UVA (320–400 nm) and UVB (300–320 nm) fluences reported in this paper are, respectively, about 3.9 and 18.5 times higher than those reported in Shick et al. (1999), which were determined for the same types of lamps but using only the IL equipment.

In experiments using metabolic inhibitors, the experimental aquarium was used as a water bath in which magnetic stirrers were placed beneath 150-ml beakers fitted with nylon mesh that held the nubbins above a stirring bar in each bea-

ker. UV-transparent plastic film prevented evaporation from the beakers. Measurements of UVR were made inside the empty beakers in situ using the IL sensors.

**Effect of continuity of UV stimulus on accumulation of MAAs**—Corals were fed *Artemia nauplii* immediately prior to, and twice weekly during, the experiment, but not for 48 h prior to extraction of corals for biochemical analysis. In addition to nubbins exposed to PAR + UVR for 3, 7, 14, and 28 d before being extracted for MAA analysis, others were subjected to these conditions for 3, 7, and 14 d but then shielded from UVR (using the Lee 226 filter) and kept in the same aquarium under PAR alone for the balance of the full 28-d exposure (i.e., for an additional 25, 21, and 14 d, respectively). The concentrations of MAAs in the latter groups of corals were compared with those in nubbins receiving PAR + UVR for the total duration of exposure to determine whether the accumulation of MAAs proceeded after their biosynthesis had been induced but the UV stimulus discontinued. Changes in primary and secondary MAAs were compared stoichiometrically.

**Metabolic conversion of MAAs**—Nubbins cut from the same parent colony as used in the UV-continuity experiment were placed in 150-ml beakers containing 100 ml of seawater (which was changed daily) and exposed to full PAR + UVR for 7 d, long enough to accumulate a pool of (largely) primary MAAs. A nylon mesh supported the nubbins above a magnetic stirrer that operated continuously. *Artemia nauplii* were provided on the day before the start of the experiment, and at day 4 of UV exposure. On day 7, one-third of the nubbins were analyzed for MAAs; half of the remaining colonies continued to be exposed to PAR + UVR for an additional 7 d, and the other half were also exposed to 1 mmol L<sup>-1</sup> N-(phosphonomethyl)-glycine (glyphosate), an inhibitor of several enzymes in the shikimate pathway (Bentley 1990), purchased from ICN Biomedicals. Glyphosate (which was replenished daily) at this concentration blocks the biosynthesis of MAAs in *S. pistillata* (Shick et al. 1999), so that colonies exposed to this inhibitor on day 7 would cease to accumulate additional primary MAAs but not necessarily secondary MAAs, if the latter arise by conversion of the existing pools of the former. On day 14 (the last seven of these involving exposure to glyphosate in half of the colonies), the remaining nubbins were analyzed for MAAs. Concentrations of MAAs were compared between baseline colonies extracted on day 7 and glyphosate-treated colonies on day 14, and between untreated and glyphosate-treated colonies on day 14.

**Effect of UV dose rate on the accumulation of MAAs**—Two groups of nubbins cut from a different colony than that used in the foregoing experiments were exposed in the experimental aquarium to the usual level of PAR (60.1 W m<sup>-2</sup>), but while half of the colonies (designated UV) experienced normal UV irradiance (UVA = 20.5 W m<sup>-2</sup>; UVB = 1.2 W m<sup>-2</sup>), the others (2×UV) received double that (UVA = 38.1 W m<sup>-2</sup>; UVB = 2.4–2.5 W m<sup>-2</sup>). This was achieved by placing a second Q-Panel UVA-340 lamp above half of the aquarium and positioning the nubbins beneath the gap be-

tween these two fluorescent tubes so that they would not be blocked from downwelling PAR from the metal halide lamps. Corals in the other half of the aquarium were positioned so that they received UVR from only the single lamp above that half of the aquarium. Exposures were for 3, 6, and 12 d.

**Effects of antioxidants on accumulation of MAAs**—Nubbins cut from what proved to be a third distinct colony were placed in 150-ml beakers and exposed to PAR + UVR for 7 d as described above. In one experiment, half of the colonies served as controls, while the others were continuously exposed to 10 mmol L<sup>-1</sup> D-mannitol, a scavenger of hydroxyl radicals, at a concentration that reduced thermally induced bleaching in a coral (Lesser 1997). In the second experiment, half of the UV-exposed colonies were controls and the others were continuously exposed to 100 μmol L<sup>-1</sup> Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of α-tocopherol that scavenges peroxy and alkoxyl radicals, and peroxyxynitrite (a reactive nitrogen species); the concentration of Trolox used was 4× that of α-tocopherol in human plasma (Halliwell and Gutteridge 1999). The antioxidants were purchased from Calbiochem and were refreshed daily.

**Biochemical measurements**—MAAs were analyzed using standard procedures (Shick et al. 1999), with the following changes. After two successive hour-long extractions each in 3 ml of 100% methanol at 4°C in the dark, nubbins were extracted a third time overnight, and the three extracts were pooled for analysis. Extraction efficiency was ≥95%. After their absorption spectra were measured, extracts were cleaned of lipids and lipophilic pigments by passage through a Sep-Pak Plus C18 cartridge (Waters Corp.). Cleaned extracts were stored at -80°C until analysis. The concentrations of all MAAs in subsamples of a cleaned extract of *S. pistillata* were unchanged during at least 6 weeks of such storage. Protein in the methanol-extracted nubbins was measured as before (Shick et al. 1999).

MAAs were analyzed on an LDC/Milton Roy CM4000 pump and SpectroMonitor 3600 detector (operated at 320 nm) using Nelson Analytical Model 2600 chromatography software (rev. 4.1, 1987). The Phenosphere analytical and Brownlee guard columns were the same as used by Shick et al. (1999), and the system was calibrated using quantitative MAA standards prepared by W. C. Dunlap (Australian Institute of Marine Science) and standards prepared from extracts of *S. pistillata* quantified in Shick et al. (1999).

## Results

**Effect of continuity of UV stimulus on accumulation of MAAs**—All 10 of the MAAs reported in *S. pistillata* by Shick et al. (1999) were found in this experiment. The *Artemia* nauplii (Utah B2005) fed to the corals contained only one MAA, mycosporine-2 glycine, at 0.08 nmol g<sup>-1</sup> wet weight. The combined concentrations of the measured mycosporine-2 glycine and the maximal estimated gadusol content in the nauplii were calculated to contribute, maximally, 4.4% of the total MAAs accumulated by the corals during

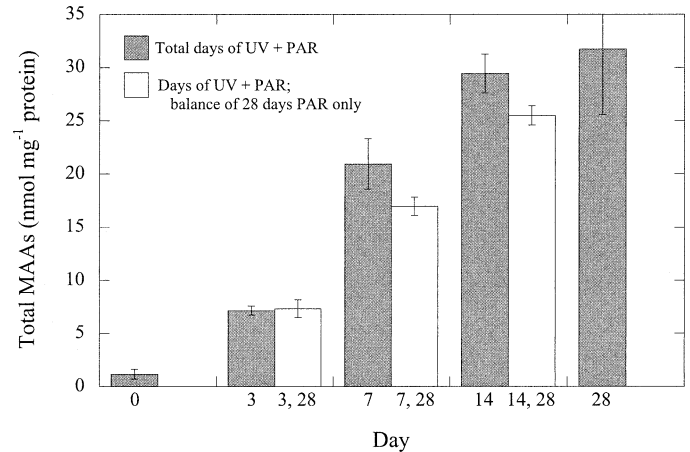


Fig. 2. Concentration of total MAAs in corals exposed to PAR + UVR for different durations up to 28 d (shaded bars) and in colonies receiving PAR + UVR for shorter durations and then shielded from UVR for the balance of 28 d (open bars). MAA concentration was the same in colonies exposed to PAR + UVR for 14 and 28 d and in those exposed to PAR + UVR for 14 d plus PAR alone for an additional 14 d ( $F_{2,6} = 0.713$ ,  $p = 0.528$ ). Values are means ± standard error (SE), where  $n = 3$  in each group.

28 d of UV exposure (J. M. Shick et al. unpubl. data). Reassuringly, by day 28, the actual increase in the total MAA concentration in control colonies unexposed to UVR was only 0.67 nmol mg<sup>-1</sup> protein, 2.2% (i.e., half of the maximum calculated to originate in the diet) of that in nubbins exposed to PAR + UVR in the same aquarium.

Analysis of variance (ANOVA) indicated that mg protein g<sup>-1</sup> skeleton did not differ among the experimental groups ( $F_{7,16} = 1.351$ ,  $p = 0.291$ ), so MAAs were expressed per mg protein. The total MAA concentration was stable by day 14, i.e., there was no net synthesis of MAAs between days 14 and 28 (Fig. 2). Moreover, colonies exposed to UVR for only the first 14 d of the 28-d period had the same concentration of MAAs as colonies extracted after 14 d and as those extracted after 28 d of exposure to PAR + UVR ( $F_{2,6} = 0.713$ ,  $p = 0.528$ ). Conversely, ANOVA ( $F_{2,6} = 5.404$ ,  $p = 0.046$ ) also indicated a difference among colonies exposed to PAR + UVR for 28 d, those exposed to PAR + UVR for 7 d and then extracted, and colonies exposed to PAR + UVR for 7 d and then to PAR alone for an additional 21 d. The last group had a significantly lower concentration of MAAs than both of the other groups (Student-Newman-Keuls [SNK] test,  $p < 0.05$ ). Those two groups did not differ (SNK,  $p > 0.05$ ). Colonies receiving only 3 d of UVR (whether or not followed by an additional 25 d of PAR alone) did not differ in their concentration of MAAs, but both of these groups had lower total MAA concentrations than did colonies exposed to PAR + UVR for the full 28 d (Kruskal-Wallis test,  $p = 0.045$ ).

Considering particularly the corals exposed to PAR + UVR for 14 and 28 d and those receiving PAR + UVR for 14 d and PAR alone for an additional 14 d (because total MAA concentration was stable during this period), temporal patterns are clearly different among the individual MAAs (Fig. 3). Concentrations of the primary MAAs shinorine,

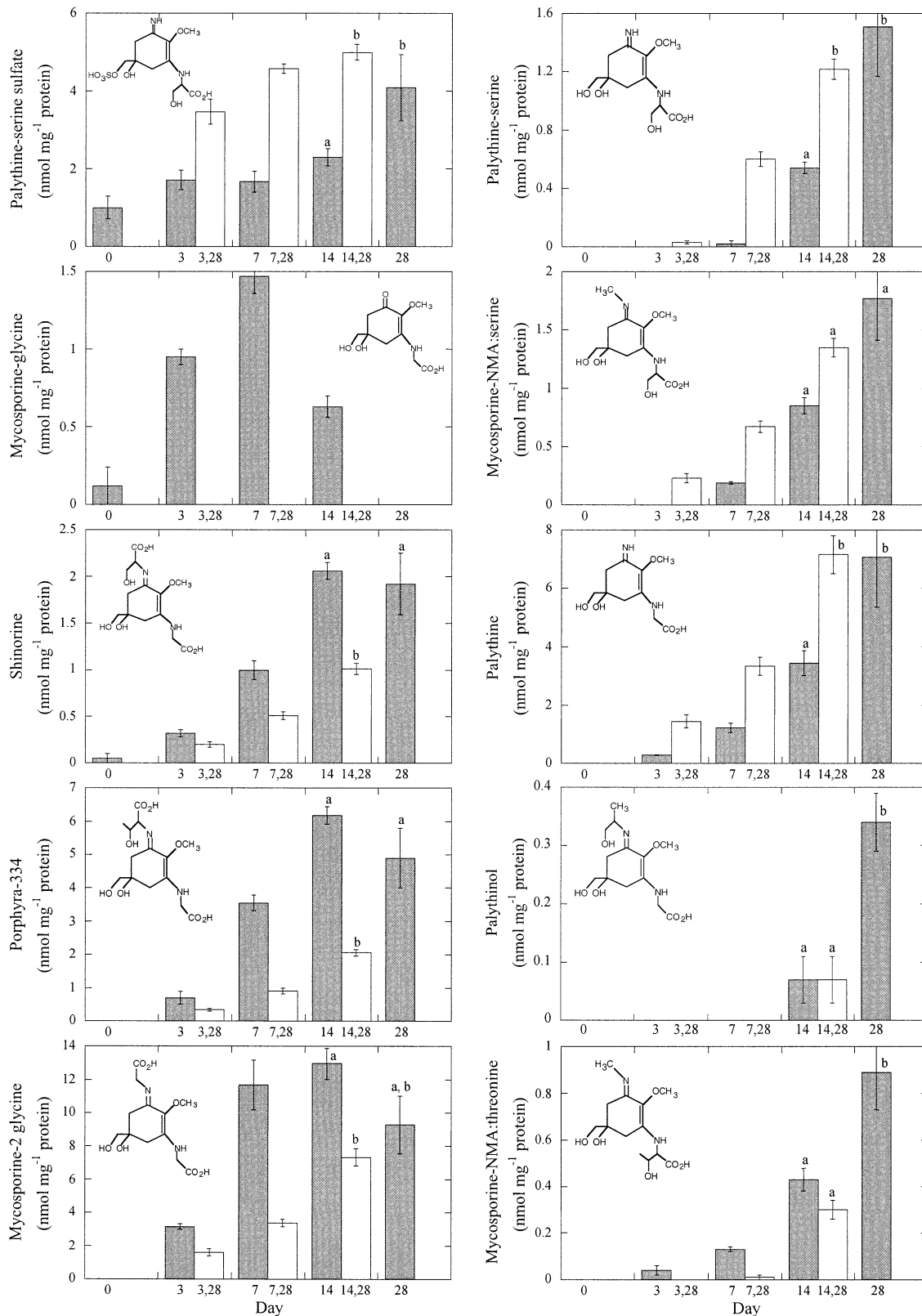


Fig. 3. Concentrations of individual MAAs in corals exposed to PAR + UVR for different durations up to 28 d (shaded bars) and in colonies receiving PAR + UVR for shorter durations and then shielded from UVR for the balance of 28 d (open bars). Differences in MAA concentrations among colonies exposed to PAR + UVR for 14 d and 28 d, and those exposed to PAR + UVR for 14 d plus PAR alone for an additional 14 d, were ascertained by Student–Newman–Keuls multiple comparison tests at  $\alpha = 0.05$  where ANOVA indicated a significant difference; groups in which means are not different have the same letter. Values are means  $\pm$  SE, where  $n = 3$  in each group. Insets show the planar structures of the MAAs.

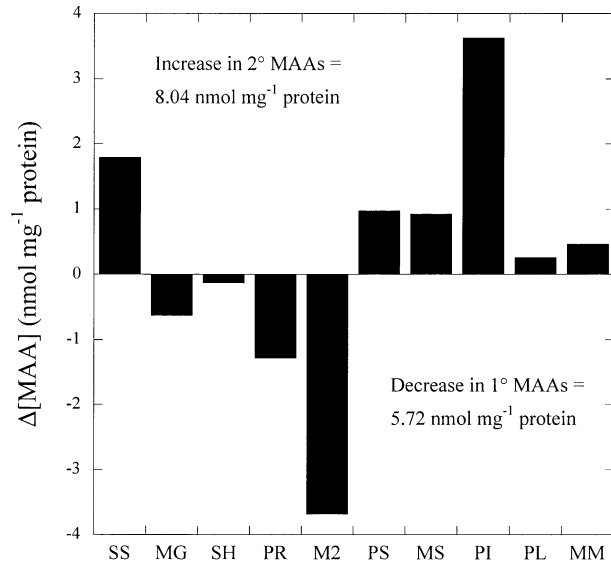


Fig. 4. Decreases in the concentrations of primary MAAs (MG, mycosporine-glycine; SH, shinorine; PR, porphyra-334; M2, mycosporine-2 glycine) between days 14 and 28 of exposure to PAR + UVR are mirrored by stoichiometric increases in concentrations of secondary MAAs (SS, palythine-serine sulfate; PS, palythine-serine; MS, mycosporine-N-methylamine:serine; PI, palythine; PL, palythanol; MM, mycosporine-N-methylamine:threonine). There was no significant net change in the total MAA concentration between day 14 and day 28.

porphyra-334, and mycosporine-2 glycine all stabilized by day 14 and were sustained to day 28 (albeit with a tendency to decline) if exposure to UVR was continued. If UV exposure was discontinued after day 14, concentrations of all of these declined significantly from the value at day 14 during an additional 14 d of exposure to PAR alone. The pattern for the secondary MAAs, particularly palythine-serine sulfate, palythine-serine, mycosporine-N-methylamine:serine,

and palythine, was distinctly different: all continued to increase between days 14 and 28, even in the absence of a UV stimulus during that time. (It is also evident that 3 and 7 d of UV exposure stimulated subsequent accumulation of these secondary MAAs, even if exposure to UVR was discontinued; Fig. 3.) However, the ongoing presence of UVR seems to be required for the accumulation of the minor MAAs palythanol and mycosporine-N-methylamine:threonine because if UVR was discontinued after day 14, these MAAs did not accumulate further and their concentrations remained unchanged. Mycosporine-glycine was transient, increasing in concentration up to day 7, decreasing thereafter, and disappearing by day 28.

Examining the two groups of corals analyzed on days 14 and 28 after those durations of exposure to PAR + UVR, as well as considering only the absolute changes in individual MAAs (Fig. 4) and not whether the changes are statistically significant (significant changes are marked by an asterisk), revealed that the only MAAs tending to decrease were the primary MAAs (mycosporine-glycine\*, shinorine, porphyra-334, and mycosporine-2 glycine), for a total decrease of  $-5.72 \text{ nmol MAA mg}^{-1} \text{ protein}$ . The other six MAAs (all secondary MAAs: palythine-serine sulfate, palythine-serine\*, mycosporine-NMA:serine, palythine, palythanol\*, and mycosporine-NMA:threonine\*) increased by  $8.036 \text{ nmol MAA mg}^{-1} \text{ protein}$ . The difference ( $+2.323 \text{ nmol MAA mg}^{-1} \text{ protein}$ ) was not significant, as indicated by the analysis of variance (ANOVA) showing no net change in total MAAs between days 14 and 28 (Fig. 2).

Grouping MAAs by class (primary and secondary) emphasizes their different kinetics of accumulation (Fig. 5A). Primary MAAs increased parabolically, eventually decreasing in concentration, while secondary MAAs showed a longer lag phase and accumulated more gradually and continuously. The first derivative of the curve describing the concentration of secondary MAAs versus time estimated the rate of accumulation on each day (the rate on day 28 was

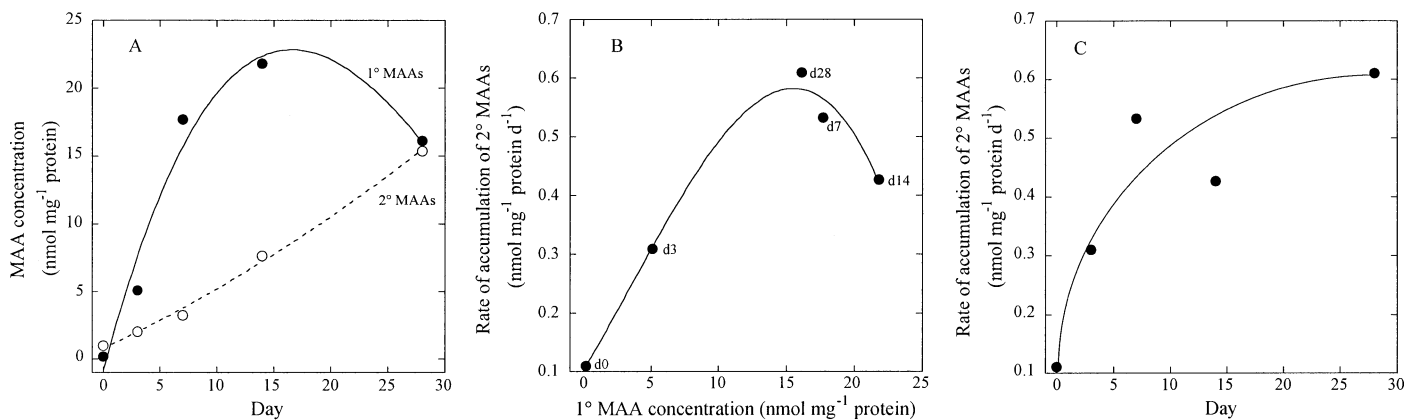


Fig. 5. (A) Pooling primary and secondary MAAs showed different temporal patterns of their accumulation. Data for primary MAAs were fitted by a third-degree polynomial ( $r^2 = 0.965$ ) and secondary MAAs by a second-degree polynomial equation ( $r^2 = 0.996$ ). (B) The first derivative of the curve for secondary MAAs estimated the rate of their accumulation on each day, which was positively related to the concentration of primary MAAs on those days, albeit not by a simple kinetic model, the curve being fitted by a third-degree polynomial ( $r^2 = 0.988$ ). Days on which the concentrations of primary MAAs occurred are shown next to the data points. (C) The rate of accumulation of secondary MAAs increased with time and not simply with concentration of primary MAAs, perhaps because of a slower up-regulation of the enzymes involved in the biosynthesis of secondary MAAs.

obtained by estimating the concentration on day 30 from the polynomial equation and using this datum to calculate the slope from day 28 to day 30). (Note that the background amount of palythine-serine sulfate present on day 0 was omitted from the polynomial-fitted curve to allow a more accurate estimation of accumulation rate on days 0 and 3; this made essentially no difference in the fit, since  $r^2 = 0.996$  if it was included and 0.992 if omitted.)

The rate of accumulation of secondary MAAs was in general positively related to substrate concentration (i.e., the concentration of primary MAAs), although the relationship was parabolic (Fig. 5B). A temporal element was also evident because the rate of accumulation of secondary MAAs increased with time and then apparently stabilized (Fig. 5C).

**Metabolic conversion of MAAs**—Colonies exposed to PAR + UVR but not to glyphosate for 7 d contained all 10 of the MAAs commonly seen in *S. pistillata* (Fig. 6A). Addition of glyphosate on day 7 prevented any further increase in total MAA concentration during an additional 7 d of exposure to PAR + UVR, whereas uninhibited colonies had higher concentrations of MAAs than glyphosate-treated colonies by day 14 (ANOVA;  $F_{2,9} = 15.506$ ,  $p = 0.001$ ). Although there was no net change in total MAA concentration after the addition of glyphosate, the concentrations of five secondary MAAs increased significantly and one primary MAA (mycosporine-glycine) decreased significantly. Other primary MAAs tended to decrease, but not significantly. Summing all changes whether or not statistically significant (Fig. 6B) showed a change of +6.001 nmol secondary MAAs  $\text{mg}^{-1}$  protein and  $-3.375$  nmol primary MAAs  $\text{mg}^{-1}$  protein; the difference (+2.626 nmol MAA  $\text{mg}^{-1}$  protein) was not significant (SNK test for total MAA concentration,  $p > 0.05$ ). The higher concentration of total MAAs in uninhibited colonies than in glyphosate-treated nubbins on day 14 resulted from significant increases in six of the ten MAAs: the primary MAAs mycosporine-glycine, porphyra-334, and mycosporine-2 glycine, and the secondary MAAs palythine-serine sulfate, palythanol, and mycosporine-NMA:threonine (Fig. 6A). The principal secondary MAA, palythine, was as concentrated in glyphosate-treated nubbins as in uninhibited colonies, as were palythine-serine and mycosporine-NMA:serine.

**Effect of UV dose rate on the accumulation of MAAs**—The accumulation of primary MAAs (except mycosporine-glycine) showed sigmoidal kinetics, with MAA concentration increasing rapidly between days 3 and 6, and the rate of accumulation decreasing thereafter (Fig. 7). Some secondary MAAs (palythine-serine, mycosporine-NMA:serine, and palythine) increased exponentially, with no accumulation evident until day 6 and rapid increases thereafter. Mann-Whitney *U*-tests indicated significantly higher concentrations of all primary MAAs in  $2\times$ UV corals on all days (except for porphyra-334 on day 6); the higher rate of accumulation in  $2\times$ UV corals was particularly evident after day 3. Compared with the normal fluence rate of UVR,  $2\times$ UV resulted in significantly higher concentrations of palythine-serine, mycosporine-NMA:serine, and palythine on days 6 and 12 (Mann-Whitney *U*-test). UV dose rate did not affect the rate

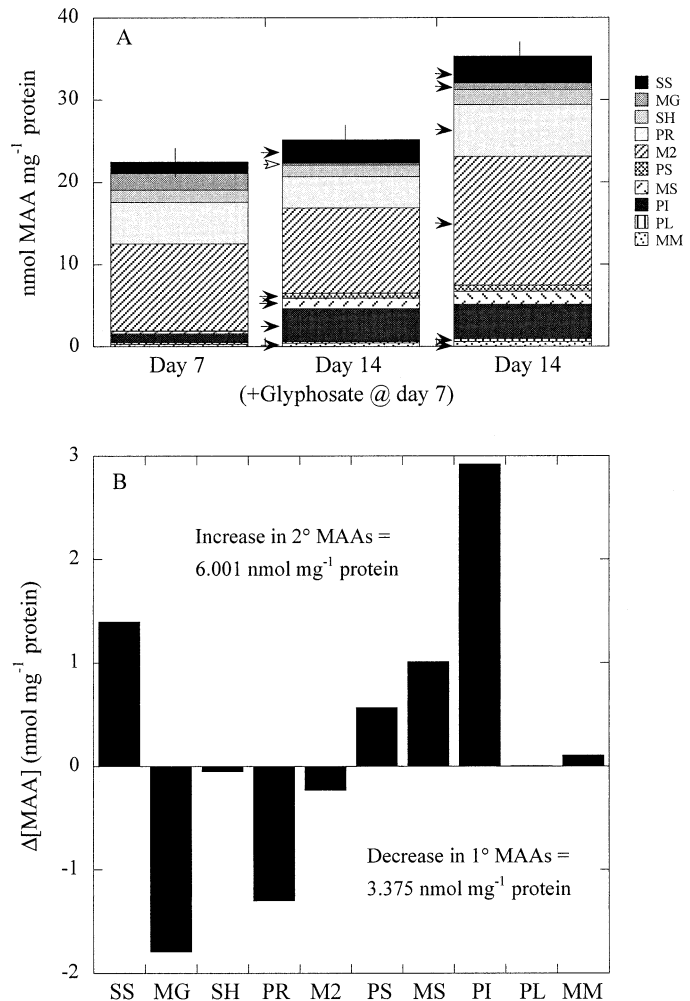


Fig. 6. (A) Concentrations of individual (see caption to Fig. 4 for abbreviations) and total MAAs varied among corals exposed to PAR + UVR for 7 and 14 d, and colonies exposed to glyphosate from day 7 to day 14 of UV exposure (ANOVA for total MAAs;  $F_{2,9} = 15.506$ ,  $p = 0.001$ ). Total MAA concentration did not increase after the application of glyphosate (SNK test,  $p > 0.05$ ), but did accumulate further from day 7 to day 14 in the absence of glyphosate (SNK test,  $p < 0.05$ ). Significant differences in individual MAAs between colonies extracted at day 7 and glyphosate-treated colonies extracted on day 14, and between the latter group and 14-d colonies unexposed to glyphosate, were ascertained by SNK tests following ANOVA, and are shown by arrows (solid arrows indicate increases, open arrow denotes decrease). (B) Following the application of glyphosate, decreases in the concentrations of primary MAAs (see caption to Fig. 4 for abbreviations) were mirrored by stoichiometric increases in the concentrations of secondary MAAs, with no net change in the total MAA concentration.

of accumulation of mycosporine-glycine or mycosporine-NMA:threonine, nor, generally, of palythanol. Concentrations of all MAAs increased with time of exposure (cumulative UV dose). Treating primary and secondary MAAs as two separate classes emphasized the difference in the kinetics of their accumulation (Fig. 8A). The decrease in the rate of accumulation of primary MAAs between early (days 0–6) and late (days 6–12) in the experiment was reflected by op-

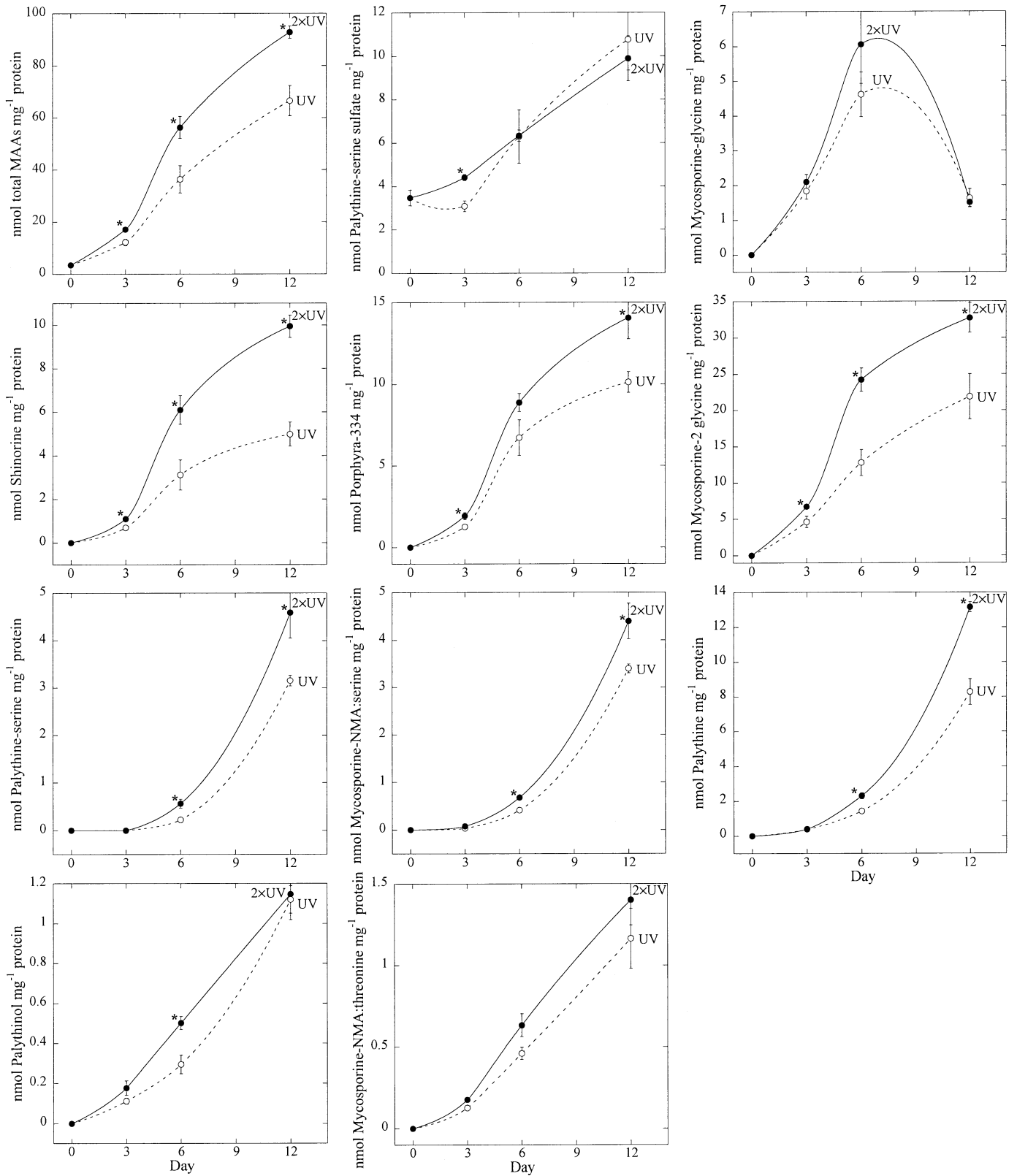


Fig. 7. Accumulation of total and individual MAAs in colonies exposed to PAR + normal levels of UVR (open circles, broken lines), and in those exposed to PAR + double irradiances of UVR (2xUV) (solid circles, solid lines). Values are means  $\pm$  SE,  $n = 4$  for each treatment and day. Significant differences (Mann-Whitney  $U$ -tests,  $p < 0.05$ ) between treatments on individual days are shown by asterisks (\*).

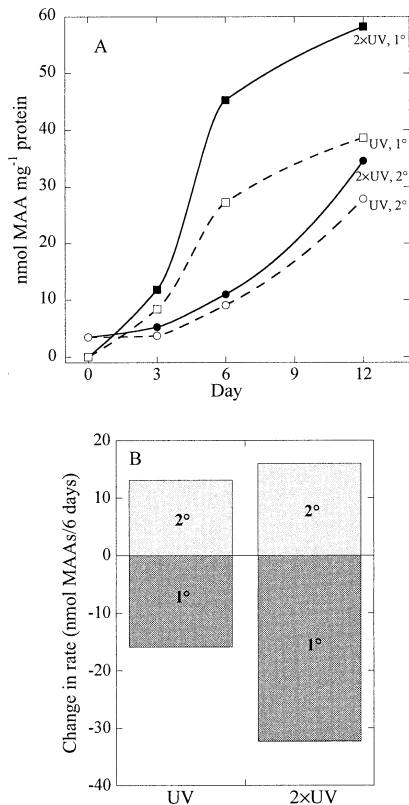


Fig. 8. (A) Accumulation of pooled primary (square symbols) and secondary (circles) MAAs in colonies exposed to UV (open symbols, broken lines) and 2×UV (solid symbols, solid lines) at the same level of PAR. For clarity, error bars are omitted (*see Fig. 7 for error bars for individual and total MAAs*). (B) Changes in the average rates of accumulation of primary and secondary MAAs between days 0–6 and days 6–12 of exposure.

posite changes in the kinetics of secondary MAAs, albeit to different extents in the two groups exposed to UV and to 2×UV (Fig. 8B).

**Effects of antioxidants on accumulation of MAAs**—After 7 d of exposure to PAR + UVR, control colonies and those exposed to mannitol contained only seven MAAs (palythine-serine sulfate, mycosporine-glycine, shinorine, porphyra-334, mycosporine-2 glycine, palythine, and mycosporine-NMA:threonine). There was no significant difference between control and mannitol-exposed nubbins in the concentration of any MAA (Student's unpaired *t*-tests,  $p > 0.05$ ; data not shown). Control and Trolox-exposed colonies likewise contained these seven MAAs after 7 d of exposure to PAR + UVR (Fig. 9). In this case, Trolox-treated corals had significantly lower concentrations of total MAAs ( $t = 3.849$ ,  $df = 4$ ,  $p = 0.018$ ) and specifically of the primary MAAs shinorine ( $t = 2.907$ ,  $df = 4$ ,  $p = 0.044$ ), porphyra-334 ( $t = 5.524$ ,  $df = 4$ ,  $p = 0.005$ ), and mycosporine-2 glycine ( $t = 2.863$ ,  $df = 4$ ,  $p = 0.046$ ).

## Discussion

The responsiveness of an organism's concentration of MAAs to changes in the photic environment is well estab-

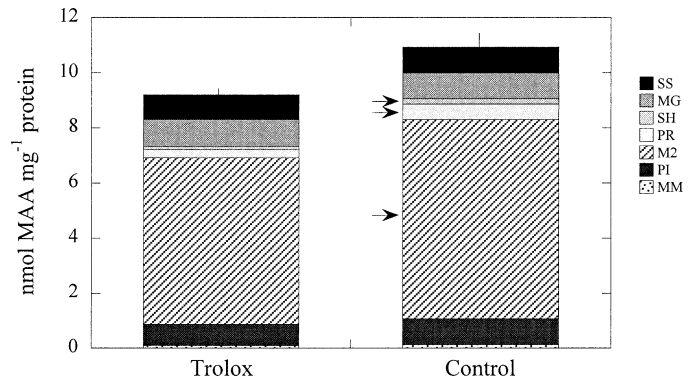


Fig. 9. Concentrations of individual (*see caption to Fig. 4 for abbreviations*) and total MAAs in colonies exposed to PAR + UVR in the absence (control) and presence of Trolox for 7 d ( $n = 3$  in each group). Total MAA concentration was significantly lower in Trolox-treated colonies ( $t = 3.849$ ,  $df = 4$ ,  $p = 0.018$ ). Significant differences (*t*-tests,  $p < 0.05$ ) in individual MAAs are indicated by arrows.

lished, but heretofore there have been no kinetic studies of the effect of UV irradiance (dose rate) on the accumulation of individual MAAs. Sequential structural modification of MAAs is suspected to contribute to the diversity in an organism's complement of these compounds, but this study provides the first extensive stoichiometric analysis of such conversions. The *de novo* biosynthesis of MAAs probably occurs via an early branchpoint of the shikimate pathway, but the enzymes of this branchpoint have not been identified. The present results are relevant to the diversity of enzymes required to catalyze conversions of primary, *Symbiodinium* MAAs to secondary MAAs, presumably in the host's tissues. The empirical data suggest a conceptual model of the regulation of MAA biosynthesis and conversion.

**Effect of continuity of UV stimulus on accumulation of MAAs**—The concentrations of total MAAs in corals transiently exposed to UVR for 3, 7, and 14 d and then maintained under PAR alone for the balance of 28 d are, respectively, the same as in colonies exposed to PAR + UVR for 3, 7, and 14 d and then extracted immediately (Fig. 2). This illustrates the persistence of MAAs after they have been synthesized. Once accumulated, MAAs have a long residence time (Adams and Shick 1996; Newman et al. 2000; Portwich and Garcia-Pichel 2003) and are photochemically stable (Adams and Shick 1996; Conde et al. 2000; Shick et al. 2000), which, in organisms that produce MAAs *de novo*, would save the metabolic cost (300 mol photons or 60 ATP (adenosine triphosphate) equivalents per mol MAA; *see Shick and Dunlap 2002*) of an ongoing biosynthesis to offset a high rate of loss or degradation. In reef corals living in sunlit, oligotrophic waters and synthesizing MAAs, there is also the issue of conserving scarce nitrogen (Gleason 2001).

The lower concentration of total MAAs in colonies exposed only for 3 or 7 d than in 14- and 28-d corals indicates the requirement for an ongoing UV stimulus if total MAAs are to continue to increase. However, the strikingly different patterns of UV-induced accumulation of individual MAAs (Fig. 3) indicate underlying differences between the kinetics

of de novo biosynthesis of primary MAAs and of their metabolic conversion to secondary MAAs, perhaps related to differences in the up-regulation and turnover of the putative enzymes involved in these processes.

The immediate increase in primary MAAs (Fig. 5A) is consistent with a rapid up-regulation of the enzymes catalyzing their biosynthesis in the zooxanthellae. The blocking of UVB-induced biosynthesis of MAAs by inhibitors of protein synthesis in cyanobacteria indicates control at the level of gene expression (Portwich and Garcia-Pichel 1999; Sinha et al. 2003). In higher plants, UVR induces mRNA that encodes 3-deoxyarabinoheptulosonate 7-phosphate (DAHP) synthase, the first enzyme in the shikimate pathway, within 2–4 h (Logemann et al. 2000). Downstream enzymes involved in the biosynthesis of flavonoids (UV defenses in higher plants) are coordinately induced by UVR within hours (Kubasek et al. 1992; Logemann et al. 2000), and their mRNAs accumulate sequentially during 2–7 d of exposure (Kubasek et al. 1992). A similar timing of the induction of early enzymes in the shikimate pathway and the branchpoint to MAAs (Favre-Bonvin et al. 1987; Shick and Dunlap 2002; Portwich and Garcia-Pichel 2003) seems likely and would be consistent with the kinetics of accumulation of primary MAAs. The discontinued accumulation and decline of primary MAAs when UV exposure ends probably results from the enzymes directly involved in their biosynthesis having short half-lives and thus a rapid decline when the stimulus for their induction is removed. Given both the protective importance of MAAs and the presumed high cost of their de novo biosynthesis, it is not surprising that the enzymes involved are quickly induced by exposure to UVR and then decline when it is removed, especially because MAAs are persistent once accumulated.

The longer lag in the increase of secondary MAAs (Fig. 5A) may be related both to a longer time until the necessary enzymes are expressed and to the necessity for the prior accumulation of those enzymes' substrates (primary MAAs). This is reminiscent of the sequential induction of genes encoding enzymes in the flavonoid pathway, where the induction is in the order of the biosynthetic steps, which may allow accumulation of sufficient concentrations of precursors (substrates) to optimize enzymic catalysis (Kubasek et al. 1992).

The continued accumulation of palythine, palythine-serine, and mycosporine-NMA:serine long after the cessation of the UV stimulus (Fig. 3) may indicate a longer turnover time for the enzyme(s) involved in the production of these quantitatively important secondary MAAs. Their continued accumulation despite the absence of UVR shows that UVR acts as a stimulus to up-regulate the biosynthetic pathway but is not required catalytically, e.g., to drive a photoenzyme involved in MAA biosynthesis. This lack of a catalytic UV requirement was also concluded for the synthesis of MAAs induced by salt stress in a cyanobacterium (Portwich and Garcia-Pichel 2000), where a putative UVB receptor further regulates a proposed "shinorine synthase" enzyme (Portwich and Garcia-Pichel 2003).

The rate of accumulation of the secondary MAAs in general increases with the concentration of the primary MAAs (Fig. 5B), as would be expected if the latter are substrates

for enzymes involved in the bioconversion of primary to secondary MAAs. There is a slight apparent decline in the rate at the two highest substrate concentrations, which may be related to the different time courses of up-regulating the enzymes involved in synthesizing primary MAAs and those converting them to secondary MAAs. The highest concentrations of primary MAAs occurred on days 7 and 14, when the concentration versus time curve indicated that the rate of accumulation of secondary MAAs was still submaximal. This is seen more clearly when the *rate* of accumulation of secondary MAAs is plotted versus time, where the rate increases with time until apparently stabilizing by day 28 (Fig. 5C). Therefore, if the enzymes necessary to convert primary MAAs to secondary MAAs are up-regulated more slowly than the enzymes catalyzing de novo biosynthesis of primary MAAs, higher concentrations of primary MAAs will be present before the enzymes that convert them to secondary MAAs have achieved their maximal titers. This would explain why rates of accumulation of secondary MAAs are not maximal when the concentration of substrate is maximal, but instead on day 28, when their substrate concentration has started to decline.

The increase in secondary MAAs between days 14 and 28 (Fig. 3), when there is no net change in total MAA concentration, involves a stoichiometric decrease in primary MAAs (Figs. 4 and 5A). This is strong evidence that the postulated conversions do occur. In taxonomically diverse hard and soft corals fresh from the field, secondary MAAs (especially palythine) tend to predominate over primary MAAs (Dunlap et al. 1986; Shick et al. 1991, 1995; Jokiel et al. 1997; Teai et al. 1997, 1998; Banaszak et al. 1998; Muszynski et al. 1998; Corredor et al. 2000; Lesser 2000; Michalek-Wagner 2001), which suggests an ongoing conversion of primary to secondary MAAs in nature, yielding a steady state that includes proportionally more of the latter. The relative concentrations of the eight MAAs resolved in a colony of *S. pistillata* freshly collected on the Great Barrier Reef (J. Wu Won pers. comm.) are decidedly more like those for 28-d corals than for 3-, 7-, or 14-d colonies in the present experiment.

Once the enzymic machinery is in place, the lower metabolic cost of producing secondary MAAs from primary MAAs compared with the higher cost of de novo biosynthesis of the latter would not select as strongly for the degradation of the secondary enzymes. Also, when total MAAs reach high concentrations intracellularly, it may be important to accumulate preferentially the less acidic secondary MAAs, which have fewer ionizable groups and would be less perturbing to cellular functions (e.g., Hochachka and Somero 2002; see also Carreto et al. 2001). Moreover, unlike the acidic primary MAAs, functionally neutral secondary MAAs such as the predominant palythine are refractory to bacterial metabolism (Dunlap et al. 1998, and W. C. Dunlap pers. comm.), so accumulating the latter might render them inaccessible to the bacteria that inhabit corals (Rohwer et al. 2002 and references therein). These additional physiological considerations may be as important as the often-assumed role of accumulating chemically diverse MAAs to broaden the absorption spectrum of the UV filter that they provide (Carreto et al. 1990; Dunlap et al. 1998; Franklin et al. 1999;

Karentz 2001), especially in view of the broad absorption spectra of certain secondary MAAs (Gleason 1993) and the considerable spectral overlap of many MAAs.

Mycosporine-glycine is structurally the simplest MAA and the one postulated to give rise to the other MAAs by amino acid substitutions (*see* fig. 3 in Shick and Dunlap 2002, and below), which has been shown directly by Portwich and Garcia-Pichel (2003) for the stoichiometric production of shinorine from mycosporine-glycine and serine in a cyanobacterium. Its decline in concentration after day 7 (Figs. 3 and 7) may be a result of increasing rates of production of shinorine, porphyra-334, and mycosporine-2 glycine outstripping that of mycosporine-glycine. The eventual disappearance of mycosporine-glycine from corals in the present study suggests that in experiments of intermediate duration, biosynthesis of this primary MAA may decrease greatly or even cease when the steady-state concentration of MAAs is achieved.

Nevertheless, mycosporine-glycine is one of the most frequently observed and often most concentrated MAAs among diverse corals in field studies (Dunlap et al. 1986; Gleason 1993; Gleason and Wellington 1995; Shick et al. 1995; Jokiel et al. 1997; Teai et al. 1997, 1998; Banaszak et al. 1998; Muszynski et al. 1998; Corredor et al. 2000; Lesser 2000). Because long-lived but highly soluble MAAs eventually show some leakage or other loss (Adams and Shick 1996; Portwich and Garcia-Pichel 1999), and because growth will produce new tissue that must be provided with UV protection, the *de novo* biosynthesis under natural conditions of primary MAAs such as mycosporine-glycine must eventually continue at some level, even if it ceases in corals in experiments lasting 1 month. Mycosporine-glycine did not disappear during 1 month of exposure to UVR in our earlier experiments, but its concentration was extremely variable among colonies by day 30 (Shick et al. 1999). Maintaining some intracellular level of this oxo-MAA may also be important because of its antioxidant properties (Dunlap and Yamamoto 1995; Shick and Dunlap 2002; Suh et al. 2003), which may account for its omnipresence among shallow-water photosynthetic corals in the field (*see* aforementioned references on the MAA complements of corals). Its variable turnover as a sacrificial antioxidant may account for the widely varying concentrations in which it occurs; in *S. pistillata* in nature, mycosporine-glycine constitutes a minor fraction of the MAA pool (J. Wu Won pers. comm.). It is unknown whether the small amounts of several MAAs (including mycosporine-glycine; Fig. 3) sometimes seen in colonies unexposed to UVR originate from constitutive biosynthetic enzymes or from uncharacterized dietary sources available to the corals during their long-term culture in the laboratory mesocosm.

*Metabolic conversion of MAAs*—An ongoing biosynthesis of primary MAAs would complicate the analysis of the stoichiometry of their conversion to secondary MAAs, so 1 mmol L<sup>-1</sup> glyphosate was used to block the shikimate pathway, presumably at the first two enzymes (Bode et al. 1984; Shick and Dunlap 2002). Once applied, glyphosate inhibited any further increase in primary, *Symbiodinium* MAAs (Fig. 6A), as shown earlier (Shick et al. 1999). This is consistent

with the prevention, by high concentrations of glyphosate, of carbon flow into the shikimate pathway and thence to the MAA branchpoint. The failure of glyphosate to prevent increases in most secondary MAAs (Fig. 6A), however, indicates that it does not affect the putative enzymes involved in the metabolic conversion of preexisting primary MAAs into secondary MAAs. This conclusion is reinforced by the stoichiometric balance between the increase in secondary and the corresponding decrease in primary MAAs after the application of glyphosate (Fig. 6B).

*Effect of UV dose rate on the accumulation of MAAs*—The steady-state concentrations of MAAs in corals in nature are related to UV irradiances prevailing at different depths (Dunlap et al. 1986; Drollet et al. 1993; Gleason 1993; Gleason and Wellington 1995; Shick et al. 1995; Jokiel et al. 1997; Banaszak et al. 1998; Corredor et al. 2000; Lesser 2000) and at differently illuminated locations within a single colony (Jokiel et al. 1997; Muszynski et al. 1998). Transplanting corals, other reef anthozoans, and macroalgae from greater to shallower depths, or exposing them to artificially enhanced UVR, generally causes increases in their concentrations of MAAs (reviewed by Shick et al. 1996 and Gleason 2001). Hard and soft corals in the field show seasonal cycles of MAA concentration related to incident solar UVR (Drollet et al. 1997; Michalek-Wagner 2001), changes that occur on the order of weeks. UV-naive corals in the laboratory quickly synthesize and accumulate MAAs over a time course similar to this when acutely exposed to UVR (Shick et al. 1999), but the kinetic response to the UV irradiance (dose rate) has not been tested heretofore.

Doubling the instantaneous UV irradiance significantly enhanced the rate of accumulation of the primary MAAs shinorine, porphyra-334, and mycosporine-2 glycine, an effect that was evident by day 3 of exposure and pronounced by day 6 (Figs. 7 and 8A). Twice-normal fluence (2×UV) also accelerated the accumulation of the principal secondary MAAs, palythine-serine, mycosporine-NMA:serine, and palythine, but with a longer lag phase than for the primary MAAs. In the absence of molecular (e.g., mRNA transcripts) or biochemical (e.g., enzyme concentrations) data regarding the putative enzymes involved, the basis for this rate-enhancing effect is uncertain. The principle of reciprocity may provide some insights. If reciprocity holds, then doubling the UV dosage rate should have the same effect in half the time, and a failure of reciprocity indicates the operation of dark processes not directly related to UVR (Jagger 1985).

Comparing the concentrations of MAAs in 2×UV-treated corals at day 3 with those in UV-treated corals at day 6 (Fig. 7) indicates that reciprocity failed in eight of the ten MAAs (including all of the primary MAAs), where the concentration at day 6 in UV-treated colonies was greater than in 2×UV-treated colonies at day 3. This probably is because at day 3, the biosynthetic enzymes were still in the induction phase, and the full titer of enzymes had not yet been synthesized. (Recall that accumulating mRNA that encodes the enzymes of flavonoid biosynthesis in higher plants requires 7 d; Kubasek et al. 1992.) This argument assumes the operation of dark processes, e.g., the biosynthetic enzymes in-

duced by UVR being produced even during the time of day when UVR is not present.

Applying reciprocity to a multifaceted process such as the biosynthesis and accumulation of MAAs is problematic, and the reciprocity seen in primary MAA biosynthesis by comparing 2×UV-treated nubbins at day 6 with UV-treated corals at day 12 may be more apparent than real. This is because by day 12, secondary MAAs have increased dramatically, drawing down the pools of primary MAAs from which they are synthesized, whereas by day 6 secondary MAAs have scarcely begun to accumulate (Fig. 8A). This would leave higher concentrations of primary MAAs in 2×UV corals on day 6 to be compared with depleted pools of primary MAAs in UV-treated corals on day 12, giving the appearance of reciprocity.

The mechanistic basis for the increased rate of accumulation of primary MAAs in 2×UV corals could involve a simple doubling of the amount of the enzymes catalyzing their biosynthesis. This might be indicated by the data on day 6, when the accumulation of secondary MAAs by conversion from primary MAAs has not yet begun to draw down the concentration of the latter: at this time, nubbins exposed to 2×UV have, on average, 1.7 times higher concentrations of primary MAAs (shinorine, 2-fold higher; porphyra-334, 1.9-fold higher; mycosporine-2 glycine, 1.3-fold higher) than do those exposed to the normal UV irradiance. Whatever the mechanism, increasing the rate of biosynthesis of MAAs could be adaptive during times of increased solar UV irradiance in nature (e.g., doldrums, with low wind, little cloud cover, and high water clarity).

Doubling the UV fluence rate is also a useful tool to examine the regulation of the biosynthesis and conversion of MAAs. The sigmoidal kinetics of accumulating primary MAAs (Fig. 8A) suggest a regulated process, but is the changing rate of accumulation owing to regulation of their de novo biosynthesis, or simply to their depletion via an increasing rate of conversion to secondary MAAs? Under normal UVR, 82% of the decrease in the rate of accumulating primary MAAs between the initial (days 0–6) and later (days 6–12) phases of the experiment can be accounted for by the increase in the rate of accumulating secondary MAAs between the initial and later phases (Fig. 8B). Under 2×UV, however, the decrease in the rate of accumulating primary MAAs is twice as great as can be explained by their stoichiometric conversion to secondary MAAs (Fig. 8B). Therefore, the accumulation is regulated, at least in the latter case.

*Possible mechanisms of inducing and controlling the biosynthesis of MAAs*—The mechanism of such regulation must remain speculative in the absence of specific information about the biosynthetic enzymes, but once accumulated, MAAs might down-regulate their own biosynthesis via a feedback loop. This could occur by their attenuating the UV signal that induces the enzymes (perhaps mediated by a UV receptor; Portwich and Garcia-Pichel 2000), followed by a depletion of the enzyme titers as rates of their degradation exceed those of replacement. Nothing is known of the UV signal-transduction mechanism in zooxanthellate corals, but like the case in higher plants, it may involve reactive oxygen species (ROS) such as superoxide radical ( $O_2^{\bullet-}$ ), hydrogen

peroxide ( $H_2O_2$ ), and nitric oxide (A.-H.-Mackerness 2000; A.-H.-Mackerness et al. 2001), as well as organic peroxides (Baier and Dietz 1999). The inverse relationship between the concentration of shinorine + porphyra-334 (which do not have antioxidant activity; Dunlap and Yamamoto 1995) and the production of endogenous ascorbate radical in the eggs of a sea urchin during UV irradiation (Shick et al. 2000) does indicate that accumulating UV-attenuating MAAs can progressively dampen a UV-induced cellular redox event.

The lowering of the UV-induced accumulation of shinorine, porphyra-334, and mycosporine-2 glycine by 100  $\mu\text{mol L}^{-1}$  Trolox (a scavenger of peroxyxynitrite and of alkoxy and peroxy radicals involved in lipid peroxidation; Halliwell and Gutteridge 1999) (Fig. 9) suggests an involvement of lipid peroxides or related products, perhaps formed in chloroplast membranes, in up-regulating the biosynthesis of these primary MAAs. Hydroxyl radical ( $HO^{\bullet}$ ), a common initiator of lipid peroxidation (Halliwell and Gutteridge 1999), seems not to be involved, because mannitol (a scavenger of  $HO^{\bullet}$ ) at 100  $\text{mmol L}^{-1}$  (a concentration that ameliorates thermally induced bleaching in the coral *Agaricia tenuifolia*; Lesser 1997) did not affect UV-induced accumulation of MAAs in *S. pistillata*. The tissue of corals exposed to 100  $\mu\text{mol L}^{-1}$  Tiron (4,5-dihydroxy-1,3-benzenedisulfonate, a scavenger of  $O_2^{\bullet-}$ ) became necrotic and sloughed from the skeleton, so it is not known whether  $O_2^{\bullet-}$  is involved in UV signal transduction (J. M. Shick unpubl. data).

Assuming that ROS are principally formed in the zooxanthellae, and particularly in the chloroplasts, during irradiation (Asada 1999; see also Dykens et al. 1992), this putative redox signal that induces up-regulation of the early shikimate-pathway enzymes (and presumably of the enzymes catalyzing MAA biosynthesis) must travel some distance and cross several membranes, since the enzymes of the shikimate pathway are encoded in the nuclear DNA (Kishore and Shah 1988; Herrmann and Weaver 1999). Candidates for such long-distance messengers include lipophilic aldehydes, which are degradation products of alkyl hydroperoxides (Baier and Dietz 1999), as well as  $H_2O_2$ , which might secondarily be involved in redox signaling in the host animal's cells (see Dykens et al. 1992), where enzymes for producing secondary MAAs presumably are located. A network of parallel and interacting redox signals, rather than a single trigger (A.-H.-Mackerness et al. 2001), may be involved in cell signaling in the host and its endosymbionts.

Regardless of the possible secondary involvement of ROS, reactive nitrogen species, organic peroxides, or other signaling intermediates generated by UVR, the kinetic data for primary and secondary MAAs suggest a simple model for their UV-induced biosynthesis and regulation (Fig. 10). In UV-naive corals, concentrations of MAAs are extremely low, and UVR applied acutely will penetrate to diverse cellular targets. The resulting signal(s) will induce the expression of enzymes catalyzing the de novo biosynthesis of primary MAAs in the zooxanthellae and of enzymes that convert primary to secondary MAAs, perhaps in the host. Increased concentrations of primary enzymes lead to the accumulation of primary MAAs, which then serve as substrates for the concentration-dependent production of secondary MAAs (in Fig. 10, two curves are shown for the

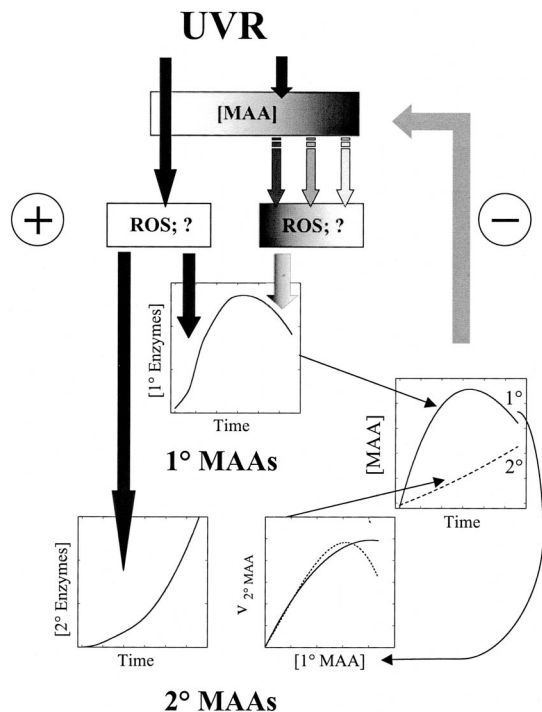


Fig. 10. Conceptual model for the UV-induced accumulation and conversion of MAAs in *S. pistillata* during 1 month, based on the empirical data in this paper and in Shick et al. (1999). See text for discussion.

relationship between the rate of production,  $v$ , of secondary MAAs and the concentration of primary MAAs because of uncertainty whether the rate declines at the highest concentrations; see Fig. 5B). The more gradual accumulation of secondary MAAs than their concentration-dependent production from primary MAAs could support (Fig. 5A,B; Fig. 8A) may be due to the later up-regulation of secondary enzymes compared to primary enzymes, as discussed above for flavonoid biosynthesis.

Rising concentrations of MAAs progressively attenuate the amount of UVR reaching cellular targets, leading to a decrease in the signal(s) governing the expression of the primary enzymes, titers of which would decline during normal turnover when the rates of their degradation exceed rates of synthesis. Consequently, concentrations of primary MAAs begin to decline as their synthesis decreases and as they are converted to secondary MAAs. Presumably the attenuated UV signal will result in a similar decline in secondary enzymes on a time scale  $>1$  month. Continued exposure to UVR in MAA-replete corals will require low activities of both classes of enzymes to replace slow losses of MAAs and to provide these UV defenses in new tissues produced during growth.

Consistent with this model are the results of the experiment in which exposure to UVR was terminated but exposure to PAR continued, where pools of accumulated primary MAAs declined while those of secondary MAAs increased stoichiometrically in the absence of a UV stimulus. Experiments lasting  $>1$  month are needed to ascertain the steady state of secondary MAAs and whether the complement and

proportions of MAAs correspond to those in corals in nature. The results of the  $2\times$ UV experiment (which used a different colony than the one in the UV-continuity study) further support the biooptical and signaling model because the higher dose rate of UVR elicits faster biosynthesis of MAAs, as well as a leveling off of MAAs at the higher concentrations required to attenuate the enhanced UV irradiance and down-regulate MAA biosynthesis. These results also suggest that it is not the intracellular MAA concentrations per se that initiate negative feedback on their biosynthesis, rather, it is their biooptical effect.

*Possible pathways of conversion of primary and secondary MAAs*—Its simplest structure among the MAAs; its similarity to the probable precursor 4-deoxygadusol (Favre-Bonvin et al. 1987); its wide occurrence among diverse phototrophs (reviewed by Karentz 2001 and Banaszak 2003), including many zooxanthellae (Banaszak et al. 2000); and its rapid appearance in response to UVR all suggest that mycosporine-glycine is the progenitor MAA (reviewed by Shick and Dunlap 2002). It probably gives rise to the other primary MAAs by forming imines with glycine, serine, and threonine, yielding mycosporine-2 glycine, shinorine, and porphyra-334, respectively (Fig. 11); the biosynthesis of shinorine by this mechanism has been demonstrated in a cyanobacterium (Portwich and Garcia-Pichel 2003). These reactions are metabolically reversible, as indicated by the bacterial formation of mycosporine-glycine (and 4-deoxygadusol) from exogenous shinorine and porphyra-334 (Dunlap et al. 1998; Dunlap and Shick 1998), so that in *S. pistillata* the primary MAAs may exist as an equilibrium pool.

Although it may have a high rate of biosynthesis, mycosporine-glycine's concentration is kept low relative to those of the other primary MAAs because it is continually consumed in their biosynthesis, and perhaps in its later conversion to palythine by reductive transamination with metabolic ammonium to form the unsubstituted imine (Whitehead et al. 2001). The other secondary MAAs are probably derived from shinorine, porphyra-334, and mycosporine-2 glycine, as follows.

Mycosporine-N-methylamine:serine and mycosporine-N-methylamine:threonine could be formed via the aminolysis of serine and threonine from the C1 position of shinorine and porphyra-334, respectively, with these amino acids replacing glycine at C3 in a conjugate addition/elimination reaction. In effect, this is a rearrangement of the positions of serine and threonine in the MAAs involved, with no requirement for additional use of these amino acids from the pool—and a return of the glycine to the pool—of free amino acids. Serine and threonine then would be replaced at C1 by imine formation with ammonium, followed by methylation of the imine (likely via methionine) or by direct transamination of porphyra-334 with constitutive methylamine (not shown). Similarly, palythine-serine could be formed from shinorine, without the final methylation step. This scheme does not account for the eventual decline in mycosporine-2 glycine (the most concentrated primary MAA in these corals) by the appearance of a direct, stoichiometric product. This decline might occur via aminolysis at C1, forming palythine, which increases to a similar extent over the same time course (Fig.

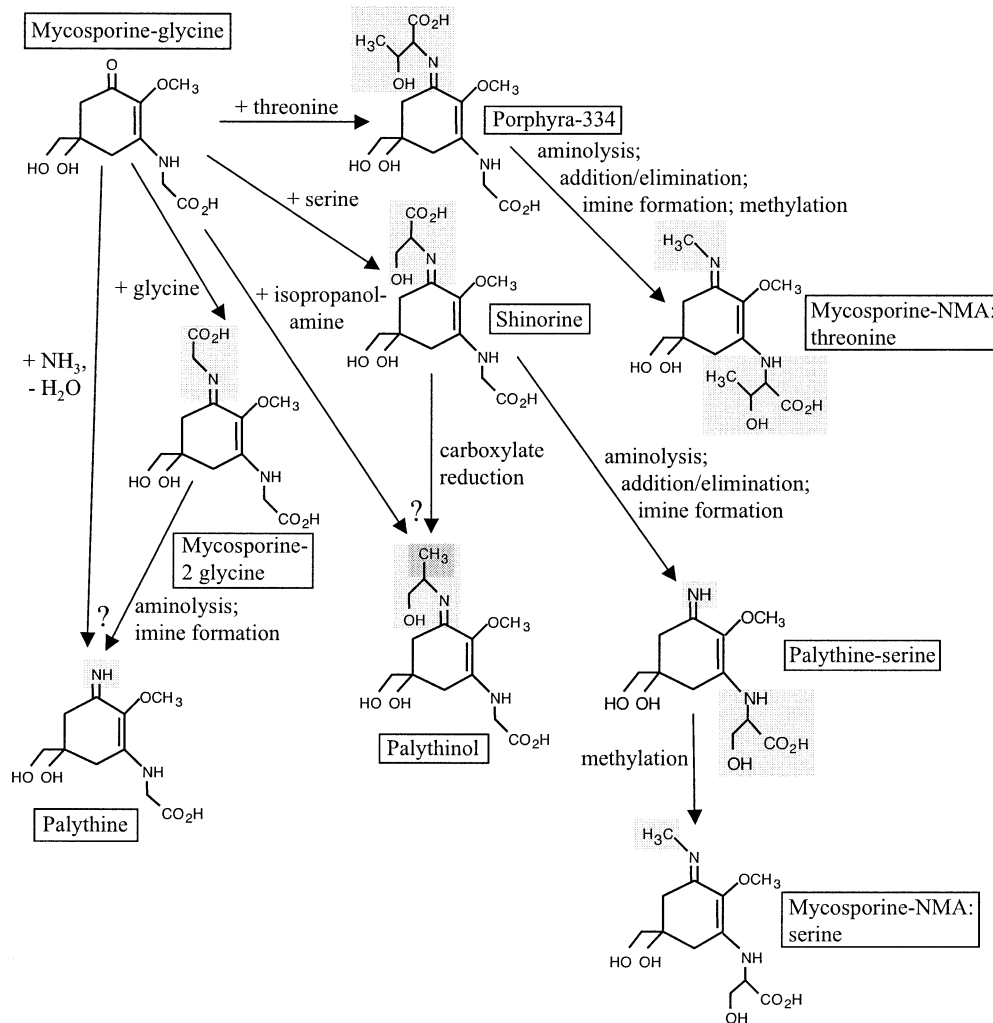


Fig. 11. Suggested pathways of biosynthesis and conversion of MAAs in *S. pistillata* during 28 d of exposure to PAR + UVR. Shaded portions of the molecules indicate changes from the previous structure in the sequence. Pathways were deduced from the kinetics observed for the individual MAAs and likely organic reaction mechanisms.

3), and might represent a means to reduce the pool of an acidic MAA by converting it to one that is functionally neutral. All of the foregoing are straightforward organic reactions and are consistent with the observed kinetics (Fig. 3).

Of the remaining secondary MAAs, palythanol (the least concentrated MAA) might plausibly be formed via carboxylate reduction of the serine residue in shinorine by a presumed reductase enzyme, by amination of mycosporine-glycine by endogenous isopropanolamine, or by transamination of other imino MAAs (not shown). In any case, palythanol is a minor MAA in this coral. The more prevalent palythine-serine sulfate remains enigmatic. This is invariably the most concentrated, and usually the only, MAA that is detectable in *S. pistillata* prior to the application of UVR. It may be formed by sulfation of palythine-serine, although we have never detected the latter MAA in UV-naïve colonies of this coral, and palythine-serine sulfate but not palythine-serine occurred in control nubbins from the coral colony used in the antioxidant experiment (Fig. 9). Nor is the biochemical

relevance of the sulfate-ester linkage in palythine-serine sulfate known, because sulfated organic compounds in general are rare in the Cnidaria but do occur in dinoflagellates (Kornprobst et al. 1998). Since palythine-serine sulfate is not reported from any free-living or cultured alga, might this MAA represent a sequestration of sulfate associated with the endosymbiotic condition?

Finally, the foregoing scheme (summarized in Fig. 11) relates primarily to the *S. pistillata* symbiosis. The ability to further metabolize primary MAAs is not unique to metazoans, and certain MAAs treated in this paper as secondary products of coral host metabolism (i.e., palythine and palythanol) also occur in free-living macroalgae and microalgae and in cyanobacteria (summarized by Karentz 2001 and Banaszak 2003). In such cases, the secondary MAAs are also derived from primary MAAs (Carreto et al. 1990; Franklin et al. 1999), albeit by the primary producers themselves. Other routes of converting primary to secondary MAAs have been suggested based on temporal changes in

MAA complements (e.g., in a red macrophyte; Franklin et al. 1999), and such differences may reflect taxonomic diversity in the specificities of the enzymes involved in the processing.

*Genotypic considerations*—Studies of genotypic effects on the complement and concentrations of MAAs in corals are in their infancy. The pioneering work of Banaszak et al. (2000) shows that different clades and species of *Symbiodinium* may produce different MAAs in culture, and field surveys (e.g., Teai et al. 1997) provide snapshots of the variety of MAAs among taxonomically diverse corals. Controlled experiments such as those reported here add insights regarding intraspecific variability in the complement of MAAs and its causes, including the host's processing of *Symbiodinium* MAAs. These results also provide tentative evidence for genotypic effects.

Pieces of the same colony were used in the experiments on UV continuity (Figs. 2 and 3) and the effect of glyphosate (Fig. 6). This colony harbors zooxanthellae of ribotype A1, the same as the culture studied by Banaszak et al. (2000). The concentrations and ratios of MAAs were the same in both experiments (cf. data for days 7 and 14 of exposure to PAR + UVR in both experiments). This shows that the biosynthetic response to UVR is consistent for this colony in different experiments conducted a year apart (and despite the glyphosate control nubbins being incubated in beakers and not in the flow-through aquarium).

The 2×UV experiment (Fig. 7) used a different colony that, although it produced the same 10 MAAs as the first colony, did so at a higher rate under the same conditions of normal exposure to PAR + UVR in the open-flow aquarium, which thus may reflect a genotypic difference. Nevertheless, the distinct kinetic patterns for primary versus secondary MAAs (Figs. 7 and 8A) were essentially the same as in the first colony (Fig. 5A); this reveals a consistency between the colonies in the underlying mechanisms of primary biosynthesis and conversion of MAAs, which may occur at different absolute rates. Such intercolony consistency, plus the multiple time points and diverse complement of MAAs in these colonies, warrants the general model shown in Fig. 10.

A third colony fortuitously used in the experiment involving Trolox (Fig. 9) produced only seven MAAs during exposure to PAR + UVR for 7 d and contained a lower total concentration of MAAs. These facts and the different ratios among the primary MAAs (notably the predominance of mycosporine-2 glycine; cf. data for control nubbins in Fig. 9 and day 7 nubbins in Fig. 6A) might reflect a genotypic difference from the other two colonies. The lack of the secondary MAAs mycosporine-NMA:serine and palythanol from the third colony may particularly indicate metabolic differences in the host.

Obviously these unplanned comparisons are not definitive, but they point the way to integrated studies involving genotypically diverse zooxanthellae and hosts to assess the metabolic responses of coral symbioses to their environmental photic regimes. The ecological and biogeographic underpinnings and the molecular genetic methods for such studies are in place (e.g., Rowan et al. 1997; LaJeunesse 2001, 2002; Shick et al. 2002, and references therein), although inte-

grating the results on culturable, diverse symbionts and the physiology of the coral microcosm (e.g., Santos et al. 2001; Rohwer et al. 2002; LaJeunesse et al. 2003) remains daunting.

Our earlier laboratory experiments on *S. pistillata* (Shick et al. 1999) established that UVB radiation is the principal stimulus leading to its accumulating MAAs and that the biosynthesis of MAAs involves the shikimate pathway. The present experiments document the biosynthetic rate-enhancing effect of increasing the UV dose rate. Short-term increases in UV irradiances reaching corals may occur during doldrum conditions of prolonged calm weather, when suspended particles settle and coral reef waters are particularly transparent to UVR, and when UV-induced bleaching may occur (see Shick et al. 1996). Therefore, the ability of corals to enhance their UV defenses quickly when the UV dose rate increases may be important under natural conditions. These experiments also provide details concerning the kinetics by which primary and secondary MAAs accumulate and how they may be metabolically related. The results suggest possible mechanisms by which the accumulation of MAAs is regulated. A model integrating the suncreening function of MAAs and UV-mediated cellular redox changes with the kinetics and stoichiometry of the differential biosynthesis of primary and secondary MAAs is proposed, based on empirical data; the enzymes involved in the biosyntheses remain unidentified, and isolating these is a priority for further mechanistic studies. More systematic studies of genotypically diverse endosymbionts and hosts offer a complementary approach.

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