Trends in Photochemistry & Photobiology

Original Communication

Diurnal changes in the composition of Mycosporine-like Amino Acids (MAA) in *Corallina officinalis*

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ABSTRACT

Thalli of *Corallina officinalis* Linnaeus were harvested at 1-h intervals during low tide in Barrancas Blancas (Patagonia, Argentina) on clear days to examine diurnal changes of the UV-absorbing mycosporine-like amino acids (MAAs) shinorine and palythine. *Corallina officinalis* is found in two growth forms in the mid and the lower intertidal zone, which were collected and analyzed separately. Recovery was measured in algal samples kept in large open containers and sampled every hour overnight. The samples were dried and the amount of MAAs analyzed by means of high performance liquid chromatography (HPLC). The average MAA concentration was 0.17 mg/g (range: 0.04 – 0.34) dry weight (DW) for shinorine and about 1.24 mg/g (0.11 – 5.2) DW for palythine in samples from the low intertidal zone and about 0.18 mg/g DW for shinorine (0.05 – 0.58) and 1.89 mg/g (0.2 – 5.76) DW for palythine in the mid intertidal algae. In the low intertidal strain the concentration of palythine, and not so pronounced for shinorine, significantly increases during the morning and shows a sudden decrease in the afternoon at around 15:00 h. Samples were taken on different days at the same time but had a different preirradiation (due to the rhythm of the tide). In most cases an increase of palythine upon longer preexposure to solar radiation was visible. In contrast, no correlation was found between MAAs and radiation dose. *Corallina* thalli growing in the mid intertidal zone did not show such pronounced daily changes in MAA composition but higher concentrations of shinorine and palythine at the same local time upon longer preexposure to solar radiation.

KEYWORDS: mycosporine-like amino acids, *Corallina officinalis*, red algae, solar radiation

INTRODUCTION

Macroalgae in the intertidal zone have to cope with severe changes in their environmental conditions. The most prominent factors are changes in irradiation, salinity, temperature and water currents. Algae growing in the upper intertidal or even in the supralitoral (above the high water mark) also have to tolerate or prevent desiccation.

Due to pronounced tidal changes of 2 – 3 m in combination with the shallow slope of the rocky abrasion terrace at the experimental site (i.e., Barrancas Blancas) near Playa Unión (Patagonia, Argentina) the intertidal zone (eulitoral) was about 200 – 300 m wide. The calcareous rhodophyte *Corallina officinalis* Linnaeus is found in two distinctly different growth forms (described by Häder *et al.* [1]). The form growing in rock pools in the mid intertidal zone has a feathery, erect, branching thallus with segmented fronds, while the low intertidal thalli near the sublitoral have a characteristic compact appearance.

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Both types of *Corallina officinalis* were shown to be very well adapted to solar radiation [1]. The photosynthetic quantum yield upon full solar exposure decreased about 50 %. The pigments were found to be partially bleached during the day, making resynthesis over night necessary. Additionally, it was shown that the mid intertidal strain shows pronounced nonphotochemical quenching which is completely lacking in the low intertidal strain.

Corallina officinalis possesses mycosporine-like amino acids (MAAs) as sunscreen compounds to protect itself from excessive UV radiation [2]. MAAs are water-soluble molecules with an average molecular weight of around 300 and consist of a cyclohexenone or cyclohexenimine chromophore conjugated with the nitrogen substituent of an amino acid or its imino alcohol, having absorption maxima ranging from 310 to 360 nm [3-5]. MAAs are assumed to be synthesized as a side branch from the shikimate pathway, originating from 3-dehydroquinate (3-DHQ) via cyclohexones (gadusol and the resonance tautomer desoxygadusol) [6]. The final mycosporine-like amino acids are formed by condensing one or more amino acids [7].

MAAs are found in many cyanobacteria, fungi and macroalgae (reviewed in [4]). In addition, MAAs were also found in many invertebrates and vertebrates; the current consensus is that these substances are accumulated by dietary uptake [8-12]. In many cases, MAA synthesis is induced by UV exposure [13-18], while other algae (e.g. *Porphyra umbilicalis*) have a high basic MAA content which does not further increase upon UV irradiation [19]. Less is known about MAA induction. In the cyanobacterium *Chlorogloeopsis* PCC 6912 a putative photoreceptor with pterin as chromophoric group had been proposed to mediate MAA induction [20]. Phenylacetic acid (PAA), a quencher of excited states of flavins and pterins, reduces the induction of MAAs in some cyanobacteria considerably [21], which is another hint for a chromophore involved in MAA induction. Action spectra in other cyanobacteria and *Gyrodinium* also speak for a possible role of photoreceptors. Induction mechanisms in seaweeds are up to know unknown. The photoprotective role of MAAs was demonstrated in several organisms [18,22]. It was shown that cyanobacteria with high MAA concentrations are more resistant to UV-A [23] and that the light-induced bleaching of the cells is considerably slower [24]. Cells of the dinoflagellate *Gyrodinium dorsum*, in which MAAs were previously induced by means of moderate UV exposure maintain motility much longer in strong UV compared to controls with low MAA concentrations [22]. In addition to the UV-screening effect, some MAAs (tested for mycosporine-glycine) show antioxidant effects, hence they very efficiently quench singlet oxygen [25]. In a recent study it was shown that MAA protect the DNA molecule by quenching the excited thymine residues [26].

The different MAAs are analyzed by means of high performance liquid chromatography (HPLC), where they can be characterized by their retention time and the absorption spectrum [8,27]. The combination between chromatography and mass spectroscopy methods has been shown to be very advantageous [28]. The chemical structure of MAA molecules can be analyzed, e.g., by means of nuclear magnetic resonance (NMR) spectroscopy.

The Patagonian *Corallina officinalis* was described to possess exclusively the MAA shinorine [2]. The species investigated during this study were also found to contain palythine. The aim of this work was to examine whether there is a diurnal or light dependent change of the MAA composition, because a possible dynamic of UV-absorbing substances in several rhodophytes was found in a recent study at the same location [29].

MATERIALS AND METHODS

Experimental site

The experiments were performed near Playa Unión, Rawson (65° 3’ W, 43° 19’ S, Patagonia, Argentina) during February and March 2004. Samples were collected in the intertidal zone of Playa Barrancas Blancas just south of the Chubut river.

Test organism and sample treatment

Corallina officinalis, which grows on rocky surfaces were found in two different modifications. At the seaward edge of the gently sloping abrasion terrace there is a pronounced step

of about 1 m downwards. Above this step *Corallina* thalli were found only growing submersed in rock pools, while the thalli below the step covered the floor and walls of crevices just above sea level during the lowest tide where they were exposed to the air but continuously sprayed by the surf. Samples were collected during low tide and dried on the beach and subsequently in the laboratory by means of pulp papers, which were periodically replaced until the samples were completely dry. During the drying procedure the thalli were protected from light, as well as during storage of the dried samples. In order to obtain comparable samples the algae were collected within a restricted area exposed to the sun in the same way with as little variation in their habitat as possible. The samples were collected on sunny days during daylight every hour. As the algae were only accessible for 2 to at maximum 5 hours, six low tides were needed to cover all necessary time points (Fig. 1). For the night samples thalli were harvested at 19:00 h and transferred into open 20-l plastic containers filled with sea water. From these reservoirs samples were taken every hour and dried. During the experimental period several time points were covered in duplicates or in triplicates. The HPLC analysis of the MAAs extracted from dried samples was performed in the Institute of Botany in Erlangen.

Radiation measurements

Solar radiation was constantly monitored during the whole experimental time period using an ELDONET [30] filter radiometer (Real Time Computer, Möhrendorf, Germany) which has three channels for photosynthetically active radiation (PAR, 400-700 nm), ultraviolet-A radiation (UV-A, 315-400 nm) and ultraviolet-B radiation (UV-B, 280-315 nm) that is permanently installed on the roof of the nearby Estación de Fotobiología Playa Unión [31].

The radiation during a number of consecutive experimental days is displayed in Fig .2, together with the collecting periods. During all collecting periods the samples the sky was completely clear.

MAA extraction and HPLC analysis

After careful removal of impurities from the thalli, including sand, small stones and shellfish, 20 mg of dry algae were extracted in 2 mL of 20 % methanol under permanent shaking inside a water bath (2 h at 45° C). To minimize the possible effects of self shading or tissue gradients, only top parts of the thalli have been used for analysis, and for each sample different branches of different

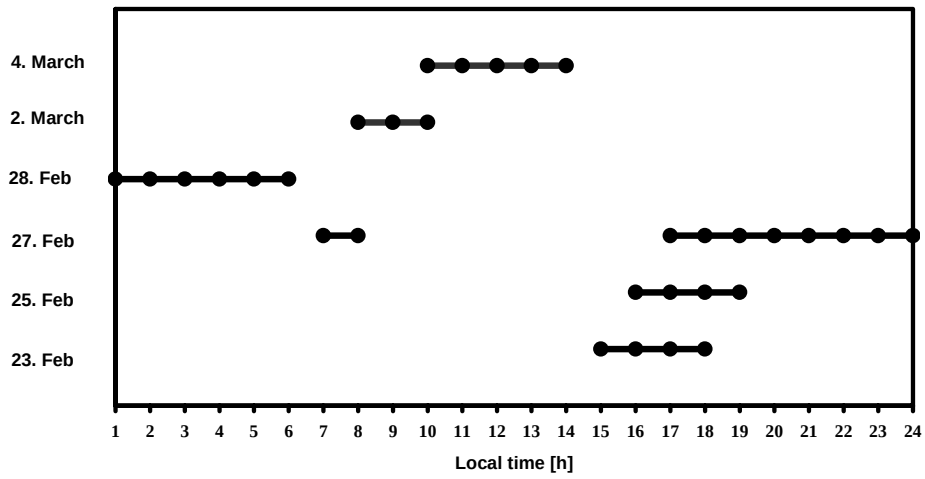

Figure 1. Collection of *Corallina officinalis* samples in the intertidal zone during low tide. The diagram shows the schedule of the sampling times covering every hour with at least one sample. Several time points were sampled in duplicates or in triplicates with different solar preexposure of the algae.

thalli have been used. After centrifugation, 1.5 mL of the supernatant was transferred into a small glass beaker (5 mL) covered with parafilm in which some small holes were pierced with a needle and lyophilized until the solvent was completely evaporated (Lyovac GT2, Leybold, Cologne, Germany). After lyophilization the residue in the beakers was redissolved in 100 % methanol and the samples were subsequently dried again on a heating plate (45 °C) under a gentle air current. After desiccation each residue was dissolved in 1.5 mL of aqua dest. and filtered (Microfilter 0.2 µm, Mikro-Spin, Roth, Karlsruhe, Germany). The samples were analyzed with a Waters HPLC

Figure 2a

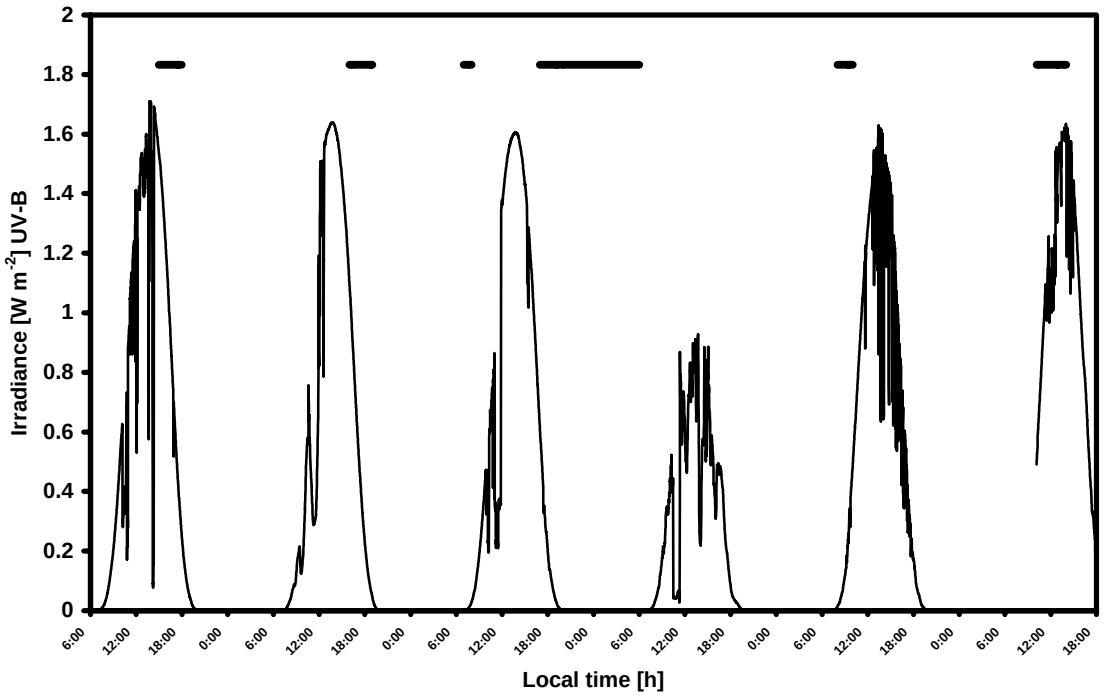

Figure 2b

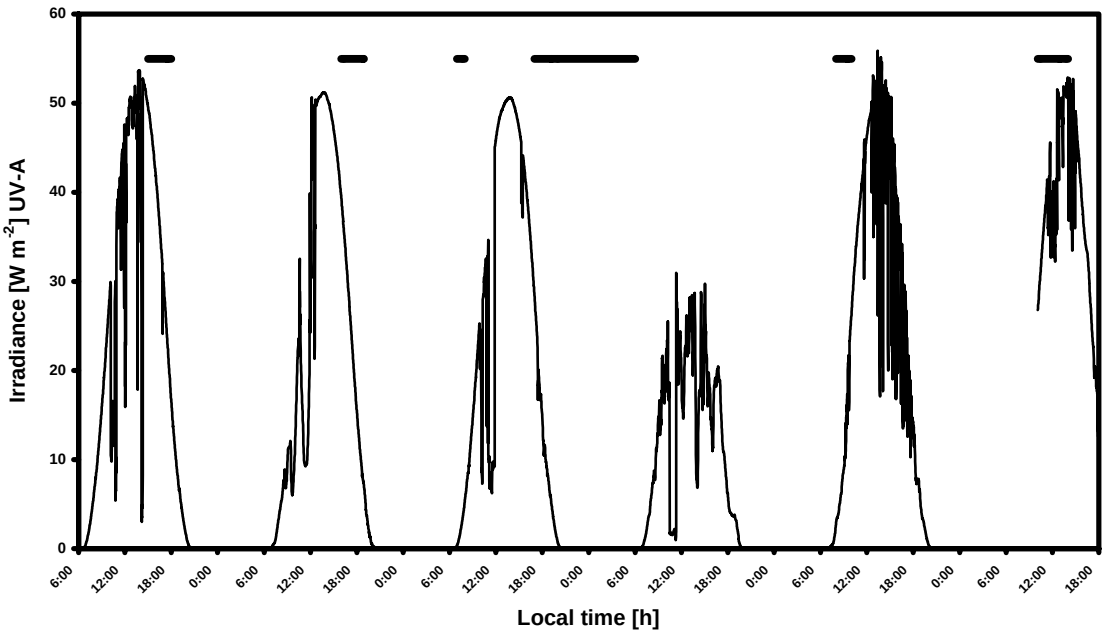

Figure 2c

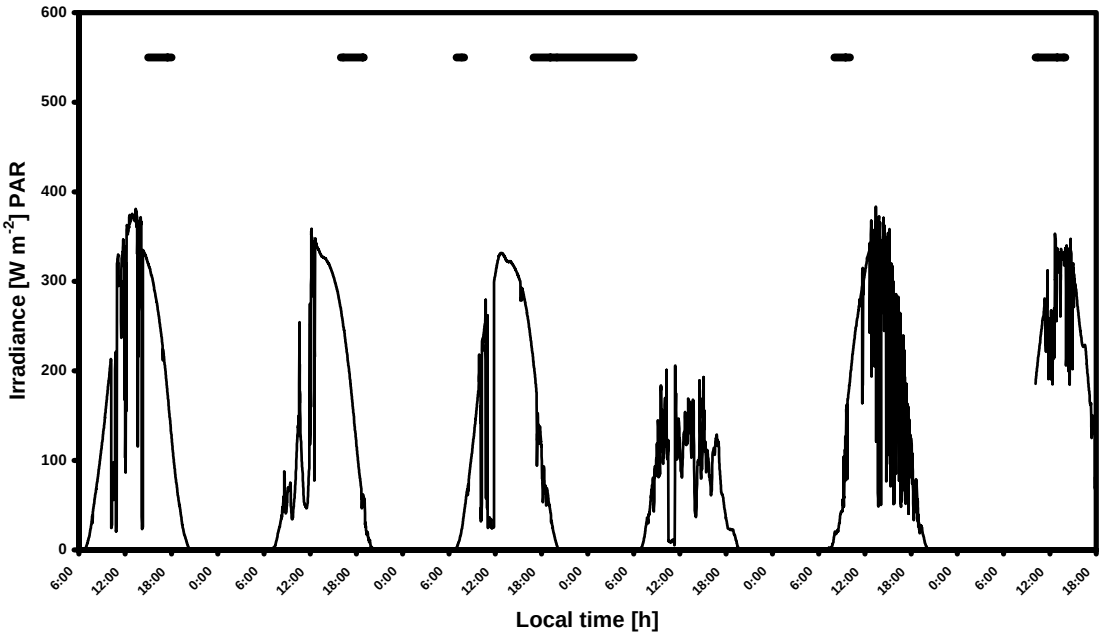

Figure 2. Solar radiation (PAR, UV-A, UV-B) at the experimental site (Playa Unión, Patagonia, Argentina 43°18' S; 65°03' W) during the sample days measured by an ELDONET instrument (European light dosimeter network, Real Time Computer, Möhrendorf, Germany). The time periods of algae collection are indicated as bars. 2.a UV-B, 2.b UV-A, 2.c, PAR irradiation.

(Waters 990), equipped with a LiCrospher RP 18 column. The running solvent was 0.02 % acetic acid in aqua dest. with a flow rate of 1.0 mL/min. The run time was 10 min for each sample. The detected wavelength range was 280 nm - 400 nm. Identification of the MAAs was based on their spectra and their retention times. Initial experiments revealed that *Corallina officinalis* contains two main MAA: shinorine (retention time about 2.25 min, λ_{max}: 334 nm) and palythine (retention time about 4.55 min, λ_{max}: 320 nm). To enable quantitative analysis of the data, calibration was performed with a high concentrated MAA extract of *Corallina officinalis*. About 300 mg of dry algae were extracted by means of the method described above. Aliquots of 200 µl of the extract were injected into the HPLC and the separated MAA fractions were collected with an auto sampler. The concentration of the isolated shinorine or palythine fraction was determined with a spectroradiometer (DU-70, Beckman, Palo Alto, USA), where published molar extinction coefficients [M^{-1} cm^{-1}] were used for the calculation (shinorine: 42000 at 334 nm [32], palythine 36000 at 320 nm [4]). Various defined volumes of the isolates were injected into the HPLC yielding defined absorbance spectra at the detector side of the HPLC. The areas of these peaks were used to plot calibration curves. The method was shown to be very exact (R^2 of calibration plot in shinorine 1 and in palythine 0.997). At a first view, the chromatograms indicate a much higher shinorine concentration compared to palythine but the calibration curves indicate a several fold higher concentration of palythine. This is due to the sensitivity properties of the HPLC sensor.

Evaluation of the HPLC data

The areas and retention times of the absorption peaks of shinorine (at 334 nm) and palythine (at 320 nm) were extracted from the chromatogram, which is provided by the software of the HPLC. By means of the values obtained with the calibration curve the MAA content was calculated in mol/g dry weight or mg/g dry weight, respectively. Because of the variability between

the different samples also the ratios between this two main MAAs was calculated. In spite of a higher UV absorbance, the concentration of shinorine was considerably smaller compared to palythine. For this reason also the ratios of the absorption peak areas were calculated. The values are plotted against the day time of sampling or in the case of duplicate or triplicate samples against the perceived irradiation time before sampling. E.g. the 18:00 h value on 23rd March was the last sampling time during this tide, that means within 4 hours of low tide the algae perceived 3 h of full sunshine before collection. On 25th March there were only two hours and on 27th of March only 1 hour of preirradiation before sampling.

Determination of inorganic matter

Several different *Corallina* samples were carefully cleaned from sand and other impurities and filled into ceramic cups. The samples were dried for about 2 h at 60°C, weighed and subsequently heated in an oven at 1500°C until all organic matter was removed. The remaining skeletons were weighed again in order to calculate the amount of organic material in the thalli.

Statistical analysis

Statistical significance was tested with the student-t test (a level of 0.05 was used in all comparisons) and one-way ANOVA tests.

RESULTS

HPLC analysis revealed that the *Corallina officinalis* samples collected in the present study contained mainly two mycosporine-like amino acids, shinorine and palythine. Although the absorbance measured by the HPLC detector was higher for shinorine the absolute concentration of palythine was about tenfold higher. The amount of MAAs in the low-intertidal samples was lower than in the mid-intertidal strain. The average concentration of all samples was about 0.17 (range 0.04 – 0.34) mg/g DW shinorine and about 1.24 (0.11 – 5.2) mg/g DW palythine in the low-intertidal samples while it was about 0.18 (0.05 – 0.58) mg/g DW shinorine and 1.89 (0.2 – 5.76) mg/g DW palythine in the mid-intertidal algae. Determination of the contents of inorganic material of the thalli revealed that the skeleton of the low-intertidal *Corallina* represents about 45 % w/w of the total dry weight and about 49 % in the mid-intertidal sample. This means that the MAA concentration in the living tissue is about twofold higher than measured.

The low-intertidal *Corallina* algae showed significant diurnal changes in their MAA concentration and in the ratio between the two MAAs. Both MAAs increase during midday (between 11:00 and 15:00 h), which is highly significant for palythine (Figs. 3a and 3b). Shinorine increased from 0.16 mg/g DW to about 0.21 mg/g DW, palythine increased from 0.98 mg/g DW to 2.33 mg/g DW.

The ratio between shinorine and palythine decreases during midday indicating a rise of palythine over shinorine (Fig. 4). Around 14:00 to 15:00 h the MAA concentrations of mainly palythine are considerably lower. During this time solar radiation was still very high. This kinetics was detected in all five independent samples.

In order to test whether the changes in the MAA composition are diurnal or dependent on the radiation, samples were compared which were drawn at the same hour but had received different doses of previous irradiation. The data indicate a decrease in the concentration of shinorine (not significant) and an increase of palythine with increasing exposure time. This was particularly obvious for the samples drawn at 18:00 h, in which the preirradiation was 1, 2 or 3 hours, respectively, on different days, depending on the time of low water (cf. Fig 1). The difference of palythine between 1 (0.77 mg/g DW) and 2 hours (1.21 mg/g DW) of preexposure was significant (one-way ANOVA: $F = 7.19$, $df = 9$, $P < 0.05$).

The samples taken at 17:00 and 19:00 h indicate a similar dependency on the radiation history, but are not statistically significant. No significant correlation between MAA concentration and accumulated radiation dose was detected.

In contrast to the low-intertidal thalli, in which all five measurements show an increase of shinorine and palythine, the mid-intertidal strain did not show such clear diurnal changes in the MAA composition (Figs. 5a, b). The data of the five

independent samples showed a higher variation than those for the low-intertidal *Corallina*. Only three of the measurements of the mid-intertidal thalli indicate a loss of shinorine as well as palythine around noon hours, in two measurements no trend was visible (data not shown).

Figure 3a

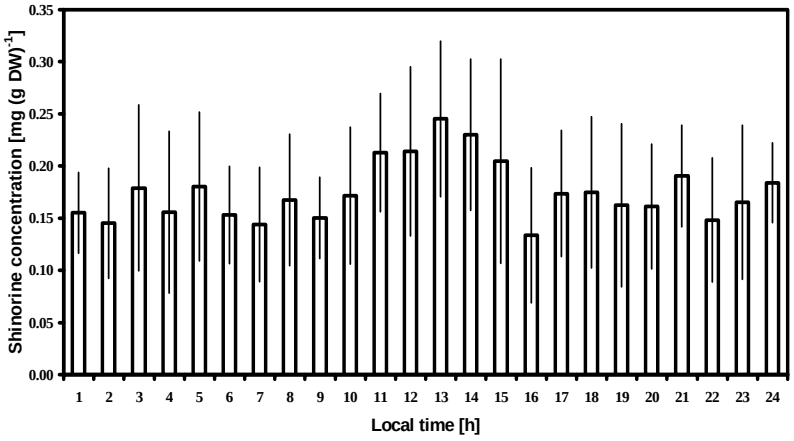

Figure 3b

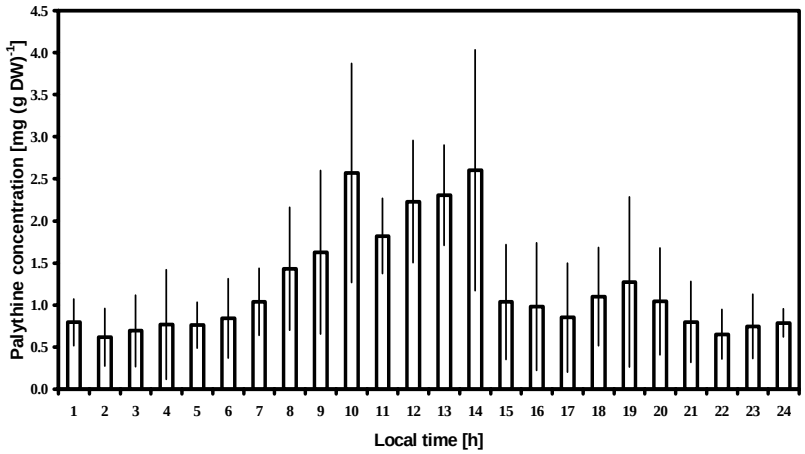

Figure 3. Diurnal changes in concentration (mg/g DW) of the MAAs shinorine (a) and palythine (b) in *Corallina officinalis* growing in the low intertidal. The graph shows the means and standard deviation of all values of five independent measurements of different samples collected at the corresponding time point. Differences between midday samples and morning or evening samples in shinorine are significant (one-way ANOVA: $F = 11.11$, $df = 145$, $P < 0.05$) and highly significant in palythine (one-way ANOVA: $F = 79.98$, $df = 145$, $P < 0.001$).

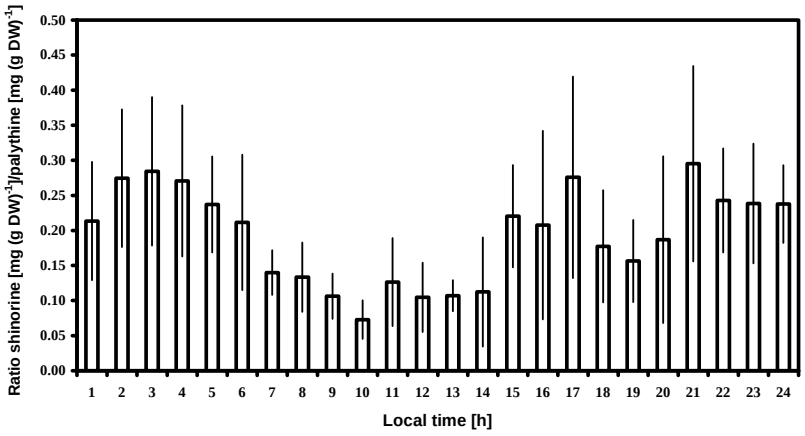

Figure 4. Ratios of the concentrations of shinorine over palythine in low-intertidal *Corallina officinalis*. The graph shows all values and standard deviation of five independent measurements of different samples collected on the corresponding time point. The differences between midday samples and morning or evening samples are significant (cf. Fig. 3, one-way ANOVA of the ratios: 30.41, $df = 145$, $P < 0.001$). Decreasing values indicate an increase in palythine relative to shinorine.

Figure 5a

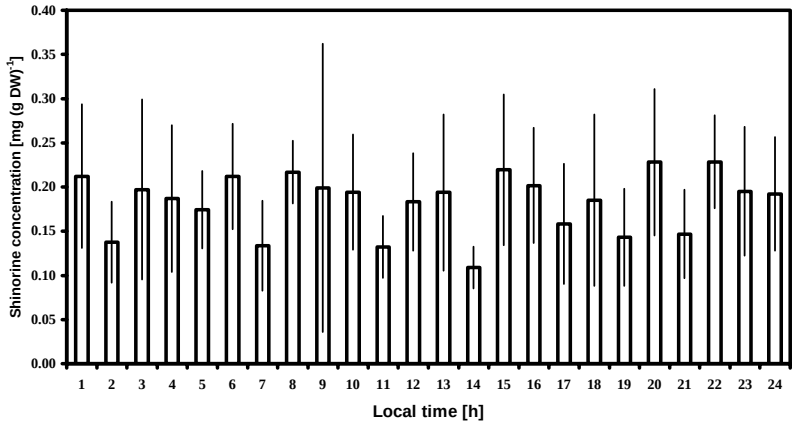

Figure 5b

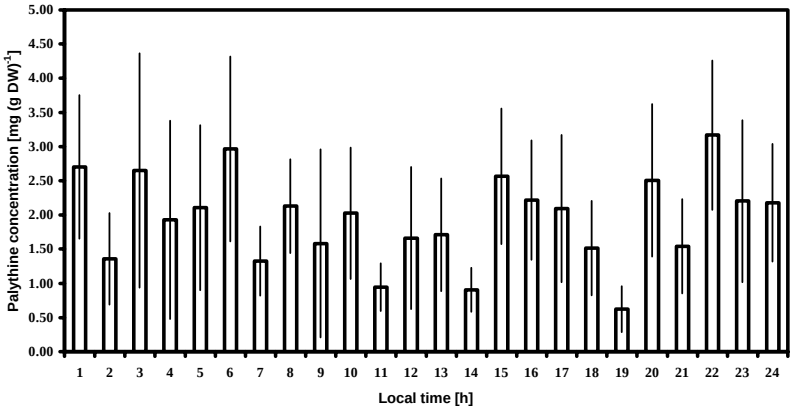

Figure 5. Diurnal changes in concentration (mg/g DW) of the MAAs shinorine (a) and palythine (b) in *Corallina officinalis* growing in the mid-intertidal. The graph shows all values and standard deviation in five independent measurements of different samples collected at the corresponding times. Differences between midday samples and morning or evening samples are only significant for palythine (one-way ANOVA: $F = 4.22$, $df = 185$, $P < 0.05$) but not for shinorine.

Data analysis revealed that only the palythine concentration is significantly lower during the midday hours (10:00 – 14:00), compared to the other samples (one-way ANOVA: $F = 4.22$, $df = 185$, $P < 0.05$). Palythine decreased from about 1.96 mg/g DW to 1.53 mg/g DW. Also the ratio between shinorine and palythine had a different daily pattern than that for the mid-intertidal strain. Around noon the palythine concentration decreased compared to shinorine (Fig 6). In the afternoon (15:00 -17:00 h measurements) the palythine concentration showed a tendency to increase and displayed a very pronounced decrease at 18:00 and 19:00 h in all analyzed samples. The analysis of samples with different preirradiation times showed that upon longer exposure in the 17:00 and 18:00 h measurements the concentration of shinorine and palythine increased with the duration of the previous solar exposure. Without preexposure the average MAA concentration was about 0.12 mg/g DW and 1.7 mg/g DW after two hours exposure to solar radiation the concentration was 0.23 mg/g DW or 3.03 mg/g DW, respectively. The difference

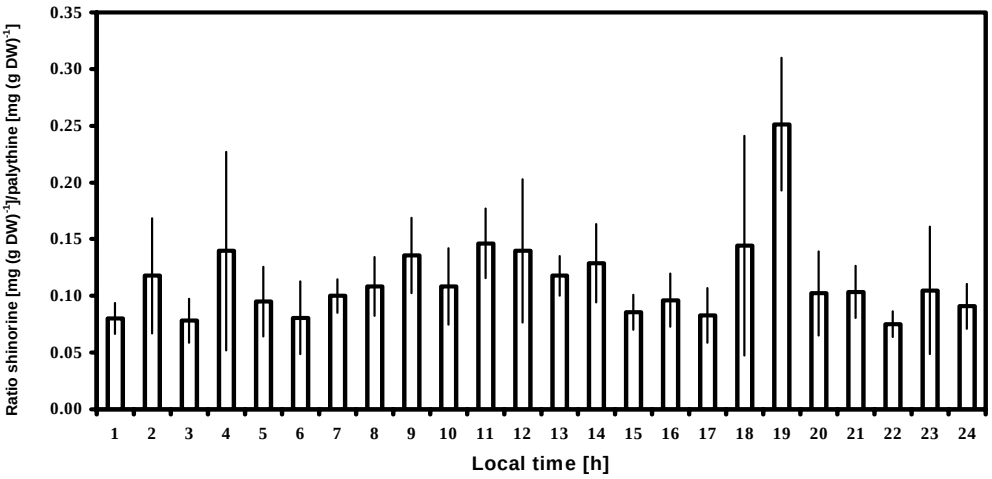

Figure 6. Ratio of the concentration of shinorine over palythine in mid-intertidal *Corallina officinalis*. The graph displays all values and standard deviation of five independent measurements of different samples collected at the corresponding times. Decreasing values indicate an increase of palythine relative to shinorine.

between 0 and 2 hours of preexposure is statistically significant for shinorine (one-way ANOVA: $F = 13.27$, $df = 10$, $P < 0.05$) and palythine (one-way ANOVA: $F = 6.18$, $df = 10$, $P < 0.05$). In the mid-intertidal strain a correlation with the applied dose can be seen. The average MAA content of 1.71 mg/g DW palythine and 0.12 mg/g DW shinorine increased to 3.03 mg/g DW and 0.22 mg/g DW, respectively after they perceived a total dose of 137 J m^{-2} UV-B (the simultaneous perceived UV-A dose was 4906 J m^{-2} and PAR 33270 J m^{-2}). The increase in shinorine is significant (one-way ANOVA: $F = 13.27$, $df = 10$, $P < 0.05$) and palythine (one-way ANOVA: $F = 6.1814528$, $df = 10$, $P < 0.05$).

DISCUSSION

Many cyanobacteria, phytoplankton and higher algae produce photoprotective substances (e.g. mycosporine-like amino acids or scytonemin [33]) in response to UV radiation. Adaptation of the MAA concentration to the UV radiation at the growth site has already been shown in a variety of studies. The amount of MAAs in algae decreases with increasing depth [34] and with increasing geographical latitudes [35]. Seasonal changes in MAA composition and concentrations have been reported for *Bangia atropurpurea*, *Chondrus crispus* and *Mastocarpus stellatus* [18,36] as well as in benthic phytoplankton [37] in which the concentrations of UV-absorbing substances are much higher during midsummer compared to spring.

In spite of the variability of the data (reasons for this will be discussed later) this study strongly indicates that solar exposure increases the MAA concentration in *Corallina officinalis* and, more important, that the concentration of mycosporine-like amino acids in *Corallina officinalis* (at least the growth form of the low intertidal) shows circadian changes. MAAs increase in the lower intertidal strain and possibly decrease in the mid-intertidal strain around local noon. The MAA increase of the lower intertidal strain, living at the border to the sublitoral, indicates an adaptation mechanism most likely in order to protect itself from excessive solar radiation. While the data for the low intertidal strain of *Corallina officinalis* show significant diurnal changes in MAA concentration and composition, the mid intertidal strain did not show such clear changes in the intracellular MAA composition (with exception of palythine). The data are more scattered, which is most likely due to a higher heterogeneity of the samples. Among others, self shading within the relatively large thalli and probably varied solar exposure inside the rock pools may have caused uneven irradiation patterns and hereby, under the

assumption that MAA synthesis is also triggered by solar radiation, leads to heterogeneous MAA induction in the samples. In contrast, the compact low intertidal thalli have been more homogeneously exposed to the sun so that the environmental effect on the algae was more consistent. This is most likely the reason for a lower variability between the samples. Three of the measurements of the mid intertidal thalli indicate a loss of shinorine as well as palythine around noon hours, while all low-intertidal thalli show a pronounced increase during this time. In order to work out possible trends in the mid-intertidal strain, more replicates are needed to overcome the problem of higher heterogeneity.

Although the samples were taken on different days, with different preexposure of the algae to solar radiation the data for the lower intertidal strain are highly significant. While in some cases the algae were collected directly after the onset of the low tide the algae were exposed to solar radiation up to 4 h of solar radiation. As the data indicate that preexposure to solar radiation affects the MAA concentration this factor may also have influenced the results.

The increase of MAAs, mainly palythine, in the lower intertidal strain of more than 1 mg per gram dry weight is remarkably high on this short time scale. Studies in cyanobacteria [14,38], phyto-plankton [39] and higher algae [40-42] have shown that MAA synthesis in order to reach high intracellular MAA concentrations is usually a long lasting process, which requires hours or even days especially in macroalgae. To the best of our knowledge this is the first time that circadian MAA variations were directly documented in macroalgae. Up to now short-term changes of MAAs have only been reported in the dinoflagellates *Alexandrum excavatum* [43] and *Scrippsiella sweeneya* [44]. In synchronized cultures (12 h light and 12 h dark) of *Scrippsiella sweeneya* under high light conditions the cells showed pronounced diurnal patterns of MAA concentration. The total amount of intracellular MAAs increased during about the first 6 h of light exposure and decreased again towards the end of the light period and in the dark period. In this species MAA production is most likely not only triggered by external light, but also by internal factors due to the daily cycle, because MAA were only produced at the first half of the light period and also similar but much smaller changes in MAA concentration was visible in low-light controls. Of course phytoplankton can not easily be compared with higher algae and up to now such fast synthesis of MAAs in higher algae was not reported. But an earlier study on four rhodophytes (among others also on *Corallina officinalis* from the sublitoral) at the some location indicates that UV-absorbing substances indeed show daily changes [29]. While in *Ceramium* a pronounced increase of UV-absorbing substances was found during the day (only upon UV radiation), *Corallina* showed only a marginal increase in the absorbance in the UV range. This study, which gave rise to the work presented in this work indicated that UV-absorbing substances can increase or decrease in the range of 100 % within several hours.

Although the data indicate a strong influence of solar radiation on MAA synthesis in *Corallina officinalis* it is still an open question, whether endogenous circadian or circatidal rhythms are also involved in this process. Such an endogenous effect might be responsible for the considerably fast decline (more than 1 mg/g DW) of the noon MAA concentrations. Degradation by UV as a reason for the decline of the MAAs is unlikely, since MAAs were found to be very stable under intensive UV radiation and other environmental factors like heat and extreme pH [45]. Even 6 hours of excessive UV-B irradiation (about 16 W/m^2) did not affect MAAs (in vitro experiments). But in vivo it might be possible that MAAs are induced as long as the organism experiences the light stress but once the stress starts to fade out there is a decrease in the amount of MAAs and the energy that was being used to synthesize the MAAs may be now diverted into the photosynthetic process. Similar results have been found in cyanobacteria, in which a circadian induction of MAAs was obvious [15]. Normally, fast induction of MAAs does not occur in seaweeds but it also depends on the habitats where it has been studied. For example, in seaweeds that are growing fully exposed to sunlight the MAAs may already have reached their maximum concentrations. But if they are growing in shaded

areas and are then suddenly exposed to full sunlight they may show a sudden increase in its MAAs content followed by a decrease in their concentration during decreasing light stress.

Future studies on these *Corallina officinalis* strains may be useful in order to learn more about the possible involvement of endogenous circadian or circatidal factors in MAA synthesis and MAA degradation.

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