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Stress metabolite pattern in the eulittoral red alga *Pyropia plicata* (Bangiales) in New Zealand – mycosporine-like amino acids and heterosides

Nora Diehl[a,d,*], Dirk Michalik[b], Giuseppe C. Zuccarello[c], Ulf Karsten[a]

[a] Applied Ecology and Phycology, Institute of Biological Sciences, University of Rostock, Rostock 18059, Germany
[b] Organic Chemistry, Institute of Chemistry, Leibniz Institute for Catalysis, University of Rostock, Rostock 18059, Germany
[c] School of Biological Sciences, Victoria University of Wellington, Wellington 6140, New Zealand
[d] Marine Botany, Institute of Biology and Chemistry, University of Bremen, Bremen 28359, Germany

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ABSTRACT

Intertidal rocky shore ecosystems are affected by steep environmental gradients such as fluctuating solar irradiation and salinity along the marine-terrestrial interface. The eulittoral red alga *Pyropia plicata* (Bangiales) is endemic and abundant to coastal regions of New Zealand and almost unstudied in terms of ecophysiological performance under radiation and salinity stress. Therefore, the acclimation potential of this species against enhanced ultraviolet radiation (UVR) and osmotic stress was evaluated in a combination of field and laboratory experiments with an emphasis on stress metabolite concentrations (UV-sunscreens, organic osmolytes). Samples of *P. plicata* were collected at the same site in the intertidal zone of Wellington, New Zealand over three seasons (April–November 2016) and used in independent UV and salt stress experiments under controlled conditions. The mycosporine-like amino acids (MAA) shinorine and porphyra-334 were the quantitatively dominant UV-sunscreen compounds, and the total concentrations varied over the year between 5 and 14 mg g^{-1} dry weight (DW), but neither UVR nor PAR had a significant impact on the total or individual MAA concentrations. A UV radiation stress experiment was conducted, but the total MAA concentrations of 6–8 mg g^{-1} DW did not change, neither did the contents of shinorine (~ 3 mg g^{-1} DW) nor that of porphyra-334 (4–5 mg g^{-1} DW). This suggests, that *P. plicata* has sufficiently high UV-sunscreen amounts and hence does not respond to changes in UV radiation. *Pyropia plicata* contained three heterosides (floridoside, D- and L-isofloridoside), which act as organic osmolytes. Seasonally the total concentrations of these compounds varied between 203 and 1226 mmol kg^{-1} DW, with L-isofloridoside quantitatively dominating all samples. A salt stress experiment showed an increase in the total heteroside concentrations in *P. plicata* with increasing salinities. However, floridoside was the most up-regulated heteroside under hypersaline conditions indicating its key role in osmotic acclimation. Our data indicate that *P. plicata* always contains various stress metabolites in consistently high concentrations which mitigate against environmental changes typical of the intertidal zone of New Zealand.

1. Introduction

Benthic habitat-forming macroalgae act as ecosystem-engineers along the coasts of rocky shores (Schiel, 2004). Intertidal rocky shore ecosystems are affected by enhanced solar irradiation and strongly impacted by steep environmental gradients along the marine-terrestrial interface with increasing abiotic stress towards the higher shore level (Thompson et al., 2002; Bischof et al., 2006). Along with mechanical stress caused by waves, intertidal species such as macroalgae are exposed to regular and extreme changes in temperature, desiccation and salinity (Davison and Pearson, 1996; Karsten and Wiencke, 1999; Karsten and West, 2000; Bischof et al., 2006).

During high tide, intertidal organisms experience aquatic conditions, while during low tide they must tolerate almost terrestrial conditions. The intertidal salinity range is complicated by tidal patterns, geographic region, adjacent estuaries and meteorological conditions (Kirst, 1989). During a sunny day at low tide, water evaporates leading to hypersaline stress, while precipitation during low tide results in hyposaline stress. However, as long as intertidal algae are submerged they typically experience rather mild salinity changes ranging from S_A 0 to full seawater strength (Kirst, 1989). In contrast, intertidal algae exposed to aeroterrestrial conditions have to cope with much higher salinities, and sometimes even salt crystals appear on the thallus during desiccation (Karsten, 2012, and references therein). Desiccation at low

* Corresponding author at: Marine Botany, Institute of Biology and Chemistry, University of Bremen, Bremen 28359, Germany
E-mail address: ndiehl@uni-bremen.de (N. Diehl).

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tide can be intensified by solar radiation and high air temperature.

In order to compensate for environmental stressors, such as salinity, algae developed different acclimation mechanisms (*e.g.*, Eggert et al., 2007; Nitschke et al., 2010, 2014; Martinez-Garcia and van der Maarel, 2016). For instance, they synthesize and accumulate organic osmolytes, that also act as compatible solutes, to conserve intracellular homeostasis and maintain cellular functions (Pade et al., 2015). Intertidal seaweeds typically can tolerate salinities from S_A 10 to 100, while subtidal species are less tolerant (Hurd et al., 2014). In red algae, heterosides used in hypersaline stress (Karsten et al., 1996), include: 1) floridoside (α-D-galactopyranosyl-(1-2)-glycerol), and its isomers 2) D-isofloridoside (α-D-galactopyranosyl-(1-1)-D-glycerol), and 3) L-isofloridoside (α-D-galactopyranosyl-(1-1)-L-glycerol) (Karsten et al., 1993; Karsten et al., 2005).

High irradiance, including ultraviolet radiation (UVR), is a further stress factor that phototrophic organisms encounter in the field. The intensity of UVR is dependent on latitude and seasons. Furthermore, diurnal solar cycles, as well as clouds and atmospheric particles have been identified as important factors determining the intensity of UVR. An increase in the emission of man-made halogenated volatile substances over the past decades has resulted in a thinning of the ozone layer in polar regions, particularly in the Southern Hemisphere (Antarctic Ozone Hole), and in this context resulted in an increase in biologically harmful UVR reaching the earth's surface (Bai et al., 2016; Franklin and Forster, 1997).

The effect of UVR on intertidal organisms has been studied for decades (*e.g.*, Cubillos et al., 2015; Franklin and Forster, 1997; Madronich, 1993; Schweikert et al., 2011, 2014; World Meteorological Organization, 2014). Particularly increases in UV-B radiation (280–315 nm) may reduce biomass production and cause changes in species composition and zonation of macroalgal communities (Zacher et al., 2007). Macroalgae growing in habitats with low irradiance, *e.g.* in the deep sublittoral, are usually most sensitive to UVR. When such organisms are exposed to higher UVR than encountered in their natural habitats, harmful effects of UV-B were detected (Hanelt and Roleda, 2009).

Macroalgae exhibit different protective mechanisms against excessive solar radiation. For instance, many species live in the shade of canopy algae and/or at depths to avoid strong irradiance, accomplish dynamic photoinhibition or regulate their polyamine metabolism and antioxidant biosynthesis (Bischof et al., 2006; Cruces et al., 2017; Schweikert et al., 2011, 2014; Yakovleva and Titlyanov, 2001). Red algae often synthesize and accumulate photoprotective secondary metabolites, like mycosporine-like amino acids (MAAs) against UVR (Karsten, 2008 and references therein). MAAs are water-soluble, low molecular weight compounds, which function as intracellular UV-sunscreens (Karsten and West, 2000; Karsten, 2008). These compounds absorb mainly UV-A (315–400 nm) and UV-B radiation (280–315 nm) (Karentz et al., 1991) and emit the absorbed radiation energy as heat without generating photochemical reactions (Bandaranayake, 1998; Cockell and Knowland, 1999; Karsten, 2008). So far about 35 different MAAs have been identified, which differ slightly in their chemical structure, and occur in different qualitative and quantitative compositions in organisms (Bischof et al., 2006; Hartmann et al., 2015). Two of the most common MAAs in macroalgae are porphyra-334 and shinorine (Hoyer et al., 2002), and their concentrations often correlate with the degree of exposure to solar radiation (Karsten et al., 1998).

Due to its location on the Southern Hemisphere, New Zealand is occasionally affected by ozone depletion and consequently enhanced UVR over the course of a year. The UV Index (UVI) has been introduced as a useful quantitative measure to describe enhanced UVR conditions. The UVI depends on various atmospheric conditions, and increases by about 1% for every 1% decrease in stratospheric ozone. Due to differences in ozone concentration, sun-earth separation and air pollution, the peak UVI in New Zealand is approximately 40% higher compared to similar latitudes in the Northern Hemisphere (McKenzie et al., 1999; McKenzie et al., 2006). Although several studies have considered the influences of UVR on New Zealand's marine environment (*e.g.*, Cubillos et al., 2015; Riemer et al., 2007), still, very little is known about UVR effects on intertidal macroalgae in New Zealand, and in particularly the seasonal variations of MAAs and heterosides. Schweikert et al. (2011, 2014) investigated the effect of enhanced UV-B radiation on the polyamine metabolism in intertidal macroalgae in New Zealand, while Lamare et al. (2004) studied the variation of MAAs in numerous marine species along a gradient of UVR.*Pyropia plicata* W.A. Nelson, an endemic red alga, is one of the most abundant Bangiales species in New Zealand (Nelson, 2013; Sutherland et al., 2011). This red alga grows in the upper eulittoral on rocky coasts, hence it is regularly exposed to solar radiation and other intertidal stressors for most of the day. It is only submerged for short periods every day and can completely desiccate at low tide with a cellular water loss of 85–90% (Blouin et al., 2011). Since *P. plicata* is so abundant in the upper eulittoral zone of New Zealand it should be well adapted to the prevailing stressful conditions. In the present study, the protective mechanisms of *P. plicata* against UVR and osmotic stress were separately investigated for the first time. The accumulation of two important groups of stress metabolites – MAAs and heterosides – were qualitatively and quantitatively assessed seasonally in field samples and in laboratory experiments, respectively. MAA concentrations and composition were expected to change and increase with increasing UVR (Karsten et al., 1998; Roleda et al., 2012).

Concentration of floridoside was also expected to increase in *P. plicata* under hyperosmotic stress (Reed et al., 1980; Reed, 1985), while the concentrations of the isomers D- and L-isofloridoside were expected to remain unchanged (Karsten and West, 2000).

2. Material and methods

2.1. Sampling

All *Pyropia plicata* samples were collected at Moa Point in Wellington, New Zealand (41°20′31.6″S 174°48′35.4″E) (Fig. 1) in the upper eulittoral zone during low tide.

2.1.1. Seasonal in situ measurements

The samples used for the seasonal measurements of the MAA and heteroside concentrations were collected every two weeks, always from the same rocks to ensure comparable abiotic conditions, between April–November 2016, which corresponds to the autumn, winter and spring austral seasons. The rocks were facing north and the samples exposed directly to the sun. The blades were checked for pathogens, mechanically cleaned under sterile seawater, oven-dried at 40–50 °C and stored in plastic bags in the dark until the analyses. The same blades were used for both, MAA and heteroside analyses.

2.1.2. Laboratory experiments

The samples for the two laboratory experiments were collected from nearby flat rocks to ensure sufficient biomass and an average sun exposure. The salt stress experiment (heteroside analysis), was conducted in June, the UV stress experiment (MAA analysis) in September 2016. Care was taken that the blades were free of visible pathogens/epiphytes and of the same size. The samples were transported to the laboratory and directly processed for the stress experiments. The respective *in situ* control samples were processed the same way, then oven-dried at 40–50 °C and stored in plastic bags in the dark until the biochemical analyses.

2.1.3. Abiotic data

The mean daily ultraviolet index (UVI) is a standard measurement of erythemal UV intensity (McKenzie et al., 2004; NIWA, 2017). Hence, the UVI was a good parameter for the solar radiation stress, which the algae were exposed to over the course of the year. In parallel, also the global radiation and ozone data were evaluated. The photosynthetically

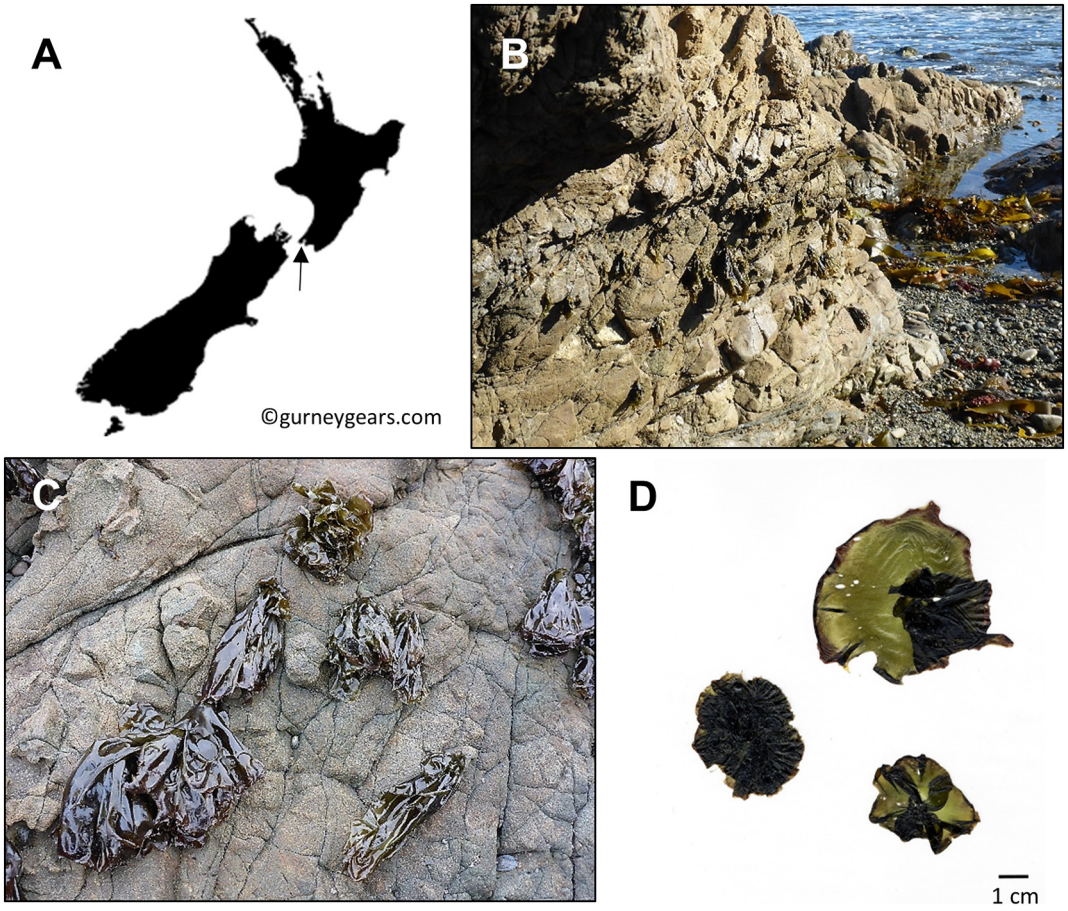

Fig. 1. A, B: Location of Moa Point, Wellington (41°20′31.6"S 174°48′35.4″E). Arrow in the map shows the location in New Zealand. C, D: *Pyropia plicata* in the field and a herbarium specimen.

active radiation (PAR, 400–700 nm) data were inferred from global radiation data and the UV-B radiation (280–315 nm) data from the UVI. All abiotic data were provided by New Zealand Institute for Water and Atmosphere (NIWA) and downloaded from the National Climate Database "CliFlo" (CliFlo, 2017). The water temperature data were downloaded from Greater Wellington Regional Council (GWRC, 2017).

2.2. UV stress experiment

Pyropia plicata blades of the same size were mechanically cleaned with sterile seawater and maintained in plastic containers filled with sterile seawater (S_A 36) at constant temperature (15 °C; Thermo Electron Corporation Haake®, EK20 and John Morris Scientific Ltd., Julabo ED) and under a 12/12 h-light/dark cycle for five days. PAR was provided as high as was possible under artificial conditions at approximately 180 µmol photons $m^{-2} s^{-1}$, provided by four Cool White fluorescent bulbs (Philips, Alto Cool White, TLD 36 W/840). UV-A was provided by a UV-A bulb (Philips, TL 40 W/03 RS) and UV-B (*ca.* 0.9 W m^{-2}) was added using a UV-B bulb (Philips, 40 W, Ultraviolet-B, TL 40 W/12 RS). As the UV-B bulb also produces UV-C (100–280 nm), the bulb was wrapped with polyvinyl chloride (PVC) film (0.13 mm, Graley Plastics & Laser Cuttings, Petone, New Zealand) to remove UV-C. The radiation levels were measured using a radiation sensor (Skye Inc., SpectroSense2+; PAR: 4-Channel Sensor, UV-A: UV-A Sensor [315–400 nm], UV-B: UV-B Sensor [280–315 nm]). The containers (n = 4) were each covered with cut-off filters to establish different radiation conditions: 1) A PVC filter provided the PAR + UV radiation (UVR) conditions; 2) Polycarbonate (Mulford Plastics, Seaview, New Zealand) film were used to provide PAR without UVR, as it cuts off all wavelengths < 400 nm.

A comparative PAR + UV-A (no UV-B) treatment was set up (filter: 0.13 mm, Mulford Plastics, Petone, New Zealand) and the constant background UV-A was measured, but the intensity was very low and not comparable to natural conditions (~ 40 W m^{-2}). The natural UV-A:UV-B ratio is 10–15:1. As the highest attained UV-A radiation in this experiment was only 1.3 W m^{-2} and the UV-B radiation was about the same value (1.7 W m^{-2} without filters; 1:1 UV-A:UV-B ratio), UV-A effects can be neglected (Diehl, unpublished data) and hence were excluded from this study. The accurate radiation values are given in the Supplementary Table 1.

Furthermore, different PAR levels (30 and 90 µmol photons $m^{-2} s^{-1}$) were tested, in combination with the same UV values, but did not result in any effect on MAA concentration and hence were not further considered (Diehl, unpublished data).

Before and after the UV stress experiment, the *in vivo* chlorophyll *a* fluorescence (F_v'/F_m') of each sample was measured with an underwater pulse-amplitude-modulated fluorometer (Diving PAM, Walz GmbH Mess- und Regeltechnik, Effeltrich, Germany). After the experiment the samples were first oven-dried at 40–50 °C, and then stored in small plastic bags in the dark until the MAA analysis.

2.3. Salt stress experiment

The collected samples were cleaned with sterile seawater and maintained in plastic containers filled with sterile seawater of eight different salinities: S_A 1, 5, 10, 15, 20, 36, 45 and 60. Salinities below 36 were produced by dilution with distilled water and higher salinities by adding sea salt (Red Sea Aquatics Ltd. (Red Sea U.S.A), Houston,

Texas, USA). As only correlations between light/temperature and heteroside concentration and not between nutrients and heterosides could be detected to date, nutrient concentrations were disregarded (Karsten and West, 2000). *Pyropia plicata* blades were kept in these containers ($n = 3$) for six days at the same temperature (~ 16 °C) and a medium artificial photon flux of 70 µmol photons $m^{-2} s^{-1}$, 12/12 h-light/dark cycle (Philips, Alto Cool White, TLD 36 W/840). The *in vivo* chlorophyll *a* fluorescence (F_v'/F_m') of each sample was measured with a Diving PAM (Walz GmbH Mess- und Regeltechnik, Effeltrich, Germany) before and after the salt stress experiment. All samples were oven-dried at 40–50 °C, and stored in small plastic bags in the dark until the heteroside analysis.

The salt stress experiment does not represent fully realistic natural conditions as the algal samples were constantly submerged. *P. plicata* will not be exposed to S_A 60 under *in situ* submerged conditions. However, at low tide and sunny/windy conditions salt crystals could occasionally be observed on *Pyropia* (Diehl, unpublished data), and hence the experimental design simulates hypersaline salt stress which under *in situ* conditions includes also desiccation. S_A 60 in the medium represents comparable osmotic stress in *P. plicata* under desiccation. The salinity range was chosen based on former salt stress studies with other intertidal algae to allow comparison with the literature, and at the same time to apply potentially extreme stress conditions to *P. plicata* (*e.g.*, Bäck et al., 1992; Karsten, 2007; Karsten et al., 1991, 1993).

2.4. Mycosporine-like amino acid (MAA) analysis

For the extraction of MAAs, 1 mL of aqueous methanol (25%) was added to approx. 15 mg of dried sample tissue in a screw-capped vial, vortexed and left in a water bath at 45 °C for 3–4 h. The extract was vortexed at regular intervals to keep the sample well dispersed. After 5 min of centrifugation (5000 rpm), 800 µL of the supernatant was transferred into new vials and evaporated until dryness (Savant Speed Vac® SPD111V; Savant™, Thermo Scientific ™ Savant, Vacuubrand GmbH & Co. KG, Wertheim, Germany). The dried pellet was re-dissolved in 800 µL of HPLC-water, vortexed for 30 s, and centrifuged for an additional 5 min (13,000 rpm). The supernatant was directly analyzed using High Performance Liquid Chromatography (HPLC, Agilent Technologies, 1100 Series, Santa Clara, California, USA), separated by a Phenomenex Synergi 4 µ fusion RP-column (C18, 4 µm, 250 × 3mm I.D., Aschaffenburg, Germany), protected by a guard cartridge of the same column material (Phenomenex, Fusion-RP 4 × 3.0 mm I.D., Aschaffenburg, Germany). The mobile phase was 2.5% aqueous methanol (*v/v*) plus 0.1% acetic acid (*v/v*, $C_2H_4O_2$) in water, run at a flow-rate of 0.5 mL min^{-1}, 150 bar, 25 °C. The identification of the MAAs porphyra-334 and shinorine was performed by absorption spectra and their retention time. Standards were extracted from the marine red macroalgae *Porphyra umbilicalis* (Linnaeus) Kützing (porphyra-334) and *Mastocarpus stellatus* (Stackhouse) Guiry (shinorine).

2.5. Heterosides analyses

Oven-dried samples were quantitatively analyzed by adding 20–25 mg of dried tissue to 1 mL of aqueous ethanol (70%) in a screw-capped vial and incubated in a water bath at 70 °C for 3–4 h to extract the heterosides. The vial was vortexed occasionally to keep samples dispersed. After 5 min centrifugation (13,000 rpm), 800 µL of the supernatant were transferred into a new vial and evaporated to dryness (Savant Speed Vac®). 800 µL of HPLC-water was added to the dried pellet and vortexed for 30 s, the vials were treated in an ultrasonic bath for 10–30 s and vortexed for another 20 s to re-dissolve the pellet completely. They were then centrifuged for 5 min (13,000 rpm). The supernatant was transferred into HPLC-vials. The heterosides were separated in a HPLC using a Phenomenex Rezex ROA-Organic acid-column (300 × 7.8 mm, 8 µm, Aschaffenburg, Germany), protected by a guard cartridge (Phenomenex, Carbo-H 4 × 3.00 mm I.D., Aschaffenburg, Germany). The mobile phase was 0.5 mmol sulfuric acid (H_2SO_4), run at a flow-rate of 0.4 mL min^{-1}, 5–40 bar, 75 °C. For calibration 0.5, 1.0, 2.5, 5.0 and 10.0 mmol standards were measured. Although this method provided the total concentration of all heterosides, it did not separate the three compounds of interest. To distinguish floridoside and both D − /L-isofloridosides, and to calculate the percentage contribution of each compound to the total concentration ^{13}C NMR spectroscopy (^{13}C nuclear magnetic resonance spectroscopy) was undertaken. 5 mL aqueous ethanol (70%) were added to 300 mg of dried algal tissue, heated in a water bath at 70 °C for 3–4 h and shaken frequently. After centrifugation for 5 min (6000 rpm), 4 mL of the extract were transferred into a new tube and dried with nitrogen (N_2). The sample was re-dissolved in 0.5 mL D_2O (99.9%) for ^{13}C NMR spectroscopy. The NMR spectra were recorded by a Bruker spectrometer (^1H: 500.13 MHz; ^{13}C: 125.8 MHz, AVANCE 500 spectrometer, Bruker BioSpin GmbH, Rheinstetten, Germany). Chemical shifts δ are given in ppm relative to the signal for internal tetramethylsilane (TMS, $\delta = 0$). The calibration of spectra was carried out externally, using the signals of acetone (5%, δ (^1H) = 2.25 ppm, δ (^{13}C) = 30.3 ppm) in D_2O. Samples were run in 5 mm diameter tubes at 300 K. For ^{13}C NMR spectra a sweep width of 30,000 Hz and a number of 10,000 scans were used.

2.6. Statistical analysis

The stress experiment data were tested for homogeneity by the Levene's test and transformed, if necessary. A one-way ANOVA was performed afterwards, using "radiation" (*in situ*/PAR/PAR + UV treatments) and "salinity" (*in situ*/salinity treatments) as independent and "MAAs" (total/shinorine/porphyra-334 concentration), "heterosides" (total/floridoside/D − /L-isofloridoside concentration) or "F_v'/F_m'" as dependent variables. If the results showed significant differences ($p < 0.05$), a *post hoc* Tukey's honest significant difference test was applied ($p < 0.05$). A test for normal distribution was not conducted as it is not expedient with small sample and replicate quantities (Underwood, 1997). If the data were not homogeneous, a nonparametric test with independent samples (Kruskal-Wallis-Test) was performed ($p < 0.05$). The statistical analyses were run using SPSS Statistics 20 (IBM, Armonk, NY, USA). To analyze the field and abiotic data correlation and trend analyses were run using Excel 2016 (Windows; Microsoft Corporation, Redmond, WA, USA).

3. Results

3.1. Abiotic conditions 2016

Strong seasonal changes in the abiotic conditions in Wellington occurred over the course of 2016 (Supplementary Figs. 1–2). The highest radiation of PAR and UVR, as well as the highest UVI values, were measured in austral summer (December–February), while it decreased towards winter followed by increasing conditions in spring again. In 2016 the ozone concentration was relatively high over Wellington and did not decline below 260 Dobson units (DU, $2.69 \cdot 10^{16}$ molecule cm^{-2}) during summer. From the end of winter until mid-spring, the ozone concentration increased to almost 350 DU. Apart from September (80 h) sunshine hours were over 100 h per month. Rainfall was lowest in summer and July. The mean monthly water temperature varied between ~ 11 and 19 °C, while the air temperature varied between ~ 9 and 20 °C.

3.2. Seasonal in situ measurements (April–November 2016)

3.2.1. MAAs

Porphyra-334 and shinorine were identified as the main MAAs in *P. plicata*, and both occurred in almost equimolar amounts. Their total concentrations varied between 8.4 and 13.7 mg g^{-1} DW (dry weight)

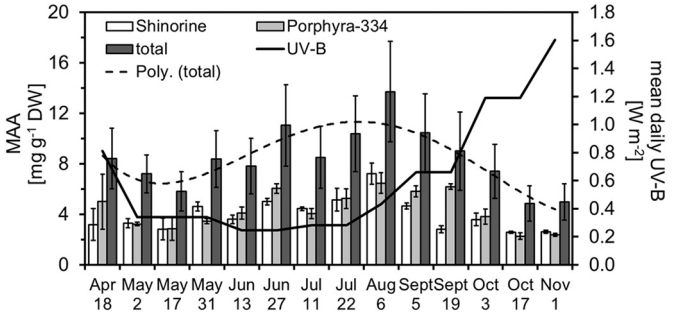

Fig. 2. Seasonal changes in mycosporine-like amino acid (MAA) concentrations of *Pyropia plicata* from Moa Point, Wellington, New Zealand in 2016. Values are means ± SD (n = 3). Columns shows the MAA concentration [mg g^{-1} dry weight (DW)]. The full line indicates the mean daily UV-B radiation. The dotted line represents the polynomial trend over the seasons. Abiotic data were downloaded from the National Climate Database (CliFlo, 2017).

(Fig. 2). Furthermore, palythine was identified in trace quantities, mostly below 5% of the total MAA concentration, and an additional, a so far unknown trace MAA could be detected with an absorption maximum at 335 nm in some samples (data not shown). These latter compounds were not considered in the present study.

There was a slight positive trend of increased total MAA concentrations from April to August (R^2 = 0.57), followed by a strong decrease from August to November (R^2 = 0.95). However, neither the individual, nor the total MAA concentrations in *P. plicata* correlated with the UV-B radiation over the seven months studied (total MAA: R^2 = 0.27; porphyra-334: R^2 = 0.17; shinorine: R^2 = 0.25). When the UV-B radiation was low during winter (~ 0.25–0.43 W m^{-2}), the highest MAA concentrations (almost 14.5 mg g^{-1} DW) were measured at that time (Fig. 2). Conversely, in November (end of spring), the UV-B radiation increased to > 1.6 W m^{-2}, and relatively low concentrations of MAAs (< 5 mg g^{-1} DW) were detected (Fig. 2).

3.2.2. Heterosides

Three heterosides, namely floridoside and D- and L- isofloridoside, could be unambiguously detected in *P. plicata* in the ^{13}C NMR spectra (data not shown). The total heteroside content varied from fall to spring between 203 and 1226 mmol kg^{-1} dry weight (DW) (Fig. 3); however, there was no trend between April and November (R^2 = 0.27) and no correlation to PAR (R^2 = 0.38) and/or UVR (R^2 = 0.33). At 117.8–766.1 mmol kg^{-1} DW, L-isofloridoside was the most abundant heteroside. It exceeded the floridoside concentrations up to 3.4 times and that of D-isofloridoside 8-fold. While the D-isofloridoside amounts in the *Pyropia* samples were the lowest, they proportionally varied the most (15.5–131.0 mmol kg^{-1} DW). A correlation between the total heteroside concentrations and only one abiotic factor such as rainfall (R^2 = 0.01) or sunshine (R^2 = 0.21) was not seen. However, some months, *e.g.*, July, show that there might be a correlation between

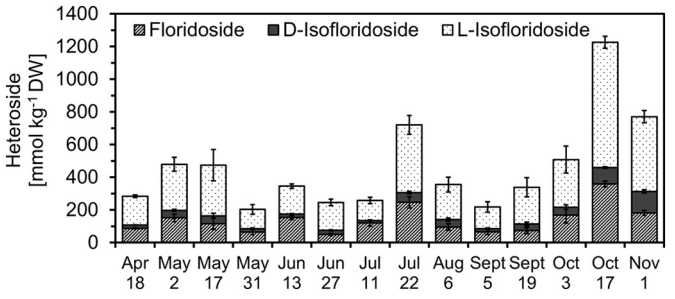

Fig. 3. Seasonal changes in heteroside concentrations [mmol kg^{-1} dry weight (DW)] of *Pyropia plicata* from Moa Point, Wellington, New Zealand in 2016. Values are means ± SD (n = 3).

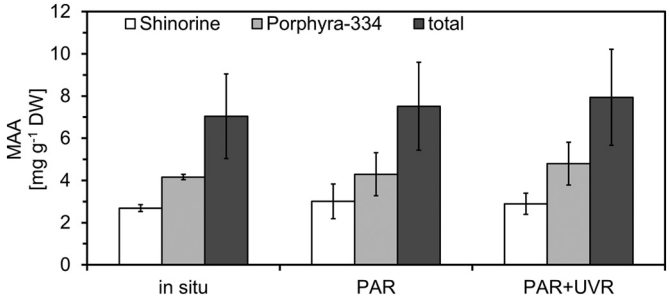

Fig. 4. Mycosporine-like amino acid (MAA) concentration [mg g^{-1} dry weight (DW)] in *Pyropia plicata* collected directly in the field (*in situ*), after 5 days treatment with PAR and 5 days exposure to PAR + UVR. Values are means ± SD (n = 4). No significant differences ($p < 0.05$; one-way ANOVA with *post hoc* Tukey's test).

sunshine hours in combination with rainfall and total heteroside concentration. The more sunshine hours and less rain result in higher heteroside concentrations. The available data do not enable any further examination.

3.3. Stress experiments

3.3.1. UV stress experiment (MAAs)

The results of the UV stress experiment did not reveal any significant change of MAA concentrations in *P. plicata* between the *in situ* samples and the treatments ($p > .05$) (Fig. 4). Porphyra-334 was the quantitatively dominant MAA in the samples (4.2–4.8 mg g^{-1} DW). The shinorine concentrations ranged between 2.7 and 3.0 mg g^{-1} DW. The photosynthetic conditions of the samples were not influenced by the UVR stress either, as the parameter F_v'/F_m' after the experiment was always between 83 and 103% of the initial values ($p > 0.05$) (Supplementary Fig. 3).

3.3.2. Salt stress experiment (Heterosides)

In contrast to the MAA concentrations in *P. plicata*, the heteroside concentrations responded to salinity stress. The total heteroside concentration of *P. plicata* under increased salinities (S_A) increased significantly (Kruskal-Wallis-Test: $p < 0.05$) (Fig. 5). Between S_A 1 and 20 (hyposaline), the mean total heteroside concentration remained unchanged (151 to 163 mmol kg^{-1} dry weight [DW]). At S_A 36, 45 and 60 (hypersaline), the mean total heteroside concentration increased up to 260–270 mmol kg^{-1} DW. All incubated samples contained less total heterosides than the non-treated *in situ* sample. While the total concentrations of all heterosides were not significantly different in the various hypersaline media (Kruskal-Wallis-Test: $p > 0.05$), the relative proportions of the three osmolytes changed (Fig. 5). The concentration of floridoside increased continuously from S_A 36 to 60, while the

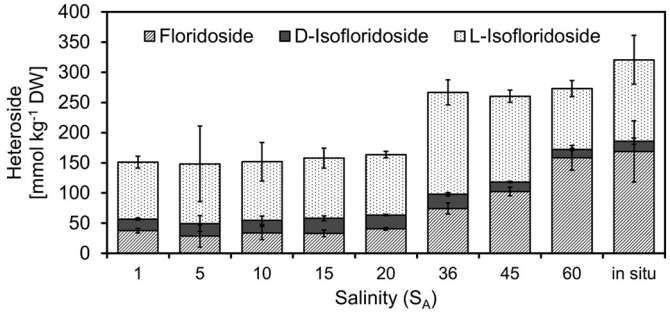

Fig. 5. The three heterosides floridoside, D-isofloridoside and L-isofloridoside in *Pyropia plicata* after 6 days treatment with different salinities. Concentrations are given in mmol kg^{-1} dry weight [DW]. *in situ* = no treatment. Values are means ± SD (n = 3).

concentration of L-isofloridoside declined (Kruskal-Wallis-Test: $p < 0.05$). The D-isofloridoside content remained unchanged (Kruskal-Wallis-Test: $p > 0.05$) (Fig. 5). The control *in situ* sample of *P. plicata* revealed an almost 50:50 ratio of floridoside to D−/L-isofloridoside.

The physiological conditions of the samples were negatively affected by salt stress, as the photosynthetic parameter F_v'/F_m' after the experiment was only 53–83% of the initial values (Kruskal-Wallis-Test: $p < 0.05$) (Supplementary Fig. 3). The strongest inhibition of photosynthesis was measured at the lowest and highest salinity applied.

4. Discussion

4.1. MAAs

While shinorine and palythine are usually the dominant MAAs in subtidal red algae, eulittoral species, such as *Pyropia plicata* typically accumulate porphyra-334 (Hoyer et al., 2001). Porphyra-334 is therefore the quantitatively most important MAA in members of the closely related genera *Bangia* and *Porphyra* (and other Bangiales), which preferentially grow in the upper littoral to supralittoral zone, often exposed to high light (*e.g.*, Karsten and West, 2000). The Bangiales contain the highest measured MAA values compared with other micro- and macroalgae, and are characterized with an apparent excess of porphyra-334, which does not increase with increased UVR (Hoyer et al., 2001, 2002). *Pyropia plicata* showed a similar response, *i.e.*, added UVR did not stimulate any increased accumulation of MAAs.

In most red algae tested, such as *Devaleraea ramentacea*, *Palmaria palmata, Bangia* spp., different radiation conditions such as PAR or PAR in combination with UVR led to variations in both, composition and specific MAA concentration (Karsten et al., 1998, 1999; Karsten and Wiencke, 1999; Boedeker and Karsten, 2005; Roleda et al., 2012). Shinorine accumulation was stimulated by UVR, while other MAAs concentrations were more affected by high PAR (Karsten et al., 1998). Similar results were discovered in *Gracilaria vermiculophylla* (Roleda et al., 2012). Neither did the total MAAs concentrations vary between the initial and after different PAR and UV treatments, nor did the specific MAA composition and concentrations. Initially only two types of MAAs were detected, while five more MAAs could be observed after the laboratory experiment (Roleda et al., 2012). These results led to the suggestion, that the formation of several MAAs at the same time or an increase of the concentration of one specific MAA supports the specific protective UV-filtering capacity in the alga.

Based on the MAA concentrations and the induction patterns after exposure to different radiation conditions red algae can be physiologically classified in three categories, according to Hoyer et al. (2002): Type I – unable to biosynthesize MAAs; Type II – MAAs inducible in variable concentrations and Type III – permanently high MAA contents. While Type I typically represents red algae of the lower sublittoral, Type II and III species grow from the mid- and upper sublittoral zone up to the eu- and supralittoral. According to this classification, *P. plicata* can be characterized as Type III, and hence there was no seasonal nor experimental effect of UVR exposure on the total or particular MAA contents.

As mentioned above porphyra-334 is the most abundant, often exclusive MAA, in eulittoral Bangiales (Hoyer et al., 2001, 2002). Although porphyra-334 occurred in high concentrations in *P. plicata*, shinorine constituted a remarkably higher proportion of the total MAAs than has been reported in other closely related species (*e.g.*, Karsten et al., 1998; Boedeker and Karsten, 2005). In a few of the seasonal *P. plicata* samples, the porphyra-334:shinorine ratio approached 1:1, while in several samples shinorine quantitatively dominated. Consequently, this indicates that shinorine has at least an equally important role as porphyra-334 in *P. plicata*. As this abundance of shinorine in Bangiales has not been reported before, its high content in *P. plicata* could be related to the local UV-B conditions in New Zealand, as it is known that UV-A and UV-B can stimulate shinorine biosynthesis (Karsten et al., 1998; Karsten and Wiencke, 1999). It could be that *P. plicata*, as an endemic species, is already adapted to these enhanced UVR conditions in the field by accumulating high concentrations of shinorine along with porphyra-334. The results of Lamare et al. (2004) support this hypothesis, as they investigated MAA variation in various marine organisms from New Zealand and also detected high shinorine concentrations compared to porphyra-334 in the red alga *Rhodymenia* sp. from Doubtful Sound (South Island). Further measurements with red algal species from New Zealand should be the subject of future research to clarify these observations.

The photosynthetic measurements of the *P. plicata* samples (F_v'/F_m') clearly indicated that UVR treatment did not lead to negative effects to photosynthesis, giving another indication that this alga was not stressed during the different radiation conditions. The high MAA concentrations probably contributed to this UVR insensitivity, as documented for many micro- and macroalgae (see review of Karsten, 2008). Upregulation of the polyamine metabolism is another protective mechanism against UVR (Schweikert et al., 2014) and the potential antioxidant activity of MAAs, like mycosporine-glycine (Dunlap and Yamamoto, 1995) should be considered in future studies.

Different factors can affect MAA biosynthesis. Not only solar radiation, but also desiccation and thermal stress can have a major impact on MAA concentration in macroalgae (Carreto and Carignan, 2011). The MAA concentrations in the thalli of desiccated *Porphyra haitanensis* (*Pyropia haitanensis*) were 90–200% higher than those from samples always submerged (Jiang et al., 2008). Nutrient availability (*e.g.*, nitrogen) in particular has positive effects on MAA concentrations in red algae (Korbee et al., 2005). These other environmental factors were not measured in the present study but warrant further investigation. Therefore, it is reasonable to assume that the seasonally changing MAA concentrations in *P. plicata* are controlled by other parameters than UVR, since nitrogen concentrations, for example, are increased over winter (Phillips and Hurd, 2003). Multiple stressor experiments (UV, salt stress, temperature, nutrients) with *P. plicata* could be of interest for future studies to more carefully investigate interactions of the different environmental stressors typical for the intertidal zone, which of course would better reflect natural conditions.

4.2. Heterosides

Pyropia plicata showed strong seasonal changes in its heteroside concentrations, which had already been reported in various members of the closely related genus *Porphyra* (Karsten, 1999, some of the *Porphyra* species in this study were later transferred to *Pyropia*), and which was explained as a response to varying light and temperature conditions. In the present study, a possible correlation between sunshine hours/rainfall and heteroside concentration in *P. plicata* was seen. More sunshine hours and less rainfall possibly increased desiccation in *P. plicata*, and therefore impacted heteroside amounts. To verify this hypothesis, further *in situ* measurements of heteroside concentrations with a local monitoring of sunshine hours, rainfall, tidal level in combination with the exact time of collections are required.

Heterosides act as organic osmolytes in red algae and additionally function as compatible solutes and carbon storage (Kirst, 1989; Karsten et al., 1993, 1996; Karsten, 1999; Martinez-Garcia and van der Maarel, 2016). Each of these organic compounds, however, cannot act as a carbon source and as an osmolyte at the same time (Raven et al., 1990). The presence of several heterosides with different functions could be a physiological advantage for living in the intertidal zone (Karsten, 1999). In the present study, L-isofloridoside was the dominant heteroside throughout the year in *P. plicata*. This has been shown in other red algae from Australia (*Porphyra columbina* (now *Pyropia columbina*) and *Porphyra lucasii*) (Karsten, 1999) compared to other *Porphyra* species from the Northern Hemisphere.

The concentrations of floridoside and D/L-isofloridoside in *P. plicata* in the salt stress experiment were similar to earlier studies (*e.g.*, Karsten

et al., 1995), with only floridoside increasing in higher salinities (Reed et al., 1980; Reed, 1985). Floridoside is the main product of photosynthesis in most Rhodophyta, and is metabolically more quickly synthesized and degraded than both isofloridosides (Reed et al., 1980; Reed, 1985). This suggests the biochemical importance of floridoside as a regulated organic osmolyte in osmotic acclimation. Red algae from higher intertidal levels contained higher concentrations of floridoside than those growing at lower levels (Chen et al., 2014). High intertidal *Porphyra/Pyropia* are extremely tolerant to desiccation. The floridoside concentration in *Pyropia haitanensis* increased with longer desiccation (Qian et al., 2014).

Desiccation typically results in increased osmotic pressure (higher water potential) and therefore, it mimics hypersaline stress. Contrary, precipitation during low tide directly exposes *Pyropia* to fresh water leading to hyposaline stress (Karsten, 2012). In the present study *Pyropia* samples were constantly incubated in various saline media for six days. The *in situ* sample exhibited a higher total heteroside concentration than the incubated experimental samples, which can be explained by the additional desiccation stressor (Qian et al., 2014). As the *Pyropia* samples were not exposed to desiccation during the experiment, they accumulated less heterosides, comparative to growing at lower intertidal levels (Chen et al., 2014).

Although raised algal nitrogen status may have a positive impact on osmotic regulation in *Gracilaria sordida* (Ekman et al., 1991), there is not much known on similar effects in other macroalgae. Whether such compounds are relevant for the process of osmotic adjustment seems doubtful, as salinity-induced changes of turgor pressure are sensed and regulated by macroalgae is solely based on differences between the cellular and external osmotic potential that are independent of any specific solutes (Kirst, 1989). Therefore, minor differences in trace element composition of the various salinity media due to preparation with distilled water or sea salt, can be disregarded for the process of osmotic adjustment in *P. plicata*.

4.3. *Pyropia plicata* in New Zealand

In summary, the endemic red alga *P. plicata,* which inhabits the upper eulittoral zone of rocky shores in New Zealand is well adapted to the prevailing strong diurnal and seasonal changes in solar radiation and salinity. The biochemical capability to synthesize and accumulate high concentration of the stress metabolites MAAs and heterosides contribute to this high tolerance. UVR might still increase in New Zealand, at least temporarily due to ongoing ozone depletion. Nevertheless, particularly high intertidal macroalgae such as *P. plicata* are expected to cope well even with such enhanced stress scenarios, because of their generally wide tolerance ranges.

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