Antioxidative properties of marine macroalgae from the Arctic

Antioxidative Eigenschaften mariner Makroalgen der Arktis

Angelika Dummermuth

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Angelika Dummermuth
Alfred-Wegener-Institut für Polar- und Meeresforschung
In der Helmholtzgemeinschaft
Am Handelshafen 12
27570 Bremerhaven

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List of abbreviations

Abbreviation	Unit in parantheses
APX	Ascorbate peroxidase (U mg^{-1} FW or U mg^{-1} DW)
BHT	butylated hydroxytoluene
CAT	catalase (U mg^{-1} FW or U mg^{-1} DW)
Chl *a*	chlorophyll *a*
CPDs	cyclobutane-type pyrimidine dimers
DCM	dichloromethane
DPPH	alpha,alpha-diphenyl-beta-picrylhydrazyl radical
Fv	variable chlorophyll fluorescence
Fm	maximal chlorophyll fluorescence of samples previously acclimated to darkness
Fv/Fm	maximum quantum yield of photosynthesis
GC-MS	gas chromatography mass spectrometry
GR	glutathione reductase (U mg^{-1} FW or U mg^{-1} DW)
GSH	reduced glutathione
GSSG	oxidzied glutathione
H_2O_2	hydrogen peroxide
MAAs	mycosporine-like amino acids
MDA	malondialdehyde
MSTFA	N-methyl-N-trimethylsilyltriflouracetamide
NADPH	nicotinamid adenine dinucleotide phosphate
nm	nanometer
$O_2^{-\bullet}$	superoxide radical
$OH^{\bullet}$	hydroxyl radical
PAM	pulse amplitude modulated
PAR	photosynthetic active radiation (400-700nm)
PSI	photosystem I
PSII	photosystem II
ROS	reactive oxygen species
SOD	superoxide dismutase (U mg^{-1} FW or U mg^{-1} DW)
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactive substances

TMS	tetramethylsilane
TSP	total soluble protein (mg g^{-1} FW or mg g^{-1} DW)
UVA	ultraviolet A radiation (320-400 nm)
UVB	ultraviolet B radiation (280-320 nm)
UVC	ultraviolet C radiation (190-280 nm)
UVR	ultraviolet radiation (190-400 nm)
W	watt

SUMMARY

The present study focuses on oxidative stress and the ability of Arctic marine macroalgae to cope with it. Oxidative stress can derive from biogenic formation of reactive oxygen species (ROS) induced by different environmental stress factors, for example ultraviolet radiation (UVR). Also high temperature, temperature changes, nutrient deficiency, heavy metals and other factors may induce oxidative stress. In aquatic environments algae may also be exposed to direct oxidative stress, where UVR induces formation of hydrogen peroxide (H_2O_2) by photoactivation of dissolved organic material (DOM), photochemical degradation and liberation of exited electrons, which initiate reduction of molecular oxygen. This process is particularly promoted in surface waters, tidal pools or flat water areas, where high concentrations of DOM and oxygen occur. Stratospheric ozone depletion leads to an increase in the short wavelengths of UVR and in consequence to an increase in H_2O_2 formation in surface waters. Elevated levels of UVR, resulting from stratospheric ozone depletion, are the major source for oxidative stress.

The investigations presented were conducted to study the effects of oxidative stress on the physiology of Arctic macroalgae in the laboratory and in the field. The results present a basis for predicting future changes within Arctic coastal ecosystems with respect to increasing UVB levels and accompanied oxidative stress.

Arctic macroalgae are subjected to strong seasonal and daily changes in the radiation climate. They are exposed to six months of darkness during polar night, but also suddenly exposed to high radiation in spring after break-up of the sea ice, especially during low tide at high water transparency, leading to oxidative stress for the algae.

When produced, active oxygen species are eliminated rapidly by efficient antioxidative systems as there are the enzymatic detoxifying systems of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), as well the antioxidant ascorbic acid and other small molecule antioxidants (glutathione, β-carotene and α-tocopherol), which were not presented in this study.

The major result of the study is that the occurrence of antioxidants in Arctic marine macroalgae is related to the vertical depth distribution along the shore. Species living

in the intertidal and upper sublittoral exhibited higher antioxidative protection, whereas species from deeper habitats showed lower levels of biochemical defence against oxidative stress. Another result is the occurrence of a different antioxidant pattern in the Chlorophyta, Rhodophyta and Phaeophyta. Green macroalgae exhibited highest enzyme activities and ascorbic acid contents, whereas red and brown algal species showed lower antioxidant activities.

These antioxidative protection mechanisms are induced or inhibited in response to the various environmental stressors. The sensitivity of photosynthesis to artificial oxidative stress derived by exposure to H_2O_2, and the antioxidative properties of several marine macroalgae under these conditions was investigated. Species exhibiting high photosynthetic rates under oxidative stress were regarded as high tolerant to H_2O_2 and equated with a high antioxidative potential. The cause of the high antioxidative potential was different in the most tolerant species. *Chaetomorpha melagonium* for example exhibited extremely high ascorbic acid contents comparable to citrus fruits and increased GR activity, indicating an active scavenging of H_2O_2 via the ascorbate-glutathione cycle. In *Chaetomorpha linum*, in contrast, photosynthetic efficiency was decreased strongly and correlated to the low SOD activity under high oxidative stress, indicating a direct toxicity effect by H_2O_2. The deep water red alga *Polysiphonia arctica* followed another strategy and increased APX and CAT activities in response to H_2O_2. Additionally, two new bromophenolic compounds with antioxidative activities were identified in this species (2,3-dibromo-4,5-dihydroxybenzyl methyl ether, and TMS derivative of 2,3-dibromo-4,5-dihydroxybenzyl alcohol).

The influence of other abiotic factors, such as salinity, was examined as well as the interactive effects of UVR and salinity on photosynthesis and MAAs evaluated. Under different salinity concentrations (15, 34 and 50 PSU) the MAA content did not rise in Arctic *Devaleraea ramentacea* and *Palmaria palmata*, but in combination with UVR an increase was observed. Optimum quantum yield in *P. palmata* was much more strongly effected by salinity than *D. ramentacea* and died under 15 PSU with or without additional UVR. While *D. ramentacea* exhibited euryhaline features and acclimated well to UVR applied, *P. palmata* can be characterised as stenohaline due to its high sensitivity under hyposaline conditions leading to cell death.

Furthermore the effects of seasonally fluctuating daylengths and additional UVR exposure on pigment concentrations, antioxidative enzyme activities, ascorbic acid, MAAs and growth was investigated in *D. ramentacea* and *P. palmata* in a long-term-culture study. Both species showed a seasonal acclimation of chlorophyll and phycobiliproteins to the fluctuating daylength. Ascorbic acid content was high throughout the whole year and especially in winter in thalli exposed to UVR in the previous summer period. Further protection was provided by the antioxidative enzyme activities, which were up-regulated in winter to prepare the algae for the coming radiation period, indicating a photoperiodic control for the antioxidative enzymes as well as for ascorbic acid. Additionally the UV-absorbing MAAs were up-regulated in response to the increasing daylength and UV-exposure from spring onwards enlarging the protection of the algae in times of high radiation stress. Slight differences between both species may be explained by the different biogeographical and depth distribution. Whereas the Arctic endemic *D. ramentacea* occupies the uppermost habitats within the Rhodophyta at Arctic Kongsfjord from 0.5 to 8m depth, *P. palmata* exhibits its northernmost distribution limit at the study site and inhabits slightly deeper habitats with 2 to 10m depth.

All data presented demonstrate distinct induction/inhibition patterns for the antioxidative activities and photoprotective mechanisms present in marine macroalgae from the Arctic. In general, the antioxidative status of marine macroalgae depends on the oxidative stress they are subjected to in their habitat. Several factors seem to be involved in regulation processes of the antioxidant status of marine macroalgae but still are not proved. Future studies should therefore focus on the regulatory processes.

ZUSAMMENFASSUNG

Der Schwerpunkt dieser Arbeit liegt in der Untersuchung oxidativen Stresses und der oxidativen Schutzmechanismen mariner Makroalgen der Arktis. Biogene Bildung reaktiver Sauerstoffspezies unter Photosynthese kann zu oxidativem Stress führen. Außerdem kann oxidativer Stress als Reaktion auf Umweltfaktoren, wie z.B. ultraviolette Strahlung (UV), Temperaturschwankungen etc. entstehen. UV-Strahlung induziert außerdem die Bildung von Wasserstoffperoxyd im aquatischen Milieu. Durch Photoaktivierung von gelöstem organischen Material, photochemischen Abbau und Freisetzung von angeregten Elektronen, welche die Reduktion von molekularem Sauerstoff initiieren, wird H_2O_2 gebildet. Dieser Prozess wird insbesondere im Oberflächenwasser, Gezeitentümpeln und Flachwassergebieten gefördert, wo hohe Konzentrationen an gelösten organischem Material und Sauerstoff vorliegen.

Der Abbau der Ozonschicht, der natürliche UV-Schutzschild der Erde, führt zu einem Anstieg der Bestrahlungsstärke im kurzwelligen Bereich, was wiederum einen Anstieg der H_2O_2-Bildung im Oberflächenwasser nach sich zieht. Erhöhte UV-Strahlung, die von der Reduktion des stratosphärischen Ozons herrührt, ist die Hauptquelle für oxidativen Stress.

Das Ziel der hier vorliegenden Arbeit war es, die Auswirkungen oxidativen Stresses auf physiologische Reaktionen von arktischen Makroalgen im Labor und im Freiland zu untersuchen. Die Ergebnisse sollen als Grundlage dienen, Vorhersagen zu Veränderungen arktischer Küstensysteme, in Hinblick auf erhöhte UV-Bestrahlungsstärken und damit verbundenen oxidativen Stresses, zu treffen.

Marine Makroalgen der arktischen Region sind starken saisonalen und tageszeitlichen Schwankungen des Strahlungsklimas ausgesetzt. Während des polaren Winters überdauern sie mehr als sechs Monate in Dunkelheit. Im Frühling, nach Aufbruch des Meereises, sind die Algen besonders erhöhten Bestrahlungsstärken ausgesetzt, insbesondere in Wasser mit hoher Transparenz und bei Ebbe.

Reaktive Sauerstoffspezies werden umgehend nach ihrer Entstehung schnell eliminiert mit Hilfe der leistungsstarken enzymatischen Entgiftungssysteme Superoxid-Dismutase (SOD), Katalase (CAT), Ascorbatperoxidase (APX) und

Glutathionreduktase (GR), sowie dem Antioxidanz Ascorbinsäure (Vitamin C).
Ein wichtiges Ergebnis der Arbeit ist das Vorkommen von Antioxidanzien in marinen Makroalgen der Arktis, welches im Zusammenhang mit der vertikalen Tiefenzonierung der Algen entlang der Küste steht. Arten, die in der Gezeitenzone (Eulittoral) und im oberen Sublittoral leben, weisen höheren antioxidativen Schutz auf als Arten aus tiefer liegenden Habitaten, die niedrigere Gehalte an biochemischen Abwehrmechanismen für oxidativen Stress aufzeigen. Ein anderes Ergebnis ist die Verteilung der verschiedenen Antioxidanzien in den Grün-, Rot- und Braunalgen. Grüne Makroalgen zeigen die höchsten Enzymaktivitäten und Ascorbinsäuregehalte, während rote und braune Makroalgenarten niedrigere antioxidative Aktivitäten aufweisen.

Diese antioxidativen Schutzmechanismen werden als Antwort auf verschiedene Umweltstressoren induziert oder inhibiert. Die Empfindlichkeit der Photosynthese sowie die antioxidativen Eigenschaften verschiedener Arten gegenüber künstlichem oxidativem Stress, hervorgerufen durch Inkubation in H_2O_2, wurden untersucht. Arten, die hohe Photosyntheseraten unter oxidativem Stress erzielten, wurden als tolerant gegenüber H_2O_2 betrachtet, was mit einem hohen antioxidativen Potential gleichgesetzt wurde. Der antioxidative Schutz wurde in den untersuchten Arten durch unterschiedliche Mechanismen gebildet. *Chaetomorpha melagonium*, z.B. verfügt über einen mit Zitrusfrüchten vergleichbar hohen Ascorbinsäuregehalt, und erhöhte unter oxidativem Stress die GR-Aktivität, was auf ein aktives Entgiften von H_2O_2 über den Ascorbat-Glutathion-Zyklus hindeutet. *Chaetomorpha linum* hingegen zeigte eine stark erniedrigte Photosyntheseeffizienz unter hohem oxidativen Stress, die mit der drastischen Abnahme der SOD-Aktivität korreliert. Dies deutet auf eine toxische Wirkung von H_2O_2 auf das Enzym hin. Die Tiefenrotalge *Polysiphonia arctica* verfolgte eine andere Strategie und erhöhte APX- und CAT-Aktivitäten infolge der Inkubation in H_2O_2. Außerdem wurden in dieser Art zwei neue Bromphenole nachgewiesen, die hohe antioxidative Eigenschaften aufweisen (2,3-dibromo-4,5-dihydroxybenzyl methyl ether, and TMS derivative of 2,3-dibromo-4,5-dihydroxybenzyl alcohol).

Weiterhin wurde der Einfluss anderer abiotischer Faktoren, z.B. Salinität, wie auch die interaktive Wirkung von UV-Strahlung und Salinität auf die Photosynthese und

die UV-absorbierenden mycosporin-ähnlichen Aminosäuren (MAAs) untersucht. Bei verschiedenen Salzgehalten (15, 34 und 50 PSU) erhöhte sich der MAA-Gehalt in *Devaleraea ramentacea* und *Palmaria palmata* nicht. Es zeigte sich jedoch ein Konzentrationsanstieg bei der Kombination von Salzgehaltserhöhung und UV-Bestrahlung. Die Photosyntheseeffizienz wurde in *P. palmata* durch Salzgehaltsänderungen viel stärker beeinträchtigt als in *D. ramentacea*. *P. palmata* starb nach Ausbleichen bei 15 PSU ab. Während *D. ramentacea* euryhaline Merkmale zeigt und sich gut auf die UV-Strahlung einstellt, verhält sich *P. palmata* eher stenohalin, da diese Art eine hohe Empfindlichkeit bei hyposalinen Bedingungen aufweist.

Außerdem wurden die Effekte von jahreszeitlich schwankenden Tageslängen und UV-Bestrahlung auf Pigmentkonzentrationen, Aktivitäten der antioxidativen Enzyme, Ascorbinsäure, MAAs und Wachstum an *D. ramentacea* und *P. palmata* in einer Langzeit-Studie untersucht. Beide Arten passten den Chlorophyll-Gehalt und die Konzentrationen der Phycobiliproteine an die schwankende Tageslänge an. Der Ascorbinsäure-Gehalt war das ganze Jahr über hoch, speziell aber im Winter in Algengewebe, das im vorherigen Sommer mit UV behandelt worden war. Zusätzlichen Schutz lieferten die antioxidativen Enzyme, deren Aktivität ebenfalls im Winter hochreguliert wurde, als Vorbereitung auf die kommende Bestrahlungsperiode, was auf eine photoperiodische Kontrolle hindeutet. Dies gilt ebenfalls für Ascorbinsäure.
Die MAAs liefern zusätzlichen Schutz, insbesondere im Frühling, wenn die Konzentrationen als Reaktion auf die steigende Tageslänge und Bestrahlung mit UV ansteigen. Kleine Unterschiede zwischen den beiden untersuchten Arten sind durch ihre unterschiedliche biogeographische und Tiefenverteilung erklärbar. Während die arktisch endemische *D. ramentacea* innerhalb der Rotalgen im arktischen Kongsfjord in Wassertiefen zwischen 0,5 und 8 m Tiefe vorkommt, erreicht *P. palmata* ihre nördlichste Verbreitungsgrenze im Untersuchungsgebiet und besetzt etwas tiefere Habitate von 2-10 m.

Die vorgestellten Ergebnisse zeigen bestimmte Induktions- und Inhibitionsmuster für die antioxidativen Aktivitäten und photoprotektiven Mechanismen in marinen Makroalgen der Arktis. Als Gesamtbild ergibt sich, dass der antioxidative Status der

Makroalgen vom oxidativen Stress abhängt, den sie in ihrer Umgebung erfahren. Verschiedene Faktoren scheinen in die Regulationsprozesse einzugreifen, sind jedoch noch nicht bewiesen. Zukünftige Forschung sollte den Schwerpunkt auf diese Regulationsprozesse legen.

1 GENERAL INTRODUCTION

1.1 The Arctic environment

The polar environments of the Arctic and Antarctica rank among the harshest environments in the world and organisms living in these habitats show a high level of adaptability. They have to endure low temperatures, long periods of ice and snow cover and variation of solar radiation ranging from 24 h daylength in summer to 24 h of darkness in winter within the polar circles.

At Spitsbergen, the coastal ecosystem is further characterized by pronounced seasonal variations in temperature, nutrients and salinity (Hop et al., 2002). At 80° North the polar day begins in mid April and lasts to mid August, and the polar night from mid October to mid February. Therefore, macroalgae have to endure at least 4 months in darkness, and this period may be prolonged by sea ice and snow cover until June (Hop et al., 2002). Consequently marine macroalgae have to cope with a long period in darkness, but also a sudden change to high radiation as soon as the ice cover breaks up in spring (Bischof et al., 1999; Hanelt et al., 2001).

In contrast to Antarctic waters, the Arctic ocean has a very limited water exchange and receives about 10% of the world river discharge (Hempel, 1987). This freshwater input results in stratification of the water masses and is responsible for the input of large fractions of sediment, strongly influencing the transparency of the water body (Wängberg et al., 1996). This is a pronounced seasonal effect, as turbid freshwater discharge is a result of snow melting and calving of the glaciers in summer (Hanelt et al., 2001). The algae are exposed to high irradiances of photosynthetically active radiation (PAR) and ultraviolet radiation (UVR) especially during low tide and periods of high water transparency.

1.2 Marine macroalgae

Macroalgae are an important component of coastal marine ecosystems and contribute to an estimated 5% of total marine primary production (Mann, 1973) and about 3 % of the global primary production (Lüning, 1985). Production in seaweed communities and kelp forests can be as high, or in many cases greater than terrestrial plant-based systems (Thomas, 2002; Smith, 1981). Macroalgal production

is important for coastal food webs, as they represent the basis of the food chain providing food for herbi- and detrivores (Dunton and Schell, 1987; Iken, 1996; 1997). They serve as habitat/nursery for juvenile fish and invertebrates (Mann, 1973) and as substrate for a large variety of epiphytes and sessile animals (Klöser, 1998; Klöser et al., 1996). Macroalgae are confined to the photic zone of the ocean and occupy diverse habitats depending on the prevailing light conditions. They occur from shallow to deep water (up to 200 m), on different substrata, along the shores of world's oceans forming distinct vertical zonation patterns. Along the vertical gradient one can distinguish major zones: the supralitoral with algae always being emerged, the eulittoral or intertidal being periodically emerged and submerged depending on the tides and the sublittoral permanently being submerged. Depending on the vertical distribution marine macroalgae experience different gradients of the prevailing environmental factors.

Eulittoral algae as e.g. *Fucus distichus*, are fully exposed to high solar radiation during low tide and therefore require strategies to cope with high radiation and desiccation. Macroalgae from the sublittoral show adaptation to lower radiation conditions, as they are protected by the water column or grow as subcanopy species, in the shade of other algae (Kirst and Wiencke, 1995). Typically low light adapted species are *Laminaria solidungula*, *Phycodrys rubens* and *Ptilota plumosa*, which grow in greater water depths.

Polar macroalgae exhibit some ecological peculiarities which enable them to prevail under the harsh conditions such as low temperatures, freezing, seasonal changes of high and low irradiance (Kirst and Wiencke, 1995). Adaptation of polar algae to cold temperatures is remarkable, showing growth optima between 0 and 5°C (Bischoff and Wiencke, 1993; Wiencke et al., 1994; Wiencke and tom Dieck, 1989).They are well adapted to low light conditions, showing low initial light saturation points and very low light requirements for the completion of their life-cycles (Kirst and Wiencke, 1995; Wiencke, 1990a; b). Physiology and life-cycles of polar algae are adapted and synchronized to the seasonal changes in this unique environment (Chapman and Lindley, 1980; Dunton, 1985; 1990; Gomez et al., 1995a; b; Henley and Dunton, 1995). In the case of *Laminaria saccharina* from the Arctic, the time of maximal growth coincides with the period of maximal water transparency in spring/early summer and therefore high irradiances of PAR and UVR (Dunton 1985). Consequently, UVR may penetrate deeply into the water column, and the question

arises how the algae can cope with this radiation stress and the accompanying oxidative stress, particularly with respect to a further increase in UVR as a consequence of increasing ozone depletion.

1.3 The "ozone hole " and ultraviolet radiation

The gas ozone is found in the stratosphere in altitudes of approximately 10 to 50 km with a maximum of ozone density (90%) within 15 to 25 km (Solomon, 1990). It builds a natural shield for the harmful ultraviolet radiation reaching earths surface. Since the 1980s a severe depletion in ozone has been described for the Antarctic (Farman et al., 1985), and later also for the midlatitudes of the Southern and Northern Hemisphere (Atkinson et al., 1989; Roy et al., 1990) and the Arctic (Austin et al., 1992; Salawitch et al., 1993). The depletion is mainly due to human impact by release of comprising compounds such as chlorofluorocarbons, bromines and nitrogen oxides (No_x) into the atmosphere (Solomon, 1990). A 30 % depletion in stratospheric ozone concentration seen pre 1980 is commonly called the "ozone hole" (WMO, 2002).

The depletion of the ozone in the stratosphere results in an increase in UV radiation at the Earth's surface. A 10% loss in ozone concentration might lead to an increase in spectral irradiance of about 50% at 297 nm, 25% at 303 nm and 0% at 325 nm (Roy, 2000). In this case the solar spectral distribution may shift to shorter wavelengths, which exhibit the highest energy, resulting in a higher UVR:PAR ratio (Smith et al., 1992). A small decrease in ozone levels may therefore cause a large relative increase in biologically effective radiation (Hollósy, 2002).

Ultraviolet radiation in the biosphere is arbitrarily divided into three components, and defined as follows by the Commission Internationale de l'Eclairage (CIE): UVA (315-400 nm), UVB (280-315 nm) and UVC (100-280 nm). UVC is extremely harmful to organisms, but not relevant under natural conditions of solar radiation, as it is completely absorbed by the ozone shield, oxygen and water vapour, as well as is the largest part of the UVB downwards from about 300 nm. UVB is of particular interest because its irradiance waveband represents only approximately 1.5 % of the total spectrum, but can induce a variety of damaging effects in plants. UVA represents

approximately 6.3 % of the incoming solar irradiance and is the less hazardous part of UV radiation (Hollósy, 2002).

1.4 Effects of UV radiation on macroalgae

Ultraviolet radiation has been shown to be harmful to living organisms, damaging DNA, protein, lipids, membranes and/or physiological processes (Hollósy, 2002). Nucleic acids, in particular DNA are the most notable targets of UVR. Exposure to UV-B and UV-C results in a multitude of DNA photoproducts which may cause mutations during replication. The most common DNA photoproducts are cyclobutane-type pyrimidine dimers (CPDs) and the pyrimidine(6,4)pyrimidone dimer (Hutchinson, 1987) as also detected in phytoplankton and macroalgae (Buma et al., 2000; Pakker et al., 2000a; van de Poll et al., 2001; 2002).

Proteins show a strong absorption peak at about 280 nm as well as at higher wavelengths of the UV-B region due to absorption by aromatic amino acids (e.g. tyrosin, phenylalanine, tryptophan) and therefore can be direct targets of UVR (Hollósy, 2002). In particular the disulfide-bonds between two cystein residues, which are important for the tertiary structure of proteins, can be split by UVR into reactive sulfhydryl groups, resulting in a loss of protein structure and function (Creed, 1984; Vass, 1997). UVR causes not only the modification and destruction of amino acid residues, but also leads to inactivation of enzymes (Grossweiner, 1984). The degradation or inactivation of any protein involved in photosynthesis (e.g. D1 protein of PS II or ribulose bisphosphate carboxylase/oxygenase = Rubisco) is likely to lower photosynthetic activity (Aro et al., 1993; Mattoo et al., 1984). In marine macroalgae deleterious effects of UVR on photosynthesis and related enzyme reactions has been shown by Bischof et al. (2000; 2002).

Lipids with isolated or conjugated double bonds can also be photochemically modified by UV absorbance. UV-induced damage to membranes is due to lipid peroxidation of the fatty acids building the membranes, as well as degradation of proteins and pigment complexes also being part of thylakoid membranes of the photosynthetic apparatus (Kramer et al., 1991).

Pigments of the photosynthetic apparatus can be destroyed by UVR, with concomitant loss of photosynthetic capacity (Jordan et al., 1994; Strid et al., 1990). Photobleaching concerns phycobiliproteins as phycoerythrin (PE) and phycocyanin

(PC), as well as chlorophylls and carotenoids and UV effects on these pigments in marine macroalgae has been studied by Aguilera et al. (2002a; 1999a).

Photosynthesis is one of the most studied processes in plants under UV-B exposure. Despite the diversity of UV targets in plants, it seems that the photosynthetic apparatus is among the main action sites of UVB. The direct effects of enhanced UVB radiation in sensitive plants are the following: Impairment of photosystem II and to lesser extent photosystem I, decrease in Rubisco activity, decreased carbon dioxide fixation and oxygen evolution, as well as reduction in dry weight, starch and chlorophyll content (Hollósy, 2002).

Marine macroalgae have evolved various biological defence mechanisms against UV damage, enabling them to minimize the deleterious effects of UVR as far as possible (Roy, 2000). The repair and protective mechanisms are manifold such as (Hanelt and Nultsch, 2003) DNA repair via photolyases and excision enzymes (Mitchell and Karentz, 1993; Pakker et al., 2000b; van de Poll et al., 2001; 2002), synthesis of UV absorbing compounds such as the MAAs (Karsten et al., 1998), which are widely distributed in aquatic organisms (Cockell and Knowland, 1999; Hoyer et al., 2002b; Karentz, 2001) and the expression and activation of detoxifying enzymes and antioxidants (Aguilera et al., 2002b; Dunlap and Yamamoto, 1995). If these mechanisms are lacking or not properly functioning, the final result can be a complete change in metabolic effects on growth, reproduction and productivity (Dring et al., 1996a; b; Aguilera et al., 1999b; Makarov and Voskoboinikov, 2001; Wiencke et al., 2000), genetic damage (Kulunscics et al., 1999; Vincent and Neale, 2000), depression of photosynthesis (Hanelt et al., 1997; Hanelt and Nultsch, 2003; Bischof et al., 2000; 2002; Gómez et al., 2001) or even an altered community structure (Madronich et al., 1995).

1.5 Oxidative stress and reactive oxygen species (ROS)

1.5.1 ROS formation in the aquatic environment

In aquatic environments, UVR induces formation of reactive oxygen species (ROS) by photoactivation of dissolved organic material (DOM), photochemical degradation and liberation of exited electrons, which initiate reduction of molecular oxygen (Cooper and Zika, 1983; Zika et al., 1985):

$$DOM + \text{UV-photon} \rightarrow DOM^{*} + e^{-}_{(aq.)} \qquad e^{-}_{(aq.)} + O_2 \rightarrow O_2^{\bullet -}$$

Superoxide anion radicals ($O_2^{\bullet-}$) abstract a second electron from DOM and protonate to yield hydrogen peroxide (H_2O_2):

$O_2^{\bullet-}$ + DOM* + 2H+ $\rightarrow$ H_2O_2 + refractory DOM.

Hydrogen peroxide is not a radical, but it shows a considerable toxicity, as the weak O-O bond increases its reactivity and activation can lead to the liberation of the hydroxyl radical ($^{\bullet}OH$):

H_2O_2 + energy $\rightarrow$ 2 $^{\bullet}OH$

This process particularly occurs in tidal pools or flat water areas in the Wadden Sea. The algal flora in these tidal pools produces high oxygen concentrations, up to 300 % oversaturation due to photosynthesis (Bridges et al., 1984). This is also seen in the free water column in times of accelerated photosynthetic production (Groth and Theede, 1989) and with increasing UVR H_2O_2 is formed (Cooper and Zika, 1983; Pamatmat, 1990; Zika et al., 1985). H_2O_2 concentrations in seawater normally range between 20 and 300nM (Pamatmat, 1990; Price et al., 1992; Szymczak and Waite, 1988; Zika et al., 1985; Zika et al., 1984). During low tide in summer, H_2O_2 was found to accumulate to micromolar (<5 µmol L^{-1}) concentrations in shallow intertidal pools on the German Wadden Sea coast (Abele-Oeschger et al., 1997). However, high H_2O_2 concentrations were also found in polar regions, where concentrations of < 2µmol L^{-1} were measured in surface or tidal pool water during the Antarctic summer (Abele et al., 1998a; Abele et al., 1999). UV-induced H_2O_2-formation in surface waters has been shown to be a temperature-independent process (Abele-Oeschger et al., 1997).

UVB photons display a 11-fold higher efficiency for photochemical H_2O_2 production compared to UVA and a 340-fold higher efficiency compared to PAR (Abele-Oeschger et al., 1997). A 10 % ozone reduction leads to a doubling of UVB surface irradiance at 300 nm, which entails a 40% increase of the apparent intertidal H_2O_2 concentrations (Abele-Oeschger et al., 1997).

1.5.2 Biogenic ROS formation

Macroalgae, as consumer of light energy, are exposed to natural changes in light with respect to duration, quality and intensity. In particular, radiation from short wavelengths, such as the ultraviolet waveband, which is particularly rich in energy, adds to the normal photosynthetic activity, leading to the formation of reactive oxygen species e.g. singlet-oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and radicals like the superoxide anion radical ($O_2^{-\bullet}$) and hydroxyl radical ($OH^{\bullet}$) (Asada, 1994a). The light-dependent generation of active oxygen species is termed photooxidative stress. It can occur in two ways: (1) the donation of energy or electrons directly to oxygen as a result of photosynthetic activity; (2) exposure of tissues to UVR (Foyer et al., 1994). When produced, active oxygen species are eliminated rapidly by efficient antioxidative systems. The chloroplast is able to use the production and destruction of H_2O_2 to regulate the thermal dissipation of excess excitation energy. This is an intrinsic feature of the regulation of photosynthetic electron transport. Photoinhibition and photooxidation only usually occur when plants are exposed to stress (Foyer et al., 1994). Production of ROS occurs especially under stress conditions as for example during exposure to excessive light or UVR as well as during desiccation, under nutrient deficiency, exposure to heavy metals, high or low temperatures and temperature changes (McKersie and Lesham, 1994; Collen and Davison, 2001; He and Häder, 2002; Okamoto and Colepicolo, 1998). Environmental stress in general leads to formation of ROS (Fourcroy, 1999) disturbing the steady-state balance of prooxidants and antioxidants (Bowler et al., 1992; Schreck et al., 1996). This imbalance in the oxidative cell metabolism towards a prooxidant state is known as oxidative stress (Sies, 1993).

Possible sources and production sites of ROS are: 1.) autoxidation reactions, including "redox cycling" by chinones, aromatic nitrocompounds and hydroxylamines, redox colours (e.g. paraquat), melanine, thiols, tetrahydropteridine, flavines and ferric complexes; 2.) Enzymatic reactions and proteins like cytochrome P-450, ferredoxine, hemoglobin, xanthine oxidase and peroxidases; 3.) Cellular sources are the mitochondrial and microsomal electron transport chains and the chloroplasts (PS I); 4.) Environmental factors include UVR, ultrasonics, X-rays, gamma radiation as well as toxic chemicals and metal ions, introduced into the environment due to human impact (Elstner, 1990).

Hydrogen peroxide occurs in important metabolic reactions, as it is formed during the process of oxygenases action in glycosysomes and peroxisomes, and during photosynthesis in chloroplasts (Asada, 1992; Ishikawa et al., 1993). Other sources for production of H_2O_2 are the pseudocyclic photophosphorylation and the Mehler reaction (Pedersen et al., 1996; Polle, 1996) under high light conditions (Collen et al., 1995).

1.6 Effects of oxidative stress

Although the release of the superoxide anion from the thylakoid membrane is suppressed, in chloroplasts even under conditions favourable to photosynthesis, the production rates of $O_2^{\bullet-}$ and its disproportionation product H_2O_2 are 240 and 120 µM s^{-1}, respectively. Those production rates are estimated maximal production rates basing on measurements with isolated spinach chloroplasts, assuming a chloroplast volume of 35 µl (mg Chl)$^{-1}$ (Asada and Badger, 1984). These amounts inhibit photosynthesis, by the inactivation of the stromal enzymes, within several seconds. The operating scavenging systems for $O_2^{\bullet-}$ and H_2O_2 are therefore essential for the maintenance of photosynthesis (Asada, 1994b).

When $O_2^{\bullet-}$ and H_2O_2 are not scavenged in chloroplasts, the metal-catalysed Haber-Weiss reaction produces the hydroxyl radical ($OH^{\bullet}$). Because of the high reactivity of the $OH^{\bullet}$ with almost any cell molecules, it interacts with chloroplast components at the molecular site where it is generated (Asada, 1994b).

The impact of hydrogen peroxide impairs photosynthesis by inhibiting a number of photosynthetic enzymes (Elstner, 1982; Elstner, 1987) such as Rubisco (Asada, 1994b; Badger et al., 1980; Bischof et al., 2000; Tanaka et al., 1982) and other enzymes such as superoxide dismutase (SOD) (Asada et al., 1975; Forti and Gerola, 1977). It has been shown that several other important enzymes, such as NADP-glyceraldehyde-3-phosphate deydrogenase, fructose-1,6-bisphosphatase and ribulose-5-phosphate kinase are very sensitive to H_2O_2 due to the oxidation of their functional thiol groups. These enzymes appear to be the target enzymes of H_2O_2 in the stroma. The inhibition of any of these enzymes would suppress the operation of the CO_2-fixation cycle in the chloroplasts (Asada, 1994b). In contrast to the stromal enzymes, the thylakoid proteins and electron carriers are comparatively unaffected by superoxide and hydrogen peroxide. At the concentration of H_2O_2 which completely

inhibits CO_2 fixation, photosynthetic electron transport is not damaged (Asada and Takahashi, 1987). Under conditions were the photon flux is higher than the capacity of the acceptor system of the chloroplast, a combination of high light and low CO_2 causes an inactivation of PSII. The inactivation site has been identified as the D1 protein of the PSII core complex (Kyle et al., 1987). In conditions of excessive irradiation, the quinone electron acceptor (Q_A) and the phaeophytin (Ph) in the PSII reaction centre become over-reduced. This induces the production of triplet-excited reaction centre chlorophyll $^3P_{680}$ and reduced phaeophytin (Ph^-). In the presence of oxygen, singlet-excited oxygen ($^1O_2$) is generated by the rapid reaction between ground state, triplet-oxygen ($^3O_2$) and $^3P_{680}$. The $^1O_2$ generated in the reaction-centre oxidizes the D1 protein itself or binding pigments, leading to degradation of the proteins.

1.7 Antioxidants

For the maintenance of all metabolic processes including photosynthesis, under stress conditions, cellular protection mechanisms are essential (Allen, 1977; Asada and Takahashi, 1987; Elstner, 1982; Halliwell, 1982). Enzymatic detoxifying systems and non-enzymatic constituents (antioxidants), belonging to different chemical groups, are known to diminish oxidative stress, by reducing ROS to less toxic and less reactive products (Pedersen et al., 1996). Common powerful detoxifying systems are the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT), as well as enzymes involved in the regeneration of low molecular antioxidants such as the glutathione reductase (GR) and dehydroascorbate reductase (DHAR) (Foyer et al., 1997). Further low molecular antioxidants belong to the group of phenolic compounds such as flavonoids, coumarins and tocopherols, nitrogen containing compounds including alkaloids, chlorophyll derivates, amino acids and amines as well as other compounds as carotenoids, ascorbic acid and glutathione (Fujimoto et al., 1985; Larson, 1988; Paya et al., 1992; Potterat, 1997). In marine algae, the antioxidative enzymes SOD, APX, CAT and GR, as well as ascorbic acid, β-carotene and α-tocopherol are well known antioxidants (Aguilera et al., 2002a; Castillo et al., 1986; Collen and Davison, 1999; Collen and Davison, 1999; Honya et al., 1994; Jayasree et al., 1985; Nakamura et al., 1994; Potterat, 1997).

The antioxidative enzymes react with distinct reactive oxygen species and convert them to less toxic compounds. Superoxid-dismutases catalyse the conversion of $O_2^{\bullet-}$ to H_2O_2 and oxygen. H_2O_2 is deprotonated by the enzyme catalase. H_2O_2 is also reduced by ascorbate peroxidase (APX) via the ascorbate-glutathione cycle. Ascorbic acid is dehydrated to monodehydroascorbate and dehydroascorbate and recycled to ascorbic acid by monodehydroascorbate reductase and dehydroascorbate reductase. Both enzymes are dependent on $NADPH+H^+$, the latter enzyme coupled with GR, converting oxidized glutathione to reduced glutathione (Elstner, 1982; Halliwell, 1982). Glutathione reductase is important in the recycling of glutathione within the ascorbate-glutathione-cycle, and was first described by Smith et al. (1989) (see Figure 1).

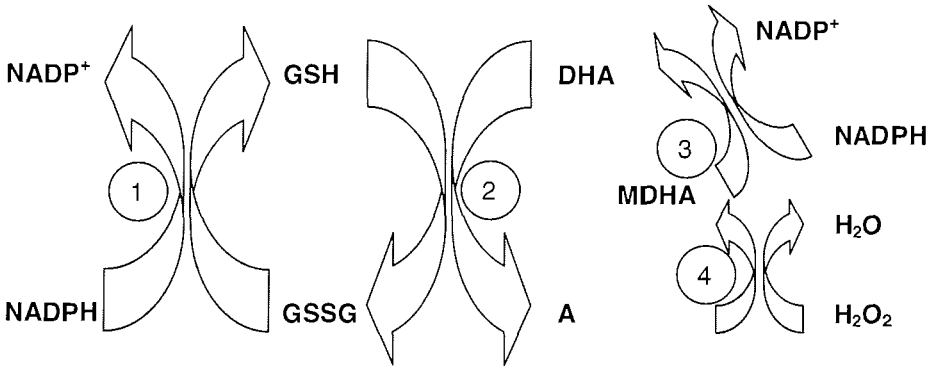

Figure 1: Glutathione-ascorbate cycle (Smith et al., 1989)
1 = Glutathione reductase; 2 = Dehydroascorbate reductase; 3 = Monodehydroascorbat reductase; 4 = Ascorbate peroxidase; A = Ascorbate; DHA = dehydroascorbate; GSH = reduced glutathione; GSSG = oxidized glutathione; MDHA = Monodehydroascorbate; $NADP^+$ = oxidized nicotinamide adenine dinucleotide phosphate; NADPH = reduced nicotinamide adenine dinucleotide phosphate

APX shows a higher affinity to H_2O_2 than CAT and is located in the chloroplasts, CAT is located in peroxisomes (Halliwell and Gutteridge, 1989; Polle, 1996).

1.8 Aims of the study

The aim of the present study was to describe the antioxidative properties of marine macroalgae from the Arctic, and to assess the induction of oxidative stress and protective antioxidative responses in relation to increasing UVR caused by the reduction in the ozone layer. Experiments were designed in order to induce direct oxidative stress by exposure to H_2O_2 and indirect stress by exposure to UVR and to describe macroalgal responses on the physiological level. In particular, the following research questions were addressed:

- What is the qualitative and quantitative distribution of antioxidants present in macroalgae from Arctic ecosystems?
- Are there specific antioxidative compounds related to Arctic macroalgae?
- How are the reaction patterns of different algal species to direct and indirect oxidative stress?
- Is there a seasonal variation in antioxidative defence mechanisms?

1.9 Thesis outline

During the last few years, much research has been directed to the potential effects of UVB on plant life. Most studies were focused on higher plants, especially crop plants, due to their economical significance (Fiscus and Booker, 1995). With regards to marine macroalgae, recently there have been a multitude of studies performed on photosynthesis, growth and respiration of Arctic species with emphasis on UV effects (Aguilera et al., 2002a; 1999a; b; Bischof et al., 1998a; 1999; 2000; 2002; Hanelt et al., 2001; Karsten et al., 2001; Wiencke et al., 2000). As the problem of ozone depletion is most drastic in polar regions of both hemispheres, and due to the fact that macroalgae play a central role in Arctic coastal ecosystems, such studies were urgently needed. However, there are still open questions concerning indirect UV effects in form of oxidative stress which form the focus of this study.
This thesis gives an overview of the antioxidative defence mechanisms present in marine macroalgae from the Arctic Kongsfjord and presents reaction patterns in response to varying abiotic factors like daylength, spectral composition of the light and salinity, in short-term and long-term changes.

The occurrence of common biochemical antioxidative defence systems in marine macroalgae from the Arctic Kongsfjord is described in the first part of **publication I**. In the second part the described effects of UVR on the enzymatic defence systems of selected species contribute to the knowledge of UV effects on growth and other physiological features, such as photosynthesis and respiration, damage in DNA, RNA and proteins.

Publication II focuses on the interactive effects of UVR and salinity on the ecophysiology of *Palmaria palmata* and *Devaleraea ramentacea* from shallow waters of the Kongsfjord (Spitsbergen, Norway). As field observations in shallow waters of the Kongsfjord during summer season showed bleaching in *P. palmata* but much less in *D. ramentacea*, although inhabiting the same location, it was presumed that UVR was a responsible factor for this phenomenon. But as former studies on the UV sensitivity of these species showed high tolerance to increasing PAR and UV doses we assumed that the decrease of salinity down to 23 PSU (Hanelt et al., 2001) as a result of the large discharge of melting water into the fjord was responsible for the bleaching of *P. palmata*. Therefore a comparative study on the two red algae was performed under controlled conditions on Spitsbergen. In hyposaline and hypersaline media in combination with artificial UVR the interactive effects of both environmental factors on photosynthetic performance, as well as on the ability to synthesise and accumulate UV-absorbing MAAs were investigated.

In **publication III** I developed an assay for the detection of the antioxidative potential of marine macroalgae to select those species with a low sensitivity to oxidative stress for further investigations on their antioxidative abilities and compounds. In a second part of the study, the common antioxidative defence systems as there are the enzymes APX, CAT, SOD and GR, as well as the pool size of ascorbic acid were investigated under H_2O_2 stress in *Polysiphonia arctica*. This species exhibited the lowest susceptibility towards H_2O_2 equated with the highest antioxidative potential and two new bromphenolic compounds were found to be responsible for this high potential.

Accumulation of H_2O_2 in seawater occurs as a result of UVR induced formation of ROS, especially in coastal waters rich in DOM. In this context, the abilities of three

different green macroalgae to cope with H_2O_2 stress was the focus of **publication IV**. Based on the methods developed and described in publication III species of green algae from the Arctic were examined for their antioxidative properties. Two *Chaetomorpha* species and *Acrosiphonia* sp. were placed under H_2O_2 stress. Their tolerance to H_2O_2 was different and could be explained by differences in antioxidative properties related to their specific growth habitats.

Publication V and VI focus again on the UV effects on a variety of physiological parameters in the two red macroalgae *Palmaria palmata* (**Pub. V**) and *Devaleraea ramentacea* (**Pub. VI**) with respect to seasonal variations in radiation regime. In a long-term culture experiment the variations in growth rates, pigment concentrations, protein content, the activity of the antioxidative enzymes APX, CAT and SOD as well as the concentration of antioxidant ascorbic acid and the UV-protective MAAs were recorded in the course of one year, simulating the fluctuating daylengths present at the original collection site of Kongsfjord (Spitsbergen, Norway). In a second treatment with additional UVR, summer conditions in the Kongsfjord were simulated. This kind of experiment shows the balance between the various damaging effects of UVR and the protective and repair mechanisms. Growth was shown to be a useful indicator to assess UV effects in long-term experiments.

2 MATERIALS AND METHODS

2.1 Study site and sampling

All field work described in this thesis was carried out at the Arctic Kongsfjord situated at the northwestern coast of Spitsbergen (78°55,5'N; 11°56,0'E, Fig. 2). The Kongsfjord is about 20 km long and 4 to 10 km wide, with an estimated total volume of the water basin of 29.4 km^3. The annual mean water temperature lies slightly above 0 °C (Ito & Kudoh 1997), and can rise in surface waters from –1 in winter to 6 °C in summer, whereas at 20 m depth about 4 °C are measured (Hanelt et al. 2001). During summer strong sedimentation and salinity gradients are formed along the main axis of Kongsfjord, in response to freshwater input of the glaciers, snow and rain. In consequence, salinity can drop from an average of 34 PSU to down to 20 PSU. The sediment input by glaciers and glacier run-offs strongly influences the water transparency and the radiation conditions in the water (Hanelt et al. 2001). Pronounced seasonal changes in light regime, salinity and temperature are characteristic for the study site, and specific for Arctic fjords in general (Hanelt et al. 2001).

Two major zones can be differentiated: 1.) the inner part with relatively shallow water less than 100 m deep, strongly influenced by large tidal glaciers and their input of sediment, ice and freshwater and 2.) the outer and deeper part (max. depth of 400 m), strongly influenced by the Spitsbergen current, streaming on the west coast of Spitsbergen (Hop et al. 2002; Svendsen et al. 2002). Because of this Atlantic influence the fjord is to be regarded as sub-Arctic rather than Arctic, despite the location at high latitude (Hop et al. 2002).

Algae were collected by SCUBA diving in summer 1999 (June to August) and summer 2000 (May to July) at different sampling sites and depths between 0-25 m. An overview on the distribution of the species used for this thesis is given in Figure 2 and Table 1, as an example *Palmaria palmata* and *Devaleraea ramentacea* are shown in Figure 3.

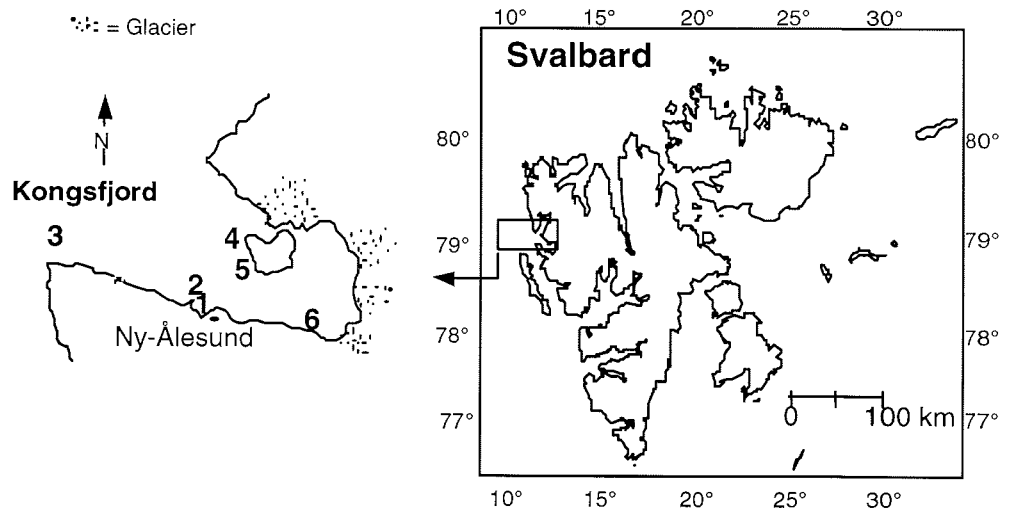

Figure 2: General view of Spitsbergen and the location of Kongsfjorden. Numbers indicate the different sampling sites: 1 Harbour and Nansen bay, 2 Brandal, 3 Kongsfjordneset, 4 Hansneset, 5 London, 6 Bird rocks

a b

Figure 3: a) *Palmaria palmata* (L.) Greville) and b) *Devaleraea ramentacea* (L). Guiry from Arctic Kongsfjord (Spitsbergen, Norway)

Table 1: List of species sampled at the different sampling sites in the Kongsfjord (see Fig.1), depth distribution and sampling depth (m)

Species	Sampling site	Depth distribution
Chlorophyta		
Acrosiphonia penicilliformis (Foslie) Kjellman	2, 4, 5	2-20
Monostroma arcticum Wittrock	1, 2	1-9, 8-20
Chaetomorpha linum (Müller) Kützing	1	6-8
Chaetomorpha melagonium (F. Weber et Mohr) Kützing	1, 3, 4	0-4, 3-6, 0-2
Prasiola crispa (Lightfoot) Knebel	6	>0
Rhodophyta		
Coccotylus truncatus (Pallas) M.J. Wynne & J.N.Heine	5	10-24
Devaleraea ramentacea (L.) Guiry	1, 2	1-8
Palmaria palmata (L.) Grev.	1, 2	2-10
Phycodrys rubens (L.) Batters	4, 5	2-20
Odonthalia dentata (L.) Lyngb	4, 5	2-20
Polysiphonia arctica J. Agardh	5	5-20
Ptilota gunneri P.C. Silva, Maggs & L.M. Irvine	4, 5	2-20
Phaeophyta		
Alaria esculenta (L.) Greville	4	3-15
Chorda tomentosa Lyngbye	1, 2	2-12
Chordaria flagelliformis (O. F. Müller) C. Agardh	1, 2	2-12
Desmarestia aculeata	2, 4, 5	1-20
Elachista fuciola (Velley) Arechoug	1	0-4
Fucus distichus L.	1	0-4
Laminaria saccharina (L.) Lamouroux	2, 4	2-15
Laminaria solidungula J. Agardh	5	8-15
Laminaria digitata (Huds.) Lamouroux	1, 2, 4	0.5-12
Sacchorhiza dermatodea (de la Pylaie) J. Agardh	2	2-15

2.2 Cultivation techniques and irradiance applied

In all laboratory experiments the following radiation sources were used: 1.) Osram L 36 W/12-950 or L58/ W19 daylight fluorescent lamps and 2.) special UV-fluorescent tubes (UVA 340, Q-panel, USA), which emit a spectrum below 340 nm, which is similar to the solar one. The emission spectrum of the fluorescent tubes was measured with a spectroradiometer Spectro 320D (Instruments systems, Munich, Germany) (Figure 4). To avoid high light effects in the experiments no additional white light sources were used in the experiments. For this reason the PAR:UVR ratio is lower in the experimental set-ups as compared to the solar radiation spectrum.

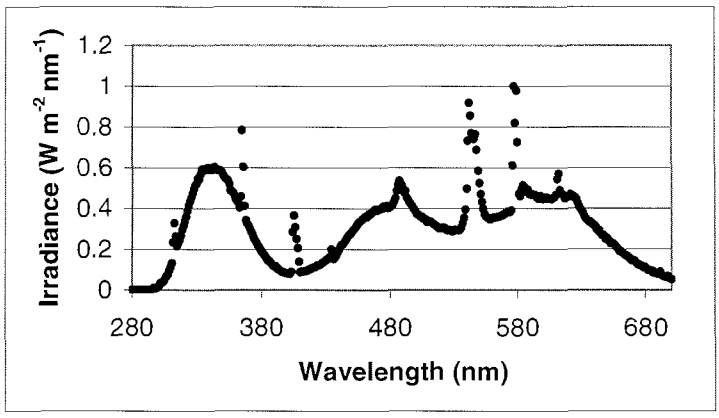

Figure 4: Radiation spectrum in the range from 280 to 700 nm emitted by daylight fluorescence lamps in combination with Q-panel UVA-340 fluorescent tubes

2.3 Measurements of photosynthesis

The direct and indirect effects of reactive oxygen species on the overall photosynthetic activity have been assessed by measuring the emission of variable chlorophyll fluorescence of PS II with the portable pulse-amplitude modulated underwater fluorometer (Diving-PAM, Walz, Germany). The physiological basis of this technique is reviewed by Schöner & Krause (1990) and Schreiber et al. (1994). Maximum quantum yield of photosynthesis, measured as the ratio of variable to maximal fluorescence (Fv/Fm), reflects the efficiency of energy transfer from the

antennae to the reaction centre. The exact determination of this parameter in Arctic marine macroalgae is described in detail by Hanelt (1998).

2.4 Measurement of growth

Growth rates were determined by recording fresh weight of algae after blotting with paper tissue using the following equation:

$$\text{specific growth rate (\% day}^{-1}) = \frac{100 \ln W_t W_0^{-1}}{t}$$

where W_0 = initial fresh weight, W_t = fresh weight on day t, and t = time interval (Wiencke & tom Dieck 1989). Five individuals per photon fluence rate were used for the growth measurements and mean values with standard deviation calculated.

2.5 Antioxidative enzymes

The respective activities of the various antioxidative enzymes under investigation were measured using the same crude extract. For this reason an average pH of 7.0 was chosen for the extracting potassium phosphate buffer. The enzyme measurements were optimised for performance in a microtiter plate spectrophotometer (Spectramax, Molecular Devices, Sunnyvale CA94089, USA) as described in detail in Pub. III.

Ascorbate peroxidase was measured according to Chen & Asada (1989). APX activity was calculated by subtracting the non-enzymatic background reaction and using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbic acid. Catalase was analysed according to Aebi (1984). Calculation of the enzyme activity was reached by subtracting the non-enzymatic reaction and using an extinction coefficient of 0.0398 mM^{-1} cm^{-1} for H_2O_2. Glutathione reductase was assayed according to Goldberg & Spooner (1983). GR activity was calculated by subtracting the non-enzymatic reaction and using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADPH. Results for CAT, GR and APX are expressed as units (U) of enzyme activity per mg of total soluble protein [1 U= 1 µmol substratum (H_2O_2, NADPH and ascorbic acid respectively) converted min^{-1}]. SOD activity was measured using the xanthine oxidase-cytochrome *c* reduction method (McCord & Fridovich 1969) as modified by Aguilera et al. (2002). One unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%.

2.6 Ascorbic acid

The measurement of ascorbic acid followed Foyer et al. (1983) and was modified for use of a microtiterplate spectrophotometer. Measurements were performed by decreasing absorbance at 265 nm after addition of 10 U mL^{-1} ascorbate oxidase and 10-50 µL sample to 1925-1965 µL sodium phosphate buffer (100 mM, pH 5.6). Quantification took place by using a standard curve with 1.25-12.5 µM of pure ascorbic acid in the reaction mixture.

2.7 Mycosporine-like amino acids (MAAs)

A 25% aqueous methanol (v/v) extraction was made from 10-20 mg dry weight (DW) of the algal samples. After evaporating to dryness under vacuum (Speed Vac Concentrator SVC 100H) dried extracts were re-dissolved in 100% methanol. Samples were analysed with a Waters high-performance liquid chromatography (HPLC) system according to Hoyer et al. (2001).

2.8 Statistics

Mean values and standard deviations were calculated from four to five independent replicates per treatment. Statistical significance was tested with either a Student's t-test or with a model 2 two-way ANOVA (Statistica) followed by a Fishers protected least significant difference test (LSD) (Sokal & Rohlf 1995). Significance level was $p<0.05$.

2.9 Overview on the experiments performed and the respective parameters under investigation

Screening for antioxidants

Quantitative and qualitative distribution of antioxidants in marine macroalgae from Arctic and cold-temperate regions

Measurements:

SOD, APX, CAT, GR, ascorbate Pub. I

Physiological response patterns to UVR and salinity

UVR exposure experiments under natural sunlight in field and under artificial UVR exposure in laboratory

Measurements: Photosynthetic efficiency (Fv/Fm), MAAs Pub. I, II

Physiological response patterns against oxidative stress

H_2O_2 exposure experiments

1.) short-term exposure as assay for the detection of the antioxidative potential

Measurements:

- Photosynthetic efficiency (Fv/Fm) as indicator for resistance against oxidative stress.
- Antioxidative activities of *Polysiphonia arctica* extract in different test system:
  a) Thiobarbituric acid reactive substances (TBARS)
  b) α,α-diphenyl-β-picrylhydrazyl radical (DPPH) assay
- Characterisation and identification of antioxidative compounds (GCMS)

Pub. III

2.) long-term exposure

Measurements:

Antioxidative enzymes, ascorbic acid Pub IV

Seasonal variation in ecophysiological patterns with emphasis on UV protective mechanisms

Measurements:

Growth, pigments (Chl a, phycobiliproteins), protein content, ascorbic acid, antioxidative enzymes, MAAs Pub. V, VI

3 RESULTS

3.1 Qualitative and quantitative distribution of antioxidants in Arctic macroalgae

A survey of the qualitative and quantitative content and activities of different reactive-oxygen-scavenging enzymes and ascorbic acid was performed in 22 macroalgal species from the Arctic Kongsfjord (**Publication I**). We detected a great variation in antioxidant enzyme activities, and found large species-specific differences. Clear differences between the three macroalgal groups were found, with green algae showing in general higher antioxidant enzyme activities than red and brown algae. SOD activities in all species tested were significantly higher ($p<0.05$) compared to GR, APX and CAT.

A relationship between antioxidant activities and depth distribution was shown, indicating the acclimation of protective mechanisms to abiotic stress along the depth gradient. UVR penetrating into the water column is attenuated with increasing water depth, therefore algae need less protection in deeper water layers. In particular, differences in SOD activity between the different algal groups were related to depth distribution on the shore (Figure 5; Table 2 in **Pub. I**). Most green algae inhabiting the upper part of the shore of the Kongsfjord, showed higher antioxidant activities. The only deviation from this pattern showed *Prasiola crispa*, living in the supralittoral, underneath bird colonies. This species exhibited very low antioxidant enzyme activities in comparison to other members of the Chlorophyta. As this alga inhabits an unusual habitat another photoprotective strategy has probably been developed, such as the biosynthesis of UV-absorbing compounds. Two new mycosporine-like amino acids (MAAs), so far uncharacterised have been reported by Hoyer et al. (2001) in this species.

Depth dependency of the antioxidative enzymes was also obvious within the group of red algae, in which *Devaleraea ramentacea* (DR) and *Palmaria palmata* (PP), typically inhabiting the upper sublittoral, exhibited highest antioxidant activities compared to other red-algal species living in deeper waters, such as *Phycodrys rubens* (PR) (Figure 6; Table 2 in **Pub. I**).

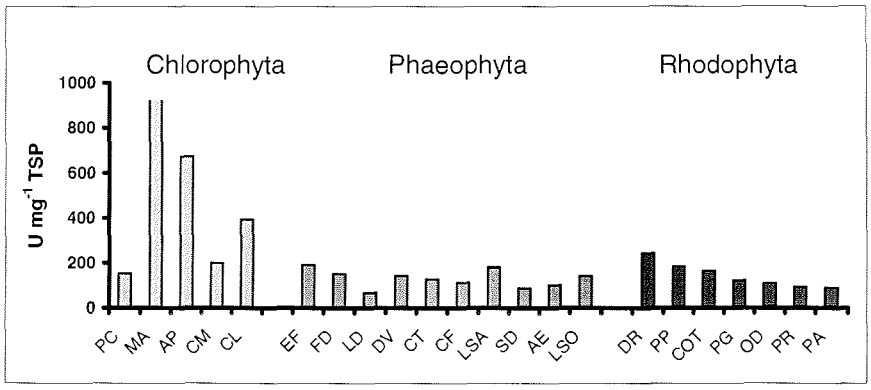

Figure 5: Activity of SOD (U mg^{-1} TSP) in the three major algal groups

Species key (from left to right): PC= *Prasiola crispa*; MA= *Monostroma arcticum*; AP= *Acrosiphonia penicilliformis*; CM= *Chaetomorpha melagonium*; CL= *Chaetomorpha linum*; EF= *Elachista fuciola*; FD= *Fucus distichus*; LD= *Laminaria digitata*; DV= *Desmarestia viridis*; CT= *Chorda tomentosa*; CF= *Chordaria filliformis*; LSA= *Laminaria saccharina*; SD= *Saccorhiza dermatodea*; AE= *Alaria esculenta*; LSO= *Laminaria solidungula*; DR= *Devaleraea ramentacea*; PP= *Palmaria palmata*; COT= *Coccotylus truncatus*; PG= *Ptilota gunneri*; OD= *Odonthalia dentata*; PR= *Phycodrys rubens*; PA= *Polysiphonia arctica*

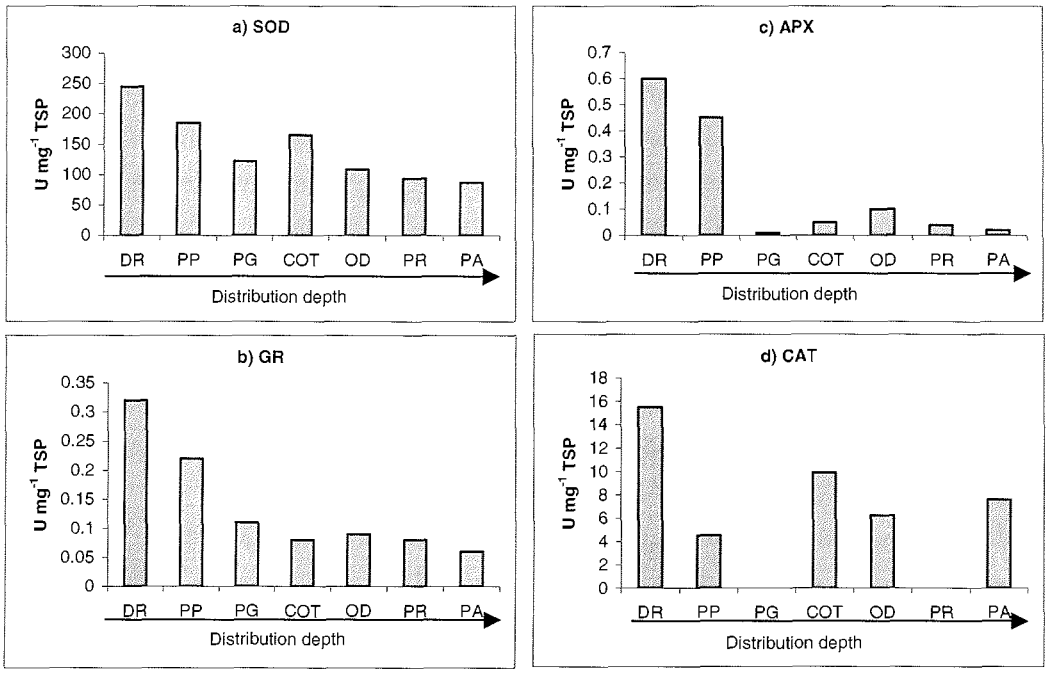

Figure 6: Enzyme activities (U mg^{-1} TSP) of a) SOD, b) GR, c) APX and d) CAT within the Rhodophyta, sorted by their distribution depth from left to right. Species key Fig. 5.

Ascorbic acid content also followed these patterns, showing high values within the Chlorophyta, as well as in species inhabiting the upper part on the shore (Table 2 in **Pub. I**).

- Antioxidant enzyme activities and ascorbic acid content show great variation within the investigated species
- SOD activities were significantly higher than GR, APX and CAT activities
- A relation between antioxidant activities and depth distribution could be shown, i.e. high activities in the eulittoral and upper sublittoral, low activities in the deeper waters indicating inverse correlation between UV exposure and antioxidative enzymes
- Species deviating from this pattern show other protective mechanisms

3.2 Effects of UVR on the ecophysiology of Arctic marine macroalgae

3.2.1 Effects of UVR on the antioxidative defence mechanisms

Ultraviolet radiation leads to a significant enhancement in enzyme activities of GR in laboratory experiments. This was shown in the green alga *Monostroma arcticum* and in the two red macroalgae *Coccotylus truncatus* and *Phycodrys rubens* (Figure1 a-c in **Pub. I**). Even in recovery phases between successive exposures GR activity increased. In two other red macroalgae, *Palmaria palmata* and *Devaleraea ramentacea,* no statistically significant UV effect on GR could be shown in permanent light but a trend to increasing activities under theses conditions was observed (Figure 2 a-b in **Pub. I**). SOD activity was either not affected by exposure to UVR as in *C. truncatus* and *D. ramentacea* or showed significant decrease in the first 24 h of exposure as measured in *M. arcticum* and *P. rubens* (Figure 1d-f, 2f in **Pub. I**) and also after further exposure in *P. palmata* (Figure 2c in **Pub. I**). For APX an inconsistent activity pattern was observed with high variation in *P. palmata* and *D. ramentacea* (Figure 2 b, e in **Pub. I**).

In a third set of radiation experiments, the effect of natural and artificial UVR was compared. In the laboratory artificial UVR was added to the PAR control, whereas in the field UVR was filtered out by cut-off foils. Artificially added UVR did not affect GR

and SOD activities in *Acrosiphonia penicilliformis*, but exclusion of UVR in the field reduced activities in both enzymes significantly (Figure 3 in **Pub. I**).

Effects of UVR on the antioxidants were also surveyed in a long-term culture study mimicking the seasonal course of daylength at the Kongsfjord (**Pub. V, VI**). In general, no or only low differences in antioxidative enzyme activities were measured between the PAR control and UV treated thalli. The seaonal pattern will be presented in section 3.4.

- UVR effects antioxidative enzymes activities
- The responses to UVR are species-specific
- Effects of artificial (laboratory) and natural (field) UVR on the antioxidant response differ remarkably

3.2.1 Interactive effects of UVR and salinity on the ecophysiology of Arctic marine macroalgae (Publication II)

The two red macroalgae *Palmaria palmata* and *Devaleraea ramentacea* were exposed to ultraviolet radiation in combination with hyposaline and hypersaline media to evaluate the interactive effects of both environmental parameters on optimum quantum yield of photosynthesis (Fv/Fm) as well as on the physiological capability to synthesise and accumulate photoprotective MAAs. *D. ramentacea* exhibited euryhaline features and acclimated well to the UV radiation applied, whereas *P. palmata* showed high mortality even under mild hyposaline conditions (15 PSU), therefore being characterised as a stenohaline type. In both species, Hypersaline conditions (50 PSU) led to a stronger decrease in Fv/Fm than hyposaline conditions, whereas the contrary was observed for the course of recovery, which was slower and incomplete under hyposaline conditions (Figure 1, 2, 5, 6 in **Pub. II**). Fv/Fm decreased even stronger in thalli treated with UVR and salinity changes compared to experiments conducted in absence of UVR, but Fv/Fm was stronger affected by salinity as by UVR as shown in Figure 3, 7 in **Pub. II**. The percentage of inhibition of Fv/Fm mediated by UVR decreased in the course of the experiment, indicating the activity of acclimation mechanisms. The effect of UVR and salinity on the synthesis and accumulation of MAAs showed a clear induction under UV in both species: *P.*

palmata exhibited an increasing MAA content in the UV treatment without salinity change nearly by factor 3 in comparison to *D. ramentacea* doubling its MAA content under 34 PSU and UV. Obviously the protection by MAAs was sufficient for *D. ramentacea* but not for *P. palmata*.

- *Devaleraea ramentacea* resists different environmental stress factors and shows high physiological plasticity
- *Palmaria palmata* shows a marked sensitivity against salinity and a limited capability to acclimate to changing radiation conditions
- UV-protection by MAAs is apparently different in the two species

3.3 Physiological response patterns against oxidative stress

3.3.1 Short-term exposure to H_2O_2

An assay for the detection of the antioxidative potential of marine macroalgae was developed (**Publication III**) to give a hint on biological interesting species, probably containing new antioxidative compounds. The assay consisted of a short-term exposure to extremely high concentrations of H_2O_2 (0-20 mM) accompanied by the measurement of the photosynthetic activity before and after the H_2O_2 exposure. A high photosynthetic efficiency, as ratio of variable to maximal chlorophyll fluorescence (F_v/F_m), after exposure to high H_2O_2 concentrations/high oxidative stress, was regarded as being indicative for a low susceptibility to H_2O_2 and equated with a high antioxidative potential.

Species maintaining a high photosynthetic efficiency under H_2O_2 stress were *Chaetomorpha melagonium* (Chlorophyta, Figure 3 in **Pub. III**), *Fucus distichus* (Phaeophyta, Figure 2 in **Pub. III**) and *Polysiphonia arctica* (Rhodophyta, Figure 1 in **Pub. III**). This study shows that measurements of the *in vivo* fluorescence of photosystem II is a suitable tool to determine the effect of oxidative stress on macroalgae. *P. arctica* exhibited the highest F_v/F_m values of all the investigated species under H_2O_2 stress although this species had rather low enzyme activities within the survey of antioxidative properties (see **Pub. I**). *P. arctica* inhabits greater water depths and may be characterised as low-light adapted, rarely being exposed to

UVR. This indicates that other antioxidative substances are present in this species, proved by the existence of two new bromophenolic compounds (Figure 9 in **Pub. III**).

- Measurement of the *in vivo* fluorescence of photosystem II under H_2O_2 is a suitable tool to determine the effect of oxidative stress on macroalgae
- Species showing a high H_2O_2 tolerance should contain high concentrations or activities of antioxidative substances
- Two new bromophenolic compounds were found in *Polysiphonia arctica*

3.3.2 Long-term exposure to H_2O_2

The effect of H_2O_2 on different ecophysiological parameters in marine macroalgae was investigated in the red macroalga *Polysiphonia arctica* (**Pub. III**). *P. arctica* was able to increase APX and CAT activities under exposure to high concentrations of H_2O_2. Although not exposed to UV-induced oxidative stress, this species is populated by a dense layer of benthic diatoms, oxidative stress may also origin from the photosynthetic activity of these epiphytes.

- *Polysiphonia arctica* increases APX and CAT activity in response to H_2O_2 stress

The effect of H_2O_2 on the antioxidative properties of three green macroalgae was investigated in **Publication IV**. *Chaetomorpha melagonium*, relatively insensible towards H_2O_2 showed good protection against H_2O_2 stress by high enzyme activities and an high ascorbic acid content. Whereas SOD and APX activity remained relatively stable with increasing H_2O_2 stress, CAT activity decreased and GR activity increased along the H_2O_2 gradient. In contrast, *Acrosiphonia* sp. and *Chaetomorpha linum*, were more susceptible to H_2O_2. *Acrosiphonia* sp. disposed of increasing SOD and CAT activity but exhibited much less ascorbic acid content as the two *Chaetomorpha* species. *C. linum* tolerated H_2O_2 stress up to 1 mM but at higher concentrations, photosynthetic activity was reduced drastically. SOD activity showed the same pattern, whereas APX and CAT activity were significantly enhanced under rising H_2O_2 stress. Ascorbic acid also served as highly effective scavenger in this species. The three species exhibit different depth and habitat preferences. *C. linum*

prefers 5-7 m depth and sheltered areas, *C. melagonium* is found in clear shallow waters with high frequency of water exchange between 1.5 and 5 m, whereas *Acrosiphonia* sp. is found from the upper eulittoral down to 10 m depth. Comparing the three investigated Arctic green algal species to each other, they have different biochemical capabilities of the enzymatic defense systems against H_2O_2 that are well reflected by the prevailing light conditions at different water depths.

- Exposure to H_2O_2 may increase or decrease enzyme activities of SOD, GR, CAT and APX in the investigated green algal species, the responses are species-specific
- Exposure to H_2O_2 decreases ascorbic acid content with increasing concentration and duration of exposure

3.4 Seasonal variation of antioxidants, pigments and UV-protective substances and growth

The seasonal variation in antioxidative properties, as well as concentrations of pigments, total soluble protein and MAAs were studied in the two red macroalgae *Palmaria palmata* (**Pub. V**) and *Devaleraea ramentacea* (**Pub. VI**). Growth was surveyed to integrate the positive and negative effects of UVR on the entire metabolism of the alga. As already seen in section 3.2 the UV effects were rather low, exhibiting statistical significance only in a few cases.

Pigment concentrations of chlorophyll a, phycoerythrin (PE) and phycocyanin (PC) were regulated depending on light demand of the algae and seasonal light supply. Highest concentrations were measured in spring and autumn, and lowest values in summer when permanent light exposure required an adjustment in the size of the photosynthetic apparatus in order to protect the algae from excessive light energy (Figure 2a-c in **Pub. V, VI**). The antioxidative enzymes APX, CAT and SOD seemed to be regulated via a photoperiodic control. High enzyme activities were maintained during winter, when no oxdiative stress was present, presumably to prepare the alga for the rapid increase in irradiance in spring accompanied with oxidative stress (Figure 5a-c in **Pub. V, VI**). Both species exhibited high ascorbic acid contents,

serving as effective scavenger in times of oxidative stress. The ascorbic acid pool was also high in winter in thalli being exposed to UV in the previous radiation period. So the ascorbic acid pool sizes adjusted to the stress, the alga was exposed to and will be exposed to in the next radiation period. In both species the synthesis and accumulation of MAAs was turned to the seasonally changing radiation conditions, exhibiting maximal concentrations in spring, when highest protection is needed (Figure 6 in **Pub. V, VI**).

Devaleraea ramentacea and *P. palmata* showed a clear seasonal growth pattern classifying both species as season anticipators after Kain (1989) and Lüning and tom Dieck (1989). Positive and negative effects of UVR on the ecophysiological parameters appear to be outbalanced since no differences between PAR and PAR+UV treated algae could be shown, indicating effectively working protection mechanisms against UVR and the accompanying oxidative stress.

- Pigment concentrations acclimated seasonally in dependency of light demand and supply
- Antioxidative enzyme activities are under photoperiodic control
- Ascorbic acid is an effectively working scavenger for oxidative stress
- MAAs are effective UV protectants
- No UV effects on growth were detected

4 GENERAL DISCUSSION AND CONCLUSIONS

4.1 General aspects on the occurrence of antioxidative substances in marine macroalgae

In this study, the qualitative and quantitative distribution of biochemical defence mechanisms against photooxidative stress was described for the first time in marine macroalgae from the Arctic (**Pub. I**). The occurrence of antioxidants in higher plants and microalgae is already well described (Larson, 1988; Mallick and Mohn, 2000), but for marine macroalgae information is very limited: For single species, data on antioxidants were already available, as e.g. for intertidal *Fucus* species and the two red macroalgae *Chondrus crispus* and *Mastocarpus stellatus* from temperate regions (Collen and Davison, 1999a; b; c). For the occurrence of ascorbic acid and α-tocopherol two screenings in macroalgae from India have been performed (Jayasree et al., 1985; Sarojini and Sarma, 1999).

Generally, the antioxidative activities measured in macroalgae are comparable to those found in higher plants and microalgae. For example, *Monostroma arcticum* exhibited the highest activity of all macroalgae investigated so far (Tab. 2 in Pub. I), which is in the same range as reported for pea leaves (Moran et al., 1994). CAT activity of *M. arcticum* is half as high as that measured in cotton fibres (Rajguru et al., 1999), whereas activities of SOD and GR were much higher. APX activities of *M. articum* and the two *Chaetomorpha* species investigated (Pub. I, IV) are comparable to those found in the symbiotic zooxanthellae of the sea anemone, *Aiptasia pallida* (Lesser and Shick, 1989) while CAT and SOD activities are much higher. Data with respect to other macroalgae are scarce.

With respect to latitudinal differences in antioxidative activities, only data measured in the Arctic *Fucus distichus* (Tab 2 in **Pub. I**) can be directly compared to those of related species from temperate waters (Collen and Davison, 1999a): CAT activity was similar to that reported by Collen and Davison (1999a), while SOD activity was much higher in the polar species and lower activities were measured for GR and APX. Enzyme activities of the green algal group are comparable to those of temperate *Ulva rigida* (Collen and Pedersen, 1996). In the group of red algae

enzyme activities of *Devaleraea ramentacea* and *Palmaria palmata*, exhibiting highest values within the Rhodophyta, are comparable to the temperate intertidal red algal species *Mastocarpus stellatus* and *Chondrus crispus* (Collen and Davison, 1999c).

The ascorbic acid concentrations found in green algae are generally very high and similar to those reported for citrus and other fruits (http://www.naturalhub.com/natural_food_guide_fruit_vitamin_c.htm). *Chaetomorpha linum*, for example, contains 0.65 mg g^{-1} FW, a concentration similar to that of orange and papaya, with approximately 0.53 and 0.62 mg g^{-1} FW. These exceptionally high values were also measured in *Fucus distichus* from Spitsbergen (**Pub. I**) and other *Fucus* species from temperate regions (Collen and Davison, 1999a).

As a general pattern this study revealed, that high antioxidative enzyme activities were found in the group of green algae and also in those algal species living in the eulittoral and upper sublittoral. These findings suggest a close relation between the respective antioxidative activity and the vertical distribution of species on the shore. This aspect will be discussed in detail in chapter 4.7.

In all species tested, SOD activities were significantly higher ($p<0.05$) compared to GR, APX and CAT, indicating a central role of this enzyme for the detoxification of ROS in marine macroalgae. As the superoxide anion radical is the primary product of the reduction of molecular oxygen, it is the most important ROS to be eliminated in the cells to suppress any further production of ROS (Asada and Takahashi, 1987). APX and CAT, which directly detoxify H_2O_2, as the second product within the oxygen reduction process, are also important, but exhibit much lower activities. Curiously, significant CAT activities could only be detected with 3.60 and 7.97 U mg^{-1} TSP in *Fucus distichus* and *Laminaria saccharina* within the brown algal species, indicating a minor role of this enzyme within the Phaeophyta. Compared to the other taxa and in contrast to the high concentration of antioxidants like ascorbic acid, the enzymatic antioxidative activities in brown algae are low, indicating that other antioxidative strategies are preferably active in this group. In this context it is referred to the typically high content of phenolic compounds present in brown algae (Ragan and Glombitza, 1986; Van Alstyne and Paul, 1990). These substances can act as

antioxidants by transferring hydrogen atoms to lipid peroxyl radicals (Foti et al., 1994), and also as UV sunscreens, as suggested by Pavia et al. (1997).

- Antioxidative activities in Arctic marine macroalgae are comparable to those of higher plants and microalgae
- The antioxidative enzymes are correlated to depth distribution
- Phaeophyta exhibit generally lower enzyme activities than Chlorophyta and Rhodophyta

4.2 Effects of UV-exposure on the antioxidative response of marine macroalgae

Within the present study, UVR was shown to be a major factor influencing the antioxidative properties of algae. GR activity was clearly induced in *M. arcticum*, *Coccotylus truncatus* and *Phycodrys rubens* (Fig. 1a-c in **Pub. I**) under artificial UV exposure similar as observed in *Pisum sativum* (Mackerness et al., 1999). In *Palmaria palmata* and *Devalereaea ramentacea*, in contrast, no increase in response to UV-exposure could be recorded (Fig. 2a, d in **Pub. I**). SOD activity, was unaffected or even reduced e.g. in *P. palmata* (Fig. 1d-f in **Pub. I**), whereas in the planktonic diatom *Ditylum brightwellii* (Rijstenbil, 2001) as well as in *Chlorella vulgaris* (Malanga and Puntarulo, 1995) SOD activity was clearly induced by exposure to UVR. APX activities were rather unaffected in *D. ramentacea* or only slightly reduced in *P. palmata* after UV-exposure (Fig 2b, e in Pub. I) as shown for zooxanthellae in the octocoral *Clavularia* sp. (Shick et al., 1991). In the seasonal studies no increase in enzyme activities could be shown during UV-exposure (Fig. 7a-c in **Pub. V, VI**), while MAA accumulation was clearly induced in response to UVR (Fig. 8 **Pub. V, VI**). Ascorbic acid showed a response to UVR, but rather seemed to be triggered by photoperiod, since high levels of ascorbic acid were maintained during winter when thalli were exposed to UVR in the previous season (Fig. 6 in Pub. V, VI). The rather moderate response of *D. ramentacea* and *P. palmata* to UV-exposure may be due to the fact, that they are well adapted to UVR as species occuring in the eulittoral and upper sublittoral. Their low sensitivity to UVR was previously shown by Bischof et al. (2002), Aguilera et al. (1999), Hanelt et al. (1997b), Karsten et al. (2001) and

explained by the presence of MAAs, as effective UV-absorbers (Karsten et al., 2001; Karsten and Wiencke, 1999; Aguilera et al., 2002a).

No effects of artificial UVR on GR and SOD have been observed in the green alga *Acrosiphonia penicilliformis*. In contrast, a significant reduction in GR and SOD actvities was observed, when natural UVR was cut off by selective filter foils in the field. This means that survival of this species in the intertidal zone is apparently supported by an enhanced oxygen-reactive scavenging system. Moreover, there are morphological strategies, as suggested by Aguilera et al. (1999b), which may additionally protect the species against UVR. While the apical part of this alga is mainly exposed to strong solar radiation, the basal cells are well protected due to self-shading. In the field yellow coloured tips were often observed, indicating lack of chlorophyll as a consequence of photobleaching of the apical parts, along with green pigmented, healthy and unstressed basal parts (**Pub. I**). Morphological alterations are also reported in the planktonic diatom *Ditylum brightwellii* (Rijstenbil, 2001) and *Chlorella* sp. (Estevez et al., 2001) as consequence of UVR. Exposure to UVA caused an increase in cell diameter, decrease in length axes and a production of vegetative resting stages in *D. brightwellii* (Rijstenbil, 2001).

Depth dependent responses to solar UVR and oxidative stress are also known for microalgae living as zooxanthellae in corals (Shick et al., 1995). Those zooxanthellae show decreasing SOD, CAT and APX activities as well as MAA contents with increasing water depth. The general baythymetric decline in the activities of the antioxidant enzymes in zooxanthellae is related to the decrease in potential for photooxidative stress with increasing depth (Shick et al., 1995).

The mechanism how UVR affects antioxidative responses appear to include endogenous photosensitization and formation of ROS (Martin and Burch, 1990). In cells there are a variety of sensitizers which absorb UVR. Interaction between excited sensitizers and triplet oxygen produces active oxygen intermediates (Estevez et al., 2001).

- UVR had no effects on SOD activity under laboratory conditions as proposed for higher plants and microalgae

- GR activity in macroalgae was induced under artificial UV exposure in some species as known for higher plants and microalgae
- APX and CAT acitivity were unaffected in the investigated macroalgae in response to UV-exposure in contrast to mostly enhanced activities in higher plants and microalgae

⇒ The regulatory processes are still unknown

4.3 Interactive effects of UV-exposure and changes in salinity

The investigation of interactive effects is of particular interest, as in the field various environmental parameters may change at the same time. Macroalgae living under fluctuating abiotic conditions, as in the Kongsfjord, require a broad physiological plasticity to acclimate to the wide range of incident solar radiation, as well as to salinity changes.

This is the first study describing the interactive effects of UVR and changes in salinity on Arctic marine macroalgae, therefore hardly any information is available from the literature, which could be used for the discussion of observed effects. Photosynthetic efficiency and MAA synthesis/accumulation were investigated in the two shallow water species *Devaleraea ramentcaea* and *Palmaria palmata* in response to UVR and changes in salinity. While in *D. ramentacea* the strongest inhibitory effect was measured under hypersaline conditions without UV (25% after 4 days), at 15 PSU only a small decrease in Fv/Fm was observed (Fig. 1, Pub II). In contrast, *P. palmata* did not survive the hyposaline treatment, and also showed strong inhibition in Fv/Fm at 50 PSU (Fig. 5,6 Pub. II). Consequently, while *D. ramentacea* can be characterised as a euryhaline species, *Palmaria palmata* rather exhibits stenohaline features. Stenohalinity with respect to growth is typical for sublittoral red macroalgae in comparison to intertidal species (Kain and Norton, 1990). These habitats are characterised with more stable environmental conditions that support development of stenohaline organisms.

Hypersaline stress had no influence on MAA synthesis and accumulation in the two species. In contrast, hyposalinity led to a decrease in MAA concentration, particularly in *P. palmata*, which bleached and died, even without the presence of UVR. In contrast to both species, stimulation of MAA and scytonemin accumulation under

enhanced salinity was observed in cyanobacteria (Karsten, 2002; Portwich and García-Pichel, 1999). Therefore *P. palmata* is a more stenohaline species in contrast to *D. ramentacea* with more euryhaline properties.

- *Palmaria palmata* shows limited capability to acclimate photosynthesis to changing PAR/UVR, pointing to a rather inflexible metabolism. It exhibits a marked sensitivity against salinity and is therefore characterised stenohaline, which is typical for sublittoral species adapted to more stable environmental conditions
- *Devaleraea ramentacea* exhibits more euryhaline properties concenring photosynthesis and is able to resist different environmental stress factors in the upper sublittoral of the Artic Kongsfjord, indicating high degree of physiological plasticity

4.4 Other factors influencing the antioxidative status of marine macroalgae

In the seasonal studies included in this thesis (**Pub. V** and **VI**) we also propose daylength to be a trigger for photoperiodic control of the activity of antioxidative enzymes: during the polar night high enzyme activities of APX, SOD and CAT were measured but not maintained throughout the entire season (Fig. 7a-c in **Pub. V** and **VI**). The ecological aspects of this seasonal response in antioxidative activity will be discussed in 4.7.2. Seasonal changes in antioxidative enzyme activities have been described for SOD and CAT in the freshwater dinoflagellate *Peridinium gatunense* (Butow et al., 1994; 1997). Increase in CAT activity was correlated with low ambient total CO_2 levels in the water. Also senescence of the cells was assumed to contribute to the increased CAT activity and variability in *P. gatunense* (Butow et al., 1994). An accumulation of H_2O_2 has been reported in aging of higher plants, in some cases this induced a temporary increase in CAT activity (Droillard et al., 1987). SOD activity increased towards the end of the spring algal bloom in Lake Kinneret simultaneously with maximal photosynthetic activity and conditions of ambient stress such as high irradiance (Butow et al., 1997). Similarily, photoperiodic control of growth and reproduction, as well as the onset of increase in pigment concentration and seasonal morphogenesis are known to be triggered by daylength in marine macroalgae (Kain,

1987; Lüder et al., 2001; Lüning, 1991; Lüning and Kadel, 1993; Lüning and tom Dieck, 1989; tom Dieck (Bartsch), 1991; Wiencke et al., 1996).

Besides the radiation conditions both temperature and nutrient concentrations show more or less strong seasonal variations in the Kongsfjord (Hanelt et al., 2001). Temperature is known to influence ROS formation: chilling, freezing and sudden temperature changes increase internal ROS formation (Collen and Davison, 2001; Wise, 1995). The defence against reactive oxygen was shown to be a response to growth at different temperatures and seasonal changes in environmental conditions in *Fucus vesiculosus* (Collen and Davison, 2001). Algae grown at low temperatures, both with and without freezing, produced less ROS after severe freezing stress than those grown at 20 °C (Collen and Davison, 2001). These differences were correlated with growth temperature-induced changes in activities of SOD, GR and APX. The content of tocopherols increased with increased cultivation temperature, whereas the activity of CAT and the content of glutathione and ascorbic acid did not change (Collen and Davison, 2001). These data suggest that elevated activities of ROS scavenging enzymes, especially SOD, increase the resistance to photoinhibition, at least at low temperatures, as well as being important for freeze tolerance (Collen and Davison, 2001) which would be of interest also in macroalgae from the Artcic Kongsfjord. As *Palmaria palmata* reaches its Northern distribution limit at Spitsbergen (Lüning, 1985), prevailing temperatures are rather suboptimal for growth. Indeed culture studies of Arctic isolates at elevated temperatures revealed an optimum growth temperature at 12 °C and temperate isolates exhibited much higher growth rates at the same optimum temperature (van de Poll et al., 2002).

Temperature dependence of UV effects on optimal quantum yield of PS II were investigated in Arctic and temperate red macrophytes (van de Poll et al., 2002). UV effects were lower at 12 and 18 °C compared with 6 °C indicating that under summer temperatures occurring in temperate regions the repair of UV-induced damage and acclimation to UVR is facilitated in theses algae compared to specimens under Arctic conditions (van de Poll et al., 2002). Temperature and light dependent repair mechanisms were previously observed for *P. palmata*, showing increased removal of CDPs at 12 and 25 °C compared with 0 °C (Pakker et al., 2000).

Nutrient limitation is also known to provoke ROS formation (McKersie and Lesham, 1994) and may lead to drastic changes in the cellular metabolism, especially the biosynthesis of proteins (Schlee, 1992). Via a "stringent control", accumulation of tRNAs can be caused due to deficiency of amino acids (Schlee, 1992). Further signals and regulation mechanisms can provoke a stress response, which may be the synthesis of definded stress proteins terminated "starving stress proteins" (SSP), also known in response to heat shock as heat shock proteins (HSP) (Jenkins et al., 1988; Spector et al., 1986).

As temperatures and nutrients vary within the seasons in the Arctic Kongsfjord, these parameters could also be involved in regulatory processes and effects on the antioxidant defence of marine macroalgae and therefore should be considered in future studies. As biogenic ROS formation is dependent on these parameters (UVR, temperature, nutrients, etc.), they may play a role in regulatory processes of antioxidant defence as proposed by Mackerness et al. (1999). Further investigations should also focus on regulatory processes and the molecular prove of induction or inhibition of antioxidative enzymes in response to different stressors (see section 4.10).

These results indicate that the antioxidant status of marine macroalgae is dependent on the environmental (stress) factors, the algae are exposed to in their habitat; that are PAR, UVR, growth temperature, temperature changes and freezing, in case of intertidal species.

- The formation of the antioxidative enzymes is presumably controlled photoperiodically by daylength

⇒ several regulatory factors seem to influence the status of antioxidants in algae, but regulatory processes are still unknown

⇒ Probably ROS are involved in the regulation processes

4.5 Direct oxidative stress derived by H_2O_2 exposure

Monitoring of the optimal quantum yield of photosynthesis (Fv/Fm) by measuring variable chlorophyll fluorescence was shown to be a suitable tool to characterise the

antioxidant potential of marine macroalgae under oxidative stress due to exposure to H_2O_2 (**Pub. III**). Chlorophyll fluorescence measurements have previously been applied in a large number of studies on different aspects of stress research in algae and higher plants (Baker, 1991; Krause and Weis, 1991; Schreiber, 1983; Bischof et al., 1998a; b; 1999; 2002 ; Hanelt et al., 1997a; b) . A high photosynthetic efficiency after exposure of experimental specimens to high H_2O_2 concentrations/high oxidative stress, was regarded as a low susceptibility to H_2O_2 and equated with a high antioxidative potential. This test system represented an important, new approach with respect to potential future applications, since species showing a high H_2O_2 tolerance may be a possible source for antioxidative substances for commercial and pharmaceutical purposes. In this respect the results obtained from *Polysiphonia arctica* were striking, since its extremely high H_2O_2 tolerance indicates a very efficient antioxidative defence system (see chapter 4.6).

Former studies have shown that biotic and environmental stresses increase the concentration of cellular oxidants that induce an increased synthesis of non-enzymic antioxidants such as glutathione, ascorbic acid and tocopherol, as well as increases in antioxidant enzymes such as SOD, glutathione peroxidase (GPX), GR, APX and CAT (Foyer et al., 1997). Induction of these antioxidant defences can be assumed to reflect a general strategy required to overcome increased oxidative stress due to imposition of environmental constraints (Foyer et al., 1997). Although plants have adapted to the respective prevailing environmental conditions during evolution, the level of "natural" resistance to oxidants varies widely among species (Bennett et al., 1984; Reinert et al., 1982). The antioxidant defence systems protect against natural and man-made stresses. Differences in resistance can be related to modifications in the constitutive levels of antioxidative enzymes or non-enzymic antioxidants. These can be age dependent, species-dependent or due to modifications in gene expression (Foyer et al., 1997). Resistance to oxidants, however is not always directly related to antioxidative protection (Foyer et al., 1997). Oxidative stress induces or enhances SOD activity (Bowler et al., 1992; Scandalios, 1990), GR (Schmidt and Kunert, 1986) and APX (Mehlhorn et al., 1987). It also leads to increases in glutathione, ascorbic acid and α-tocopherol (Mehlhorn et al., 1987).
In *Chaetomorpha linum* it was found that the decrease in SOD activity after exposure to H_2O_2 concentrations >1 mM correlates to the changes in Fv/Fm (Fig. 2 **Pub. IV**),

which might be explained by a direct toxicity effect as observed by Collen and Pedersen (1996) in similar experiments with the green alga *Ulva rigida*. Exposure to high levels of H_2O_2 (3 mM and higher) in this species caused intolerable, and finally lethal, oxidative stress. This was also observed in *C. linum* after 8 days of exposure to 2 and 5 mM H_2O_2 (data not shown). A small incremental decrease in SOD activity was found in *C. melagonium* and *Acrosiphonia* sp. after H_2O_2 treatment. In *C. linum*, however, H_2O_2 seemed to directly affect SOD activity. H_2O_2 concentrations > 1 mM fully inhibited SOD, supporting the hypothesis of a direct toxicity effect discussed by (Collen and Pedersen, 1996). Inhibition of gene expression may be a possible explanation for this negative effect as observed in *Pisum sativum* under UV exposure (Strid, 1993), but also oxidative stress may lead to inhibition of genes as e.g. of the photosynthetic enzymes (Mackerness et al., 1999). *Zea mays* leaves, in contrast, showed increased APX and SOD activity after 12 h incubation in 1 mM H_2O_2 (Pastori and Trippi, 1993) which is in agreement to the increased APX activity in *Acrosiphonia* sp. and *Chaetomorpha linum*.

GR activity was stimulated in *Chaetomorpha melagonium* indicating an active scavenging of H_2O_2 by means of the ascorbate-glutathione-cycle. It has been shown that plants increase GR activity in response to stress (Edwards et al., 1994). For example in *Arabidopsis* GR activity was enhanced under UVR (Kubo et al., 1999) as well as in several Arctic macroalgae under artificial and natural UV stress (**Pub. I**). There is evidence that a high level of GR alone does not correlate with oxidant resistance, and that increases in GR can only be effective if they accompany increases in a second antioxidative enzyme such as SOD (Malan et al., 1990).

CAT and APX activity were significantly increased after 6 days of H_2O_2 exposure of *Polysiphonia arctica* (**Pub. III**), indicating an induction by H_2O_2 as it is proposed by Foyer et al. (1994) as a general response to oxidative stress. Low CAT activity in combination with enhanced APX activity after the H_2O_2 treatment, as shown for *C. linum* (**Publ. IV**), is consistent with low levels of CAT found in *Euglena gracilis*, which primarily uses APX to reduce H_2O_2 in the cell (Shigeoka et al., 1980). The advantage of using APX rather than CAT is, besides H_2O_2 reduction, that NADPH-dependent ATP-production is promoted, which serves as an additive sink for energy. CAT is not a robust enzyme, as it is susceptible to photoinactivation and degradation

(Feierabend and Enger, 1986; Feierabend et al., 1992; Streb et al., 1993). It is also limited by its relatively poor affinity for H_2O_2 and its subcelluar localisation in the peroxisomes (Foyer et al., 1994). In spite of its relatively poor performance, CAT is essential for the destruction of photorespiratory H_2O_2 in C3 plants. In C4 plants, where photorespiration is efficiently suppressed, catalase may not be essential for survival (Foyer et al., 1994). As photorespiration in marine macroalgae is hardly affected by UVR (Aguilera et al., 1999b; Larkum and Wood, 1993), CAT should only play a minor role in these species. As an exception, *Acrosiphonia* sp. showed an extremely low APX activity and an intermediate CAT activity, which slightly increased under H_2O_2 stress. This would suggest that *Acrosiphonia* sp. rather uses CAT as scavenger when exposed to H_2O_2 indicating an inhibition or degradation of APX in this species.

- Biotic and environmental stress increases the concentration of cellular oxidants similar as in higher plants
- Oxidative stress induces or enhances antioxidative enzyme activities similar as in higher plants
- Differences in constitutive levels of antioxidants are species-specific

4.6 Macroalgae as source of hitherto unknown antioxidative substances

Biochemical examination of marine organisms chronically exposed to high levels of solar UV radiation often reveals the presence of unknown compounds, which may potentially form part of a protective/acclimation strategy (Dunlap & Yamamoto, unpublished observation). For the present study, oxidative stress was also artificially induced in order to screen macroalgae for the presence of new antioxidative compounds (**Pub. III**).

Tropical marine organisms, represent a rich source of new antioxidants, which have potential biomedical applications, or which may be used as nutriceuticals or in food-processing and other related applications (Dunlap et al., 1999). With the discovery of 4-deoxygadusol, the presumed precursor of the MAAs, with strong antioxidant properties, a novel, marine-derived antioxidant has already been successfully

extracted (Dunlap, 1998). Further search and discovery of new antioxidantive compounds is a future objective. This task is facilitated by the assay developed in **Pub. III** in combination with biological observations, which lead to the finding of two new bromophenolic compounds with antioxidative properties in *Polysiphonia arctica* (**Pub. III**). The identified compounds (2,3-dibromo-4,5-dihydroxybenzyl methyl ether, and TMS derivative of 2,3-dibromo-4,5-dihydroxybenzyl alcohol) resemble bromophenolic substances with antioxdiative properties, earlier described for several *Polysiphonia* species (Glombitza et al., 1974; Fujimoto et al., 1985) differing only in the side chains of the phenolic ring (Kurata and Amiya, 1980).

It is well known, that also phenolic compounds contribute to antioxidant properties. The antioxidant activities of beverages prepared with marine seaweeds was correlated to the contents of polyphenols within these algae. The antioxidant activity was tested in different test systems, including autoxidation test, superoxide anion radical, α,α-diphenyl-β-picrylhydrazyl (DPPH) radical (see also **Pub. III**) and hydroxyl radical scavenging (Nagai and Yukimoto. T., 2003). These beverages have a potential pharmaceutical application as health drinks with functional properties and for patients suffering cancer, cardiovascular diseases, and diabetes (Nagai and Yukimoto. T., 2003).

- The two bromophenolic compounds (2,3-dibromo-4,5-dihydroxybenzyl methyl ether, and TMS derivative of 2,3-dibromo-4,5-dihydroxybenzyl alcohol) found in *Polysiphonia arctica* have antioxidative properties

4.7 Antioxidative responses and species ecology

4.7.1 Vertical zonation patterns

Ecophysiological studies of the influence of different abiotic factors show a general correlation between stress tolerance and vertical distribution of marine macroalgae (Davison and Pearson, 1996; Hanelt, 1998; Levitt, 1980). In a large number of studies it was demonstrated, that the species sensitivity to solar radiation stress is a function of depth distribution (Dring et al., 1996b; Larkum and Wood, 1993; Bischof et al., 1998b; Hanelt et al., 1997a; 1997c). For example photoinhibition after exposure to high PAR for 2 h as well as recovery at dim white light were studied in Arctic

macroalgae and response kinetics of inhibition and recovery were calculated and related to the depth distribution of each algal species (Hanelt, 1998). The half-time (τ) of the inhibition and recovery phases. i.e. the time necessary to reach half maximum response, was clearly related to the depth distribution of the investigated species (Hanelt, 1998). Algae collected close to the water surface show a fast reaction of both photoinhibition and recovery, whereas the reactions became slower with increasing sampling depth (Hanelt, 1998). Depth zonation patterns are also reflected in UV-effects on photosynthesis in Antarctic macroalgae (Bischof et al., 1998b). The green algal group exhibited less inhibition of chlorophyll fluorescence after artificial UV-exposure than brown and red macroalgae and recovered faster. In sequent exposure and recovery periods the responses on the different algal species were more variable but only the intertidal green algae survived experimentally applied subsequent UV-exposure, whereas the brown and red algae from sublittoral died after the treatment (Bischof et al., 1998b).

Some authors regard solar UVR as one of the most important factors controlling the upper distribution limit of macroalgae in the field (Maegawa and Kida, 1993). Therefore it is reasonable to assume that increased UVB, penetrating deeper into the water column, and increasing the H_2O_2 formation in the surface layers, results in a shift of the upper distribution limit of single species to greater water depths. Results from Wiencke et al. (2000) support the idea that UVB can affect zonation patterns as zoospores are highly susceptible to UVB radiation, thus preventing recruitment in shallow waters.

These findings are strongly supported by the present study: the conducted experiments on the effects of UVR and salinity changes on marine macroalgae clearly show that the antioxidative potential as the sum of all the biochemical protection mechanisms present in a species is clearly dependent on the UVR driven oxidative stress the alga encounters in its habitat. In general, species inhabiting the eulittoral and upper sublittoral exhibit higher antioxidative enzyme activities and ascorbic acid content as algae from the lower sublittoral. In particular, differences in SOD activity between the algal groups are related to depth distribution on the shore (Fig. 5, Tab. 2 in Pub. I). Most green algae showed higher antioxidative enyzme activities and typically inhabit the upper shore of the Kongsfjord. The only exception within the green algae is the supralittoral species *Prasiola cripsa*, growing underneath bird colonies, which exhibited comparably low antioxidative enzyme

activities. In this plant, probably another photoprotective strategy seems to be developed, such as the biosynthesis of UV-absorbing compounds, that are known to prevent radiative damage (Dunlap and Shick, 1998). *P. crispa* contains high amounts of two still uncharacterised MAAs (Hoyer et al., 2001). Antioxidative properties of these compounds are yet not characterised, but may be possible as described for other MAAs (Dunlap and Yamamoto, 1995).

Within the group of red algae, also species occurring in the upper sublittoral, as *Devaleraea ramentacea* and *Palmaria palmata*, exhibited higher SOD activities compared to typical deep water species, as e.g. *Phycodrys rubens* (Pub. I). In summary, species more exposed to drastic and rapid changes in environmental radiation conditions have developed an efficient biochemical defence system to resist/avoid oxidative stress.

This relation between antioxidative activities and depth distribution is due to the fact, that UVR is rapidly attenuated when penetrating the water column and algae in the upper water layer are exposed to higher irradiances of PAR and UVR (Hanelt et al., 2001). UVR has direct and indirect effects on the algae and the surrounding environment, as oxidative stress is produced within the algae itself by excessive absorption of high energy radiation (Collen and Davison, 2001) as well as by photodynamic formation of H_2O_2 in the water surrounding the algae (Abele et al., 1998a; Abele-Oeschger et al., 1997; Cooper and Zika, 1983). Especially in tidal pools with high loads of dissolved organic matter and high oxygen concentrations due to photosynthetic production by algae, high concentrations of H_2O_2 will be formed (Abele et al., 1998a). This means that in surface waters algae are exposed to different kinds of stress leading to increased ROS formation and oxidative stress.

Another species, deviating from the general pattern described above, is the deep water species *Polysiphonia arctica*, exhibiting a high antioxidative potential, which probably derives from two new bromophenolic compounds, firstly described in **Pub. III**. This alga may be exposed to oxidative stress not as consequence of exposure to high UVR but probably due to a dense population of photosynthetic active diatoms populating this alga as epiphytes (own observations).

In general, protective defence mechanisms against UVR and accompanied oxidative stress have been evolved thousend of years ago and are correlated to the stress conditions in the different water depths as it is also known for the accumulation of UV protective substances. In laboratory and field studies the presence of mycosporine-like amino acids (MAAs) in marine organisms of different taxa is strongly correlated to the intensity of UVR, and therefore to the depth gradient at the natural growth site. Micro- (Garcia-Pichel and Castenholz, 1991) and macroalgae are known to synthesis and accumulate MAAs in relation to depth gradients (Karsten et al., 1998b), but also organisms with symbiotic algae, like corals follow this pattern (Ishikura et al., 1997; Karentz, 1992; Lesser, 1996; McClintock and Karentz, 1997; Shick et al., 1995).

The capability of dynamic photoinhibition in Arctic macroalgae is related to their depth distribution (Hanelt, 1998) and may therefore be regarded as a mechanisms of acclimation to the respective radiation conditions in the different water depths. The photoprotective mechanism only dissipates excessively absorbed energy (PAR) as physiologically harmless thermal radiation (Hanelt and Nultsch, 2003; Hanelt et al., 1997a; Osmond, 1994) and not UVR, but as high doses of solar radiation include both wavebands, any protective mechanisms reducing stress on photosynthesis may also support the protection from UVR.

- Vertical zonation patterns are reflected in the antioxidant defence mechanisms in marine macroalgae
- Species exhibiting lower antioxidant defence mechanisms as usually found in its depth zone have evolved additional protection mechanisms
- species more frequently exposed to drastic and rapid changes in environmental radiation conditions have developed an efficient biochemical defence system to withstand stress

⇒ the protective mechanisms are adapted to the stress, the alga encounters in its habitat, depending on different biotic and abiotic factors

4.7.2 Seasonality

The results presented in this thesis indicate marked seasonal effects on the respective antioxidative response (**Pub. V, VI**). Circannual rhythms and

photoperiodisms may trigger seasonal development (Wiencke, 1990a; b; Gomez and Wiencke, 1997; 1998; Gomez et al., 1995; Kain, 1987) and prepare the alga for upcoming UV and oxidative stress in early spring (Lüder et al., 2001). Therefore, enzyme activities may be up-regulated before the onset of stressful conditions (**Pub. V, VI**). Similar phenomena are described for invertebrates, e.g. land snails activate their antioxidant defence during dormancy in dryness (estivation) to be prepared for oxdiative stress during wake-up (arousal) (Hermes-Lima. M. et al., 1998). Increased activities of antioxidant enzymes have also been observed under other kind of stress situations in which the actual production of oxyradicals should decrease e.g. anoxia exposure in snakes or freezing in frogs (Hermes-Lima. M. et al., 1998; Joanisse and Storey, 1996).

As the synthesis and accumulation of MAAs is only induced in response to radiation, this protection mechanism may take some time until it provides sufficient protection. Therefore, in early spring, as soon as the radiation period starts, the algae already require another protection system, which might be provided by the antioxidative enzymes (see Pub. V, VI). Under field conditions, with substantially higher irradiances, a further increase in enzyme activities might be necessarily induced by UVR, as shown for *Acrosiphonia penicilliformis* (Fig. 3d in Pub. I), *Monostroma arcticum* and *Palmaria palmata* from the Arctic (Aguilera et al., 2002a). As in our study, PAR intensities were low, oxidative stress deriving from superoxide and H_2O_2 via the Mehler reaction is unlikely. The antioxidative enzymes are probably down-regulated and MAAs appear to provide sufficient protection under the current radiation conditions. Seasonal up-regulation of SOD and CAT activities in response to decreasing CO_2 concentrations in the freshwater dinoflagellate *Peridinium gatunense* were described by Butow et al. (1994; 1997). Also senescence and high O_2 concentrations deriving from the photosynthetic activity of the algal bloom were considered to play a role in the induction of the antioxiative enzymes (Butow et al., 1994; 1997).

It seems likely that several regulatory factors interact to regulate the status of antioxidants in algae. Oxidative stress conditions are postulated to trigger an antioxidant response that includes an increase in the activity of antioxidant enzymes (Estevez et al., 2001).

- The status of antioxidant defence in marine macroalgae seems to be triggered by the seasonally fluctuating daylength ⇒ photoperiodic control
- Other factors may play a role in induction of antioxidant activities (e.g. CO_2- and O_2 concentrations, nutrients, aging) and interact in regulatory processes

4.8 Methodological aspects

Our laboratory experiments were monitored in a controlled temperature and irradiance environment, thus allowing the comparison of UV effects between the species. However, the use of artificial light sources has drawbacks because irradiance conditions still differ from those of the natural solar spectrum even if UVR doses were chosen similar. Particularly, the low irradiance of PAR within the laboratory experiments may have altered the apparent UV sensitivity (Teramura, 1986). Secondly, because UV:PAR ratios were not natural, our experiments allow no direct estimation of the contribution of the various wavebands with regard to natural UV exposure. Under natural irradiance, effects of high PAR may hide UVR effects (Dring et al., 2001; Hanelt et al., 1997b).

In various experimental set-ups, UV effects on the antioxidative enzymes SOD, APX, CAT and GR in different species from the Kongsfjord gave a contradicting view. A large discrepancy occurred between data obtained under laboratory conditions and field data. Whereas in the field, UVR confers inducing effects on SOD activity in *Acrosiphonia penicilliformis*, under laboratory conditions no UV effects could be shown in the same species (Pub. I). Also in the seasonal studies on *Palmaria palmata* (Pub. V) and *Devaleraea ramentcacea* (Pub. VI) no UV driven increment of SOD and other antioxidative enzymes could be shown, whereas a seasonal field study from (Aguilera et al., 2002a) showed a clear increment in SOD activity under higher UVR irradiances after sea ice break up in *P. palmata* and *Monostroma arcticum* but not in *D. ramentacea*. GR activity was clearly induced in *M. arcticum*, *Coccotylus truncatus* and *Phycodrys rubens* (Pub. I) under artificial UV exposure in the field, in contrast, in *Monostroma sp.*, *P. palmata* and *D. ramentacea* GR activity decreased with increasing UVR in spring (Aguilera et al., 2002a). It is likely that the different radiation conditions and the oxidative stress induced by UVR, are responsible for the discrepancies between field and laboratory studies. Even if chosen UV doses were similar to the conditions in the field, the ratio of PAR to UVR

was substantially lower as under the natural solar spectrum. Due to the low PAR intensities, photosynthesis may be not saturated and ROS formation negligable and therefore the oxidative stress caused by UVR is scavenged by the present defence mechanisms. Since under the solar spectrum high PAR and UVR intensities exhibit interactive effects, it requires improved protection mechanisms, which may be induced either by PAR or by UVR as it is known for the induction of MAAs (Hoyer et al., 2002a; Karsten et al., 1998a; b). The trigger mechanisms of the induction of MAA synthesis/accumulation and photoreceptors involved are still unknown but it is assumed that they are light regulated (Kräbs et al., 2002; Weaver and Hermann, 1997). Mackerness et al. (1999) propose ROS being involved in the UVB signal transduction pathway leading to a down-regulation of the expression of photosynthetic genes in pea leaves, but also to a pronounced up-regulation of those genes which are related to protective mechanisms, as e.g. APX and SOD genes (Mackerness et al., 1999) and a gene encoding for chalcon synthase, a key enzyme involved in flavonoid biosynthesis (Brosché et al., 1999; Mackerness et al., 1997). However, the expression of the genes encoding for these enzymes does not always reflect changes in activity (Mackerness et al., 1998). Similar regulatory processes are also likely to occur in marine macroalgae, where the transcript levels of the photosynthetic genes rbcL and psbA were up-regulated in the first two hours of UV-stress and psbA as well as under long-term-exposure (Poppe, personal communication). Comparing low and high UV irradiances transcript levels of both genes were significantly lower after high UV exposure (Poppe, personal communication). These results indicate that irradiances of UVR as well as duration of exposure, and UV-induced oxidative stress are involved in the regulatory processes.

Discrepancies in antioxidant enzyme reactions are also known for higher plants and microalgae. In peas (*Pisum sativum*) Strid (1993) showed the inhibitory effect of UVR on the gene expression of SOD, whereas Mackerness et al. (1999) described increases in the enzyme activities in APX, SOD and GR in pea leaves without a measurable effect on transcript levels. Different irradiances applied were responsible for different responses in antioxidant activities in *Chloralla vulgaris.* Whereas moderate UVB radiation (0.3 Wm^{-2}) increased enzyme activities of SOD and CAT by 40 and 500%, respectively, high doses (4.59 W m^{-2}) lead to a 50 % decrease in SOD activity (Malanga et al., 1997; Malanga and Puntarulo, 1995). Concerning low

molecular antioxidants like α-tocopherol and β-carotene, mild UVR stress resulted in an increase, while high irradiances of UVR further increased α-tocopherol content but not β-carotene in *Chlorella vulgaris* (Malanga et al., 1997; Malanga and Puntarulo, 1995).

All enzyme assays were conducted at 25 °C and thus represent normalizied activities. It can be inferred that real tissue antioxidant defence at ambient temperatures (0-6 °C) are substantially lower. This may imply that tissue antioxidant defence in polar macroalgae from permanently low temperature environments cannot be sufficiently increased, to effectively prevent oxidative damage during times of stress.

⇒ The different radiation conditions in the laboratory and the field are responsible for the discrepancies between laboratory and field studies

⇒ Enzyme activities under laboratory conditions may be overestimated due to measurement at standardised temperature higher than the ambient temepratures

4.9 Concluding remarks and future perspectives

In conclusion, a diagram (Figure 7) is presented to summarise the responses of macroalgae to oxidative stress deriving from solar radiation. High irradiances of PAR and UVR, as they occure in spring in the Arctic Kongsfjord, have to be regarded as abiotic fators, which influence the physiology of Arctic macroalgae. Biogenic ROS formation is induced by excessive light energy in the photosynthetic apparatus. Formation of H_2O_2 in surface waters due to photoactivation of DOM by UVR acts additionally as oxidative stress on the algae. Other abiotic factors, such as salinity, nutrients and temperature, also fluctuating seasonally, may induce biogenic ROS formation within the algae. In consequence of ROS in the algal cell biomolecules are oxidised and genes encoding for photosynthetic enzymes are down-regulted, leading to inhibition of photosynthesis and production. The antioxidative enzymes are activated in response to the oxidative stress and ROS are eliminated effectively by

scavengers such as ascorbic acid, and the antioxidative enzymes. The deleterious processes and the protecting mechanisms are counteracting and depending on the extent of the oxidative stress an equilbrium is found or not. A future increase in UVR as consequence of increase in ozone depletion may result in a possible shift in depth zonation, when oxdiative stress is not scavenged sufficiently.

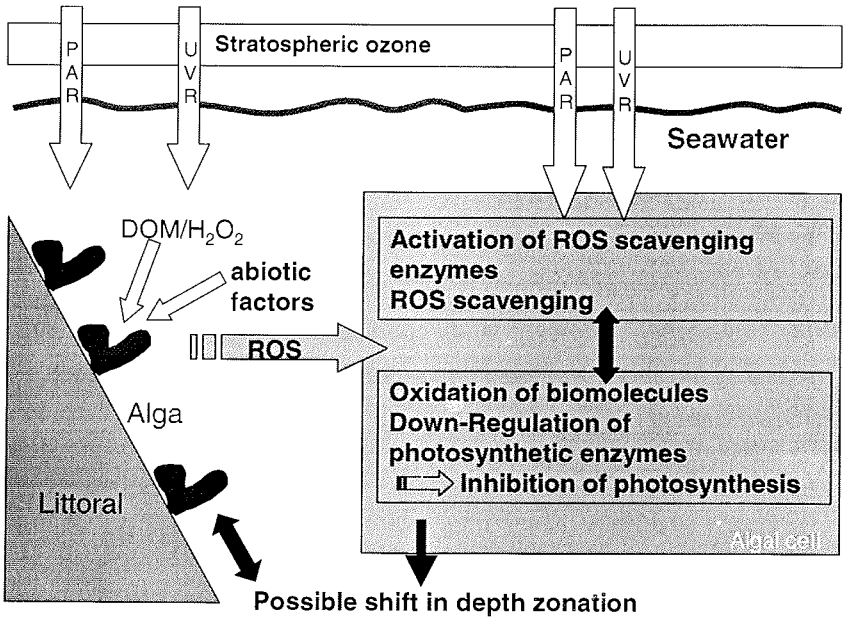

Figure 7: Responses of macroalgae to oxidative stress deriving from natural solar radiation

This study was directed to answer some basic questions related to antioxidative properties of marine macroalgae. The results clearly show, that the antioxidative status of marine macroalgae is depending on the prevailing environmental stress conditions. Although it was demonstrated that photosynthesis is sensitive to UVR (**Pub. II**) and artificial oxidative stress (**Pub. III**), effective defence meachanisms are present in the different species (**Pub. I**), reducing the harmful effects of UVR and ROS.

The sensitivity of photosynthesis to H_2O_2 was a successful tool in finding macroalgae with a high antioxidative potential and new antioxidative compounds present in one striking species. (Pub. III).

Further assays pointing on the pro- and antioxidant properties of algal extracts could be useful in future search for new antioxidative compounds.

The parallel application of different test-systems covers the greatest possible bandwidth of chemical conditions (oxidants and verification systems) to detect antioxidants with different modes of action. Two more assays have been performed in **Pub. III**. There are also other test systems to prove antioxidative properties which could be applied also for algal extracts (see below).

As every test systems has its peculiarities/specification it would be interesting to compare the antioxidative properties measured applying the different test systems.

1.) Iodine Test: Antioxidative activity in presence of photoactive pigments (photosensitiser), which are the source of singlet oxygen and/or superoxide, is detected by oxidation of iodine J^- to J^{3-} (Mosinger and Mosinger, 1995).

2.) Fenton-system: Superoxide anion provokes reduction of the Fe (III) complex to Fe (II) complex, which reduces hydrogen peroxide (Elstner, 1990).

3.) decomposition of α-keto-χ-methylthiol buturic acid which in presence of ROS generates ethylene

4.) Diene conjugation of linoleic acid (Yin et al., 1992)

The antioxidative defence mechanisms are correlated to the vertical zonation of the algae (**Pub. I**) as it is known for other acclimation and protective mechanisms against UVR. The different taxa exhibit preferences in using distinct defence mechanisms (Pub. I) which are not specific for Arctic species.

> Future work should enlarge the knowledge on antioxidants in marine macroalgae, as are still missing values for glutathione and β-carotene, as well as phenolic compounds.

Brown algae are known to contain high amounts of phenolic compounds with UV protecting properties. Also antioxidative properties are discussed (Nagai and Yukimoto. T., 2003). Phenolics are especially found in the cell walls and in physods, young tissues and early developmental stages (Schönwälder, 2002; Schönwälder and Clayton, 1999; Schönwälder and Wiencke, 2000).

> Combining ultrastructural, microscopic and analytical information about the phenolic compounds involved in the protection from UVR and oxidative stress would be a useful in future addition.

The reaction patterns of different algal species to direct and indirect oxidative stress were various. Inducing and decreasing antioxidant activities have been demonstrated in divers experimental approaches (Pub. I, Pub. III, IV, V, VI). A general approach to explain the reaction patterns is that the antioxidative status of marine macroalgae is depending on the prevailing environmental stress conditions leading to oxidative stress. Factors and regulation processes involved in the promotion of oxidative stress are: irradiance (PAR, UVR), daylength, temperature, nutrient limitation, dessication, age and aging processes and others. Daylength was considered to trigger the antioxidative enzyme activities and ascorbic acid content in two red algal species acclimating the antioxidant status within the seasonal course of the year.

> Only few of the factors were included in this study, and therefore the remaining factors should be included in future experimental and field investigations.

In preliminary experiments we found differences in antioxidative defence mechanisms within tissue gradients in brown algae. Tissue gradients are also described with respect to the concentrations of MAAs (Hoyer 2001). As the meristem is the young and physiologically most active tissue within the alga, also age may play an important role for the respective antioxidant status. The process of aging in higher plants, as leaf senescence was correlated with increased lipid peroxidation and decreased levels of SOD and CAT in tobacco (Dhindsa and Plumb-Dhindsa, 1981). Oxidative processes are coupled with aging (Droillard et al., 1987; Skulachev, 2001).

The processes regulating the amounts and activities of enzymatic enzymes are species specific and still unclear. Additional information on the regulation mechanisms could be provided in experimental designs including polyacrylamid gelelectrophoresis (PAGE) to separate the proteins followed by the identification of the enzyme by Western Blot and its quantification via densitometry to give molecular prove of enzyme induction/inhibition.
Characterisation of putative signal transduction molecules together with the identification and cloning of the regulatory genes involved in this response are necessary towards a better understanding of the photooxidative stress responses in plants/algae.

A missing link in the present study was the correlation of the antioxidative status of marine macroalgae to the actual internal ROS production and destruction/degradation processes by ROS.

The internal ROS production can be quantified by a technique, based on oxidation and fluorescence of 2',7'-dichlorohydrofluorescein diacetate (DCFH-DA) described by Collen and Davison (1997). Dihydrorhodamine 123 conversion to rhodamine 123 is a sensitive method for the detection of internal peroxide production, especially hydrogen peroxide (Windsor and White, 1993).
The lipid-peroxidation is a good marker for destruction of biomolecules by ROS and can be measured by a thiobarbituric acid test as described by Heath and Packer (1968) and Salama and Pearce (1993). DNA damage due to ROS is detectable within a system of copper (II) complexes and biological

reductants (Ueda et al., 1998) as well as in a new biochemical assay by Sattler et al. (2000). These aspects should be considered in future studies.

These remarks may show the advance of our knowledge through this study and also point to numerous possible future studies which will give an even deeper insight into antioxidative defence mechanisms of marine macroalgae.

PUBLICATIONS

The below listed publications form this thesis and my share of each publication is explained

Publication I

José Aguilera, Angelika L. Dummermuth, Ulf Karsten, Rainmund Schriek, Christian Wiencke

Enzymatic defences against photooxidative stress induced by ultraviolet radiation in Arctic marine macroalgae

2002. Polar Biology 25:432-441

The experimental set-up and measurements of the UV exposure experiment with *Devaleraea ramentacea* and *Palmaria palmata* (Fig 2a-f), as well as all measurements for ascorbate peroxidase and ascorbate were performed in close cooperation with the first author (Tab. 2). I wrote parts of the manuscript and contributed to the discussion.

Publication II

Ulf Karsten, Angelika L. Dummermuth, Kirsten Hoyer, Christian Wiencke

Interactive effects of ultraviolet radiation and salinity on the ecophysiology of two Arctic red algae from shallow waters

2003. Polar Biology, in press

The experiment was designed in close cooperation of the first author and myself. I performed the measurements of the photosynthetic efficiency (Fv/Fm) and participated in writing the discussion section.

Publication III

Angelika L. Dummermuth, Ulf Karsten, Katja M. Fisch, Christian Wiencke, Gabriele M. König

Responses of marine macroalgae to hydrogen-peroxide stress
2003. Journal of Experimental Marine Biology and Ecology, in press

I developed the idea of the experimental set-up in close cooperation with the second author. I conducted the elementary short- and long-term H_2O_2 exposure experiments, and the attributed measurements of the antioxidative enzymes and ascorbate. The third and fifth author provided the results for the antioxidative potential of *Polysiphonia arctica* by means of TBARS and DPPH assays as well as the structural identification of the bromophenols. The first version of the manuscript was written by myself and then revised in close cooperation with all co-authors.

Publication IV

Angelika L. Dummermuth, Ulf Karsten, Christian Wiencke
Antioxidative properties of three Arctic green macroalgae
2003 Phycological Research, submitted

I created the experimental set-up, conducted the experimental and computational work and wrote the first version of the manuscript which was then improved in cooperation with the co-authors.

Publication V

Angelika L. Dummermuth, Kirsten Hoyer, Ulf Karsten, Christian Wiencke

Seasonal variation in ecophysiological patterns of two red macroalgae from Arctic Kongsfjord (Spitsbergen, Norway) in a long-term culture study with emphasis on UV protective mechanisms. I. *Palmaria palmata* (L.) Greville
2003 Marine Ecology Progress Series, submitted

The long-term culture experiment in combination with UV exposure was initiated by me and the second author. Measurement of growth, enzymes, ascorbate as well as protein were conducted by myself. Data for MAAs and pigments were performed by

the second author. The first version of the manuscript was written by myself and then refined in cooperation with the co-authors.

Publication VI

Angelika L. Dummermuth, Kirsten Hoyer, Ulf Karsten, Christian Wiencke

Seasonal variation in ecophysiological patterns of two red macroalgae from Arctic Kongsfjord (Spitsbergen, Norway) in a long-term culture study with emphasis on UV protective mechanisms. II. *Devaleraea ramentacea* (L.) Guiry

2003 Marine Ecology Progres Series, submitted

See publication V

Publication I

Enzymatic defences against photooxidative stress induced by ultraviolet radiation in Arctic marine macroalgae

AGUILERA J[1], DUMMERMUTH AL, KARSTEN U[2], SCHRIEK R[3], WIENCKE C

Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12
27570 Bremerhaven

[1] Present address: Aguilera J
Universidad de Málaga,
Faculdad de Ciencias,
Departamento de Ecología,
Campus Universitario de Teatinos
S/n. 29071 Málaga, Spain
Email: jaguilera@uma.es

[2] Present address: Karsten U.
University of Rostock,
Institute of Aquatic Ecology,
18059 Rostock, Germany

[3] Present address: Schriek R.
University of Hohenheim,
Institute 140
Fruwirthstr. 12
70593 Stuttgart, Germany

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ORIGINAL PAPER

José Aguilera · Angelika Dummermuth
Ulf Karsten · Raimund Schriek · Christian Wiencke

Enzymatic defences against photooxidative stress induced by ultraviolet radiation in Arctic marine macroalgae

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Abstract The activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX) and catalase (CAT), as well as the content of the antioxidant compound ascorbic acid, were determined in five green, seven red and ten brown macroalgal species from the Kongsfjord (Spitsbergen, Svalbard, Norway). In general, higher antioxidant enzyme activities and a higher content of ascorbic acid were measured in green algae in comparison to red and brown algae. Species from the eulittoral and upper sublittoral (*Acrosiphonia penicilliformis*, *Monostroma arcticum*, *Chaetomorpha linum*, *Chaetomorpha melagonium*, *Devaleraea ramentacea*, *Palmaria palmata*) showed higher antioxidant enzyme activities compared to species from the lower sublittoral, indicating a more efficient biochemical protection in algae exposed to higher stress conditions in the field. The activity of GR was stimulated by artificial ultraviolet radiation in the green alga *M. arcticum*, and in the red algae *Coccotylus truncatus* and *Phycodrys rubens* after 84 h under continuous exposure. GR activity was even higher when the UV exposure was followed by incubation in darkness for 24 h, indicating a higher elimination rate of toxic oxygen radicals under these conditions. *D. ramentacea*, *P. palmata* and *A. penicilliformis* did not show any significant effect of UV radiation on CAT, APX and SOD activities after 8 days of culture under laboratory conditions. However, a significant reduction in activities of GR and SOD was observed in *A. penicilliformis* when solar UV radiation was cut off by selective filter foils in the field, indicating a lower oxidative stress in the absence of UV radiation. Overall, the ecological success of macroalgae in the eulittoral and upper sublittoral is supported by an enhanced oxygen-reactive scavenging system, allowing fast acclimation to the changes in environmental radiation conditions.

J. Aguilera (✉) · A. Dummermuth · U. Karsten · R. Schriek
C. Wiencke
Alfred Wegener Institute
for Polar and Marine Research,
Am Handelshafen 12,
27570 Bremerhaven, Germany

Present address: J. Aguilera
Universidad de Málaga,
Facultad de Ciencias,
Departamento de Ecología,
Campus Universitario de Teatinos
s/n. 29071 Málaga, Spain,
e-mail: jaguilera@uma.es,
Fax: +34-95-2132000

Present address: U. Karsten
University of Rostock,
Institute of Aquatic Ecology,
18059 Rostock, Germany

Present address: R. Schriek
University Hohenheim,
Institute 140, Fruhwirthstraße 12,
70593 Stuttgart, Germany

Introduction

Studies on the ability of living organisms to cope with enhanced levels of ultraviolet radiation become more and more important due to the increasing depletion of stratospheric ozone. Recent data show a dramatic trend of ozone depletion over the Antarctic regions, with a temporal decrease in springtime below 25–30% of the undisturbed conditions (NASA http://toms.gsfc.nasa.gov/ozone/ozone.html). Strong reduction of the stratospheric ozone is now also evident in the northern hemisphere (Ott and Amanatides 1994; Schulz et al. 2001), and predictions indicate a gradual increment of ultraviolet (UV) radiation in the northern polar regions similar to the southern hemisphere (Stolarski et al. 1992).

Accurate information is needed to assess the potential effects in organisms caused by UV radiation reaching the earth's surface. The effects of UV radiation on growth and other physiological features, such as damage in DNA, RNA, proteins and photosynthesis, in a range of higher and lower plants including phytoplankton are relatively well documented (Aguilera et al. 1999a, b;

Bischof et al. 2000; Buma et al. 1995; Clendennen et al. 1996; Figueroa et al. 1997; Häder and Figueroa 1997; Karentz et al. 1991; Smith et al. 1992; Strid et al. 1990; Tevini and Teramura 1989) while investigations on the effects of UV radiation on benthic marine macroalgae are scarce, although this group plays an important ecological role in the marine environment.

Photosynthesis can be damaged due to high photosynthetically active radiation (PAR) or UV radiation, as a result of an overreduction of the photosynthetic electron transport when not enough electrons are drained off by $NADP^+$ to NADPH from reduced ferredoxin of PSI. UV radiation can affect the draining-off system by damaging proteins of the Calvin cycle like Rubisco (Allen 1977; Bischof et al. 2000). Thus, in the absence of $NADP^+$, the reduced ferredoxin can also reduce oxygen, leading to superoxide radicals (O_2^-). Consequently, photosystem II is inactivated by UV and finally damaged due to degradation of the reaction centre proteins, mainly the D1 protein (Aro et al. 1993; Ohad et al. 1984). Under such conditions, singlet oxygen ($^1O_2$) can be formed from triplets of chlorophyll of the antenna. Reactive oxygen species can produce lipid peroxidation, damage proteins and have many other harmful effects (Asada and Takahashi 1987; Fridovich 1986).

Cellular mechanisms of protection against such toxic oxygen species are essential for the maintenance of photosynthetic activity and other metabolic functions (Allen 1977; Asada and Takahashi 1987; Eltsner 1982; Halliwell 1982). Plants and algae are equipped with an array of defence mechanisms that eliminate toxic oxygen radicals produced as by-products of photosynthesis and photooxidative events. Superoxide radicals are eliminated by the enzyme superoxide dismutase (SOD), yielding H_2O_2 and oxygen. Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but it is probably an intracellular precursor for more reactive oxidants, such as hydroxyl radicals. H_2O_2 is deprotonated by the enzyme catalase and by specific scavengers such as ascorbate and glutathione, mediated by ascorbate peroxidase and glutathione peroxidase, respectively. The resulting oxidized reactants, namely monodehydroascorbate and oxidized glutathione, are regenerated via enzymatic reductions by monodehydroascorbate reductase and glutathione reductase, respectively, thereby closing the antioxidant scavening cycle (Polle 1996).

Studies related to UV-induced photooxidation to the scavenging mechanisms for protection against oxidative damage are rare for macroalgae. Thus, the ability to resist high radiation stress may be one of the major factors controlling vertical macroalgal zonation patterns in communities (Bischof et al. 1998a; Hanelt 1998), and may be mediated by a higher biochemical potential against oxidative stress. Long-term exposure under UV radiation has been demonstrated to induce the activity of superoxide dismutase and ascorbate peroxidase in microalgae (Lesser 1996a, b; Malanga and Puntarulo 1997). Activities of antioxidant enzymes were higher in shallow-water coral zooxanthellae than in specimens collected from deeper waters (Shick et al. 1995). In addition, analysis of the antioxidant spectrum in selected alpine plant species collected at different altitudes proved that the total amount of antioxidants is positively correlated with altitude (Wildi and Lütz 1996). In one of the few publications on macroalgae, it was postulated that the differential stress tolerance associated with the vertical zonation of different *Fucus* species is strictly related to the antioxidant status of the plant, based mainly on species-specific differences of antioxidant enzyme activities (Collen and Davison 1999a, b).

Despite the potential importance in providing an alternative sink for excessively absorbed radiation energy and their role in scavenging, little is known about the capacity or inducibility of macroalgal antioxidant enzyme systems. The present study was designed to characterize the oxidative stress tolerance in field material of different green, red and brown macroalgae from the Arctic by the analysis of a set of antioxidant enzyme activities and the ascorbic acid content, as well as by the response to UV radiation.

Materials and methods

Algal material and study site

The macroalgal species studied and their depth distribution are listed in Table 1. Plants were collected by scuba divers in summer 1998 at the study site in the Kongsfjord (Ny-Ålesund, Spitsbergen, Norway 78°55.5′N; 11°56.0′E) from depths between 0 and 20 m. Algal samples were collected in black bags to avoid exposure to high irradiance during transport. Material for enzymatic activities and for ascorbic acid determination was immediately frozen in liquid nitrogen and kept at −30°C prior to analysis. Samples were kept for at least 48 h under white fluorescent lamps (35 μmol m^{-2} s^{-1}) in running seawater pumped directly from the fjord before laboratory experiments started.

For laboratory experiments, approximately 10 g fresh weight (FW) of algae was incubated in 5-l plastic tanks in running seawater at a temperature of 2°C and exposed to 38 μmol m^{-2} s^{-1} PAR provided by one Osram daylight fluorescence tube, 8 W m^{-2} UVA (320–400 nm) and 0.36 W m^{-2} UVB (280–320 nm) provided by two Q-Panel UVA-340 fluorescence tubes (Q-Panel, Cleveland, Ohio). Total PAR + UVA + UVB (295–700 nm) radiation treatment was obtained by covering the tanks with Ultraphan cut-off filter foil (cut-off wavelength < 295 nm; Ultraphan, Digefra, Munich, Germany). For the PAR treatment, the tanks were covered with polyester cut-off filter foil (cut-off wavelength < 395 nm; Folex, Dreieich, Germany). Radiation measurements were carried out with a Li-Cor LI-190-SB cosine corrected sensor connected to a Li-Cor LI-1000 datalogger (Lambda Instruments, Lincoln, Neb.) for PAR (400–700 nm), and with an RM-21 broad-band UV radiometer (Dr. Gröbel, Ettlingen, Germany).

In a first set of experiments, the red algae, *Coccotylus truncatus*, *Phycodrys rubens* and the green alga, *Monostroma arcticum*, were exposed for 3 days under continuous PAR and PAR + UVA + UVB radiation. Samples were taken at the start of the experiment and after 24 h and 84 h of exposure. In parallel, at the same time, subsamples were taken and maintained in darkness for 24 h under otherwise identical culture conditions, in order to characterize the possible recovery processes in darkness after exposure to UV radiation.

In a second set of experiments, thalli of the red algae, *Palmaria palmata*, *Devaleraea ramentacea* and the green alga, *Acrosiphonia penicilliformis*, were exposed for 8 days under continuous PAR and

Table 1. Investigated macroalgal species from the Kongsfjord on the Arctic island of Spitsbergen (Svalbard, Norway) and their occurrence in the eulittoral, upper sublittoral (0–3 m) and lower sublittoral (3–20 m) zone according to Svendsen (1959) and Klekowski and Weslawski (1990)

Species	Habitat
Chlorophyta	
Acrosiphonia penicilliformis (Foslie) Kjellman	Eulittoral-upper sublittoral
Monostroma arcticum Wittrock	Upper-lower sublittoral
Chaetomorpha linum (Müller) Kützing	Upper-lower sublittoral
Chaetomorpha melagonium (F. Weber et Mohr) Kützing	Upper-lower sublittoral
Prasiola crispa (Lightfoot) Meneghini	Eulittoral
Rhodophyta	
Coccotylus truncatus (Pallas) M.J.Wynne & J.N.Heine	Lower sublittoral
Devaleraea ramentacea (L.) Guiry	Eulittoral-upper sublittoral
Palmaria palmata (L.) Grev.	Upper-lower sublittoral
Phycodrys rubens (L.) Batters	Lower sublittoral
Odonthalia dentata (L.) Lyngb	Lower sublittoral
Polysiphonia arctica J. Agardh	Lower sublittoral
Ptilota gunneri P.C.Silva, Maggs & L.M.Irvine	Lower sublittoral
Phaeophyta	
Alaria esculenta (L.) Greville	Lower sublittoral
Chorda tomentosa Lyngbye	Upper-lower sublittoral
Chordaria flagelliformis (O. F. Müller) C. Agardh	Upper sublittoral
Desmarestia aculeata (L.) Lamouroux	Lower sublittoral
Elachista fucicola (Velley) Areschoug	Eulittoral-upper sublittoral
Fucus distichus L.	Eulittoral-upper sublittoral
Laminaria saccharina (L.) Lamouroux	Upper-lower sublittoral
Laminaria solidungula J. Agardh	Lower sublittoral
Laminaria digitata (Huds.) Lamouroux	Upper-lower sublittoral
Saccorhiza dermatodea (de la Pylaie). J. Agardh	Upper-lower sublittoral

PAR + UVA + UVB. Parallel to the laboratory experiments, thalli of *A. penicilliformis* were covered in situ by means of an 80×80 cm^2 UV transparent Plexiglass plate, wrapped with the 395 nm cut-off filter foil to avoid UVA plus UVB. These samples were compared with subsamples exposed to full solar radiation at the same part of the rocky shore.

Enzyme activities

Samples (0.2–0.3 g FW) of the studied species were ground in liquid nitrogen and extracted with 1–1.5 ml 50 mM potassium phosphate buffer (pH 7.0) containing Complete protease inhibitor cocktail (Boehringer, Mannheim, 2 tablets in 100 ml buffer). Extracts were centrifuged for 15 min at 15,000 rpm at 4°C. Catalase was analysed according to Aebi (1984); 10–40 µl extract was added to 810–840 µl potassium phosphate buffer (50 mM, pH 7). The reaction was started by the addition of 150 µl of H_2O_2 solution in phosphate buffer (15 mM final concentration in the cuvette) and followed by monitoring the decrease in absorbance at 240 nm at 20°C for 1–2 min. Catalase activity was calculated by subtracting the non-enzymatic reaction and using an extinction coefficient for H_2O_2 of 0.0398 mM^{-1} cm^{-1}. Glutathione reductase (GR) was assayed according to Goldberg and Spooner (1983); 10–40 µl extract was added to 960–990 µl of a buffer containing 80 mM Tris buffer (pH 8), 1 mM EDTA, 0.1 mM NADPH, and 0.5 mM GSSG, and oxidation of NADPH was followed at 340 nm at 20°C. GR activity was calculated by subtracting the non-enzymatic reaction and using an extinction coefficient for NADPH of 6.22 $mM^{-1}cm^{-1}$. Samples for ascorbate peroxidase (APX) activities were extracted with the same protocol as for the other enzymes with the modification that 0.5 mM of ascorbate was added to the extraction buffer for the stability of the APX (Chen and Asada 1989). Enzyme activities were assayed according to the same authors and the decrease of absorbance at 290 nm was followed for 1 min after adding 10–40 µl extract to 960–990 µl 50 mM phosphate buffer (pH 7) containing 0.1 mM of H_2O_2, and 0.5 mM ascorbate. All assays were performed at 20°C. APX activity was calculated by subtracting the non-enzymatic reaction and using an extinction coefficient for ascorbate of 2.8 mM^{-1} cm^{-1}. Results for catalase, GR and APX are expressed as units (U) of enzyme activity per milligram of total soluble protein [1 U = 1 µmol substratum (H_2O_2, NADPH and ascorbate, respectively) converted min^{-1}]. SOD was measured using the xanthine oxidase-cytochrome c reduction method (McCord and Fridovich 1969). In this coupled reaction, SOD inhibits the reduction of cytochrome c by superoxide anions generated from xanthine. The assay mixture (860–1,000 µl) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA, 10 µM cytochrome c and 50 µM xanthine. Xanthine oxidase (Merck) was added to give an increase of absorbance at 550 nm of 0.025 ± 0.003 min^{-1} at 20°C. Samples (10–50 µl) were added to the reaction mixture and the rate of reduction of cytochrome c was followed spectrophotometrically at 550 nm, and 1 unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome c reduction by 50%.

Ascorbic acid was measured according to Foyer et al. (1983). Thalli of 0.2–0.4 g fresh weight were ground in liquid nitrogen and extracted with 1–1.5 ml 100 mM potassium phosphate buffer (pH 5.6) containing 5 mM dithioerythritol (DTE). Extracts were centrifuged for 15 min at 15,000 rpm at 4°C. The ascorbate content was determined by the disappearance of absorbance at 265 nm after addition of 10 U ml^{-1} ascorbate oxidase and 50 µl sample to 925 µl sodium phosphate buffer (100 mM, pH 5.6). Amounts were quantified using a standard curve with 1.25–12.5 µM of pure ascorbate in the reaction mixture.

Protein assay

Total soluble proteins of the crude extract for antioxidant enzyme activities were determined using a commercial Protein Assay (BioRad), based on the Bradford method (Bradford 1976). Protein content was determined spectrophotometrically at 595 nm and concentrations were calculated compared with a standard of bovine serum albumin (SIGMA).

Statistics

Mean values and their standard deviations were calculated from the different replicates per treatment. Statistical significances of means were tested with a model 1 one-way ANOVA, followed by a

multi-range test by Fisher's protected least significance difference (LSD) (Sokal and Rohlf 1995), and a $P < 0.05$ was considered to be statistically significant.

Results

Antioxidant enzyme distribution in Arctic macroalgae

To investigate the protection mechanisms against oxidative stress, a total of 22 species of green, red and brown Arctic macroalgae were analysed for the presence of the activity of superoxide dismutase, glutathione reductase, ascorbate peroxidase and catalase (Table 2). Clear differences were found between the three macroalgal groups, with green algae showing in general higher antioxidant enzyme activities than red and brown algae. Independent of the reference parameters tested to express enzyme activity (fresh weight or protein content), these taxon-specific differences remained similar. Maximum SOD activities were found in *M. arcticum* and *A. penicilliformis*, exhibiting values of 1,004 and 674 U mg TSP^{-1}, respectively.

SOD activities in all species tested were significantly higher ($P < 0.05$) compared to GR, APX and CAT. Maximum GR activities were measured again in the green algal group, with the highest value of 2.3 U mg TSP^{-1} found in *A. penicilliformis*. Red and brown algae showed species-specific GR activities ranging from 0.07 to 0.32 U mg TSP^{-1}. Similar results were obtained in APX and CAT activities, and in both cases *M. arcticum* and *Chaetomorpha* species exhibited highest values. *D. ramentacea* showed exceptionally high values of APX and CAT activities, being 6 times higher in APX and almost 2 times higher in CAT compared to the other investigated red algae (Table 2). The internal concentrations of ascorbate in the different species varied from traces in brown algae up to values of 1.63 mg ascorbate gFW^{-1} in *Chaetomorpha melagonium*.

Ultraviolet radiation effects on antioxidant enzymes

Ultraviolet radiation leads to a significant enhancement ($P < 0.05$) of the enzymatic activities of GR in laboratory experiments (Fig. 1a, b, c: note the different species-specific scales). Exposure to 8 W m^{-2} UVA and 0.36 W m^{-2} UVB promoted an increase of 22% in GR activity in the green alga *M. arcticum* after 24 h of continuous irradiation compared to the control under PAR radiation (Fig. 1a). GR activity in subsamples kept for 24 h in darkness increased further. After 84 h exposure, GR activity rose under both radiation conditions, especially after exposure to PAR and UV radiation. After 84 h exposure, followed by 24 h of darkness, GR activity was slightly higher ($P < 0.05$) in specimens previously exposed to UV radiation.

In the red algae *Coccotylus truncatus* and *Phycodrys rubens*, 24 h of continuous UV radiation did not result in significant differences of GR activity with respect to the control (Fig. 1b, c). However, after 84 h of contin-

Table 2. Enzymatic activities of superoxide dismutase (*SOD*), glutathione reductase (*GR*), catalase (*CAT*), ascorbate peroxidase (*APX*) and the content of ascorbic acid in different green, red and brown algae from the Kongsfjord (Spitsbergen). Results are expressed as units (*U*) of enzyme activity per milligram of total soluble proteins (*TSP*) where 1 $U = 1$ µmol substrate converted min^{-1}. Standard deviations were less than 20% (– not measured)

Species	SOD (Umg TSP^{-1})	GR (Umg TSP^{-1})	APX (Umg TSP^{-1})	CAT (Umg TSP^{-1})	Ascorbate (mg gFW^{-1})
Chlorophyta					
Acrosiphonia penicilliformis	674	2.30	0.2	1.0	0.19
Monostroma arcticum	1004	1.58	0.97	27.11	1.63
Chaetomorpha linum	395	0.10	0.778	0.77	0.65
Chaetomorpha melagonium	200	1.54	0.5	30.00	1.57
Prasiola crispa	153	0.10	0.12	3.86	–
Rhodophyta					
Coccotylus truncatus	165	0.08	0.05	9.91	0.27
Devaleraea ramentacea	245	0.32	0.60	15.5	0.2
Palmaria palmata	185	0.22	0.45	4.5	0.43
Phycodrys rubens	94	0.08	0.04	–	–
Odonthalia dentata	109	0.09	0.10	6.24	0.43
Polysiphonia arctica	87	0.06	0.02	7.58	0.18
Ptilota gunneri	122	0.11	0.01	–	0.2
Phaeophyta					
Alaria esculenta	102	0.07	0.05		0.06
Chorda tomentosa	128	0.11	0.07	–	0.75
Chordaria flagelliformis	112	0.10	0.03	–	–
Desmarestia viridis	143	0.10	0.08	–	0.31
Elachista fuciola	191	0.10	0.05	–	–
Fucus distichus	151	0.09	0.07	3.60	0.29
Laminaria saccharina	181	0.18	0.04	7.97	0.17
Laminaria solidungula	142	0.08	0.01	–	traces
Laminaria digitata	68	0.10	0.05	–	traces
Saccorhiza dermatodea	88	0.09	0.06	–	0.58

uous UV radiation, a significant increment ($P < 0.05$) in GR activity from 0.18 to 0.31 U mgTSP^{-1} for *C. truncatus* and from 0.25 to 0.32 U mgTSP^{-1} for *P. rubens* was measured. Moreover, the GR activity increased in subsamples of both species cultured in darkness following the UV exposure.

In contrast to GR activity, SOD in the three algal species studied seemed not to be positively affected ($P > 0.05$) at the end of the exposure to the radiation treatments (Fig. 1d, e, f). In *M. arcticum* and *P. rubens*, UV promoted a significant decrease of SOD activity after 24 h of exposure and after 24 h in darkness ($P < 0.05$), but after 84 h exposure and the following 24 h of darkness, no significant difference ($P > 0.05$) between the two radiation conditions could be detected. The red alga *C. truncatus* showed an unchanged SOD activity under all treatment conditions.

The red algae, *Palmaria palmata* and *Devaleraea ramentacea*, were kept for 8 days under continuous UV radiation under the same conditions as described above. For both species, no significant UV effect was observed ($P > 0.05$) in CAT, APX and SOD activities (Fig. 2a–f). Although CAT actvities in both plants increased within 8 days, significant differences between the radiation treatments could not be detected (Fig. 2a, d). For APX, an inconsistent activity pattern was observed. After UV exposure, variation was high in both species, with no specific pattern or effect (Fig. 2b, e). In *P. palmata*, SOD activities markedly increased

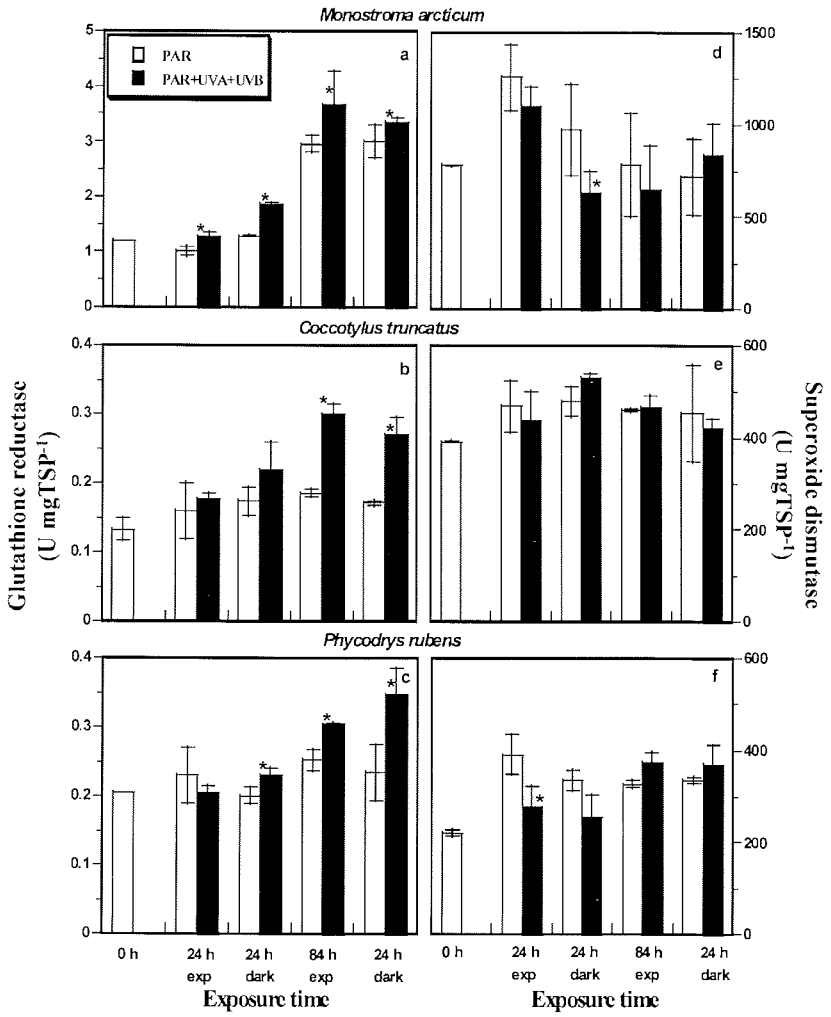

Fig. 1a–f. Influence of exposure under artificial PAR (*grey bars*) and PAR + UVA + UVB (*black bars*) radiation on enzymic activities of glutathione reductase (*GR*) and superoxide dismutase (*SOD*) in the red algae *Coccotylus truncatus* and *Phycodrys rubens* and the green alga *Monostroma arcticum* from Spitsbergen. Plants were exposed for 84 h under continuous irradiation. Subsamples were cultured for 24 h in darkness after 24 and 84 h exposure. Data are given as mean values ± SD ($n = 3$) and expressed as Units mg^{-1} total soluble proteins (*TSP*). Mean values with different *asterisks* are significantly different (at $P = 0.05$) to the control

under PAR within the 8 days of exposure, whereas activities under UV radiation were lower (Fig. 2c). In *D. ramentacea*, no significant changes in SOD activities could be recorded (Fig. 2d).

In a third set of radiation experiments, the eulittoral green alga *Acrosiphonia penicilliformis* was kept for 8 days under continuous UV irradiation under the same laboratory conditions as before (Fig. 3a, b). In this case, no significant differences were observed in GR and SOD activities when the algae were maintained under PAR and PAR+UVA+UVB radiation ($P > 0.05$). In contrast, in the field experiment, algal thalli were exposed in situ to the full solar spectrum and compared with thalli where UVA+UVB was filtered out of the natural solar radiation spectrum (Fig. 3c, d). Samples exposed for 8 days to UV filtered radiation showed a significant decrease ($P < 0.05$) in GR and SOD activity with respect to the fully exposed subsamples.

Discussion

The present study provides a survey of the qualitative and quantitative content and activities of different reactive-oxygen-scavenging enzymes in 22 macroalgal species from the Arctic. We detected a great variation with respect to the antioxidant enzyme activities and found strong species-specific differences. High activities of antioxidant enzymes found in green algae were

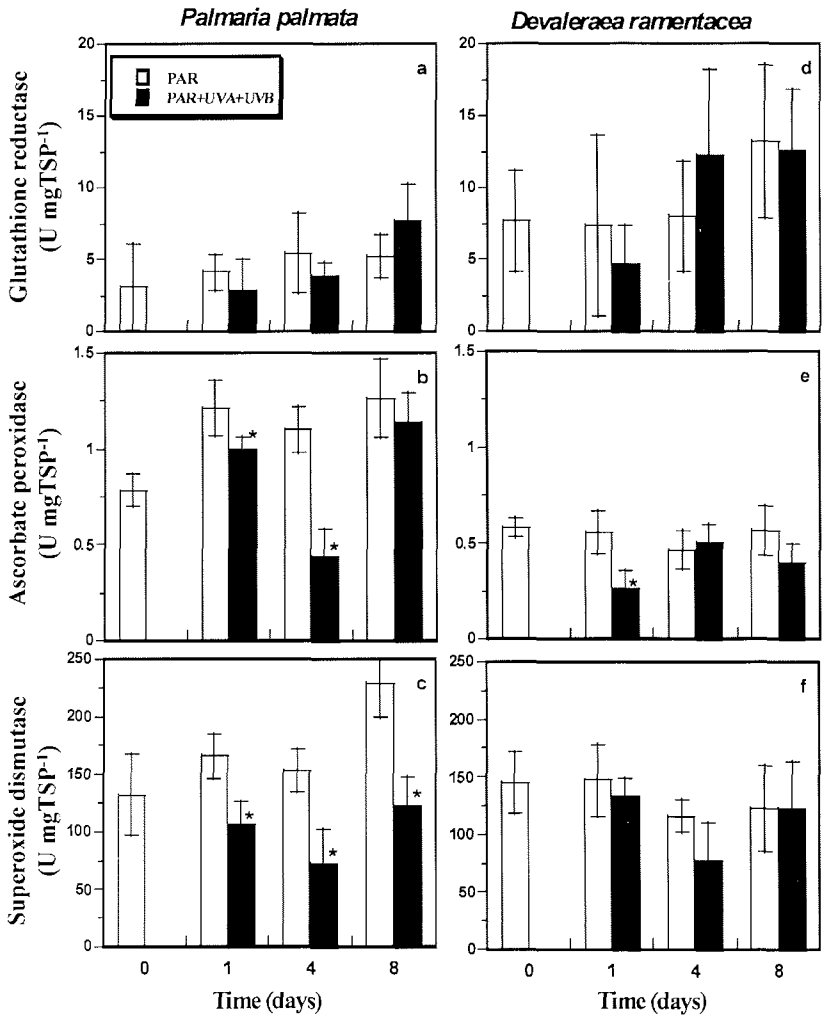

Fig. 2a–f. Influence of continuous exposure under artificial PAR (*grey bars*) and PAR+UVA+UVB (*black bars*) radiation on enzymatic activities of glutathione reductase (GR), ascorbate peroxidase (*APX*) and superoxide dismutase (*SOD*) in the Arctic red algae *Palmaria palmata* and *Devaleraea ramentacea*. Data are given as mean values ± SD ($n = 5$) and expressed as Units mg^{-1} total soluble proteins (*TSP*). Mean values with different *asterisks* are significantly different (at $P = 0.05$) to the control

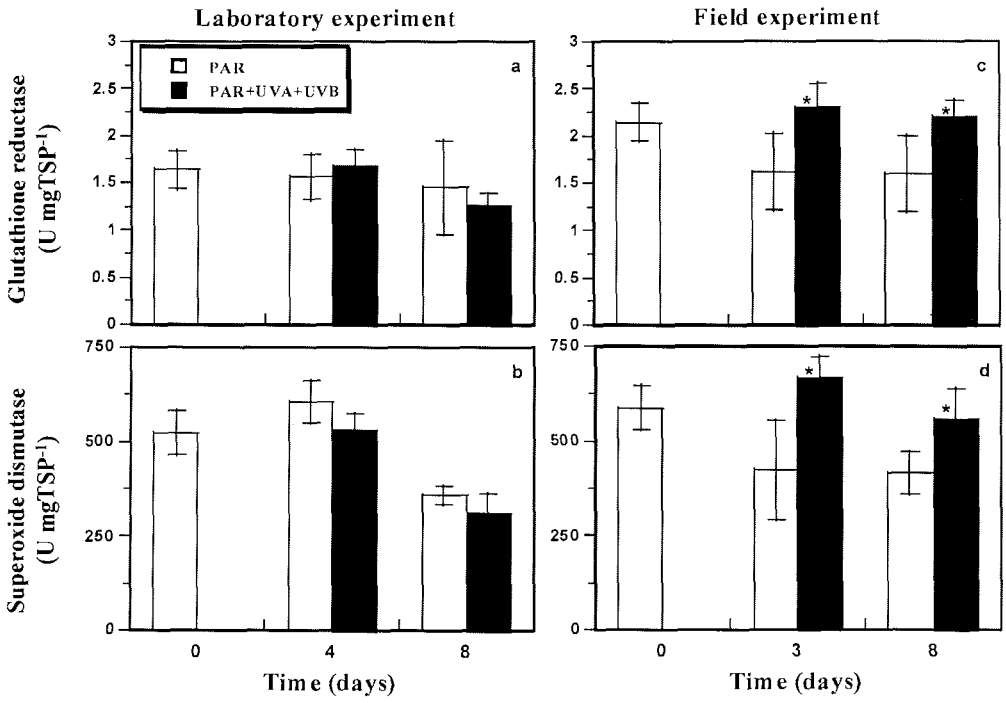

Fig. 3a–d. Changes of the activities of glutathione reductase (*GR*) and superoxide dismutase (*SOD*) in the Arctic green alga *Acrosiphonia penicilliformis*. **a**, **b** Laboratory experiment: continuous exposure under artificial PAR (*grey bars*) and PAR + UVA + UVB (*black bars*) radiation; **c**, **d** Field experiment: exposure under total (*black bars*) and UVA + UVB cut-off solar radiation by means of a selective UV cut-off filter (cut off at < 395 nm.). Data are given as mean values ± SD ($n = 3$) and expressed as Units mg^{-1} total soluble proteins (*TSP*). Mean values with different *asterisks* are significantly different (at $P = 0.05$) to the control

comparable to those of higher plants and microalgae. For example, *M. arcticum*, which showed the highest activity in SOD within the investigated macroalgae, is in the same activity range as reported for pea leaves (Moran et al. 1994). CAT activity of *M. arcticum* is half as high as that of cotton fibres (Rajguru et al. 1999), whereas activities of SOD and GR were much higher. In comparison with other algae, APX activities of *M. arcticum* are comparable to those of the symbiotic zooxanthellae of the sea anemone, *Aiptasia pallida* (Lesser and Shick 1989) while CAT and SOD activities are much higher. Data with respect to other macroalgae are scarce and only the data on the Arctic brown alga *Fucus distichus* (Table 2) can be directly compared to those of similar species from temperate water (Collen and Davison 1999a). CAT activity of *F. distichus* was similar to that reported by other authors for the same species while SOD activity was much higher in the polar species and lower activities were measured for GR and APX. The ascorbic acid concentrations found in green algae are very high, similar to those reported, in general, for lemons and oranges. *Chaetomorpha linum*, for example, contains 0.65 mg ascorbic acid g^{-1} FW, a concentration similar to that of lemon, with approximately 0.69 mg g^{-1} FW. *Fucus distichus* from Spitsbergen contained ascorbic acid values in the same range as in this and other *Fucus* species from temperate regions (Collen and Davison 1999a).

A closer analysis points to the relation between antioxidant activities and depth distribution. In particular, differences in SOD activity between algal groups are related to the depth distribution on the shore. Most green algae, which showed higher antioxidant activities, typically inhabit the upper part of the shore at the Kongsfjord. Similarly, the red algae, *Devaleraea ramentacea* and *Palmaria palmata*, occurring frequently in the upper sublittoral, exhibited higher SOD activities compared to red-algal species living in deeper waters, such as *Phycodrys rubens*. Additionally, green algae contain relatively high amounts of ascorbic acid. Therefore, species more exposed to drastic and rapid changes in environmental radiation conditions have developed an efficient biochemical defence system to withstand the stress.

Although directly exposed to solar radiation, the supralittoral species *Prasiola crispa* growing underneath bird colonies exhibits very low antioxidant enzyme activities in comparison to the other green algae. In this plant from such an unusual habitat, it seems that

another photoprotective strategy is developed, such as the biosynthesis of UV-absorbing compounds that are known to prevent radiative damage (Dunlap and Shick 1998). In *Prasiola crispa* ssp. *antarctica* from Antarctica, high amounts of two new, so far chemically uncharacterized, mycosporine-like amino acids (MAAs) have been reported (Hoyer et al. 2001). MAAs represent a group of compounds with a potential role as UV sunscreens, exhibiting absorption maxima between 310 and 360 nm (Karentz et al. 1991). Their accumulation is positively correlated to the extent of UV exposure, as shown in laboratory and field studies (García-Pichel and Castenholz 1991; Karsten et al. 1998; Lesser 1996b; Shick et al. 1995). *Prasiola* is the only genus within the green algae containing MAAs. At present it is unknown whether the uncharacterized MAAs in *Prasiola crispa* also exhibit antioxidative properties as described for other MAAs (Dunlap and Yamamoto 1995).

Compared to other algal taxa, antioxidant enzyme activities in brown algae are low. However, there is a strong adaptation and/or acclimation potential of photosynthesis (Bischof et al. 1998a, b, 2000) and growth (Aguilera et al. 1999a) to UV radiation in this macroalgal group. In this context we refer to the typically high content of phenolic compounds in brown algae (Ragan and Glombitza 1986; Van Alstyne and Paul 1990), since these substances can act as antioxidants by transferring hydrogen atoms to lipid peroxyl radicals (Foti et al. 1994). However, their role as antioxidants may be questionable because they are accumulated in special compartments, the physodes (Schoenwaelder 2001), rather than uniformly distributed in the protoplasm. Another explanation for the high adaptation and acclimation potential of brown algae to UV radiation may be the ability of phenolic compounds such as phlorotannins to act as UV sunscreen pigments, as suggested by Pavia et al. (1997).

The investigation of the effects of the ultraviolet waveband of the solar spectrum on polar marine ecosystems has become an important ecological issue as a result of a gradual depletion of the ozone layer in both hemispheres. Exposed organisms have developed different strategies for protection against this biologically harmful radiation. However, almost no studies on the mechanisms of production of reactive oxygen species by UV radiation, and the biochemical defence strategies against this reactive species, have been performed for macroalgae. In order to analyse the ecophysiological importance of the total UV region (UVA + UVB) of solar radiation, laboratory and field experiments have been performed. Our study has clearly shown that the activity of antioxidant enzymes is stimulated by UV radiation in several Arctic macroalgae. The combination of artificial UVA + UVB radiation increased the GR activity in *arcticum*, *Coccotylus truncatus* and *Phycodrys rubens* after 84 h under continuous exposure. GR stimulation under UV radiation indicates an active scavenging of H_2O_2 by means of the ascorbate-glutathione cycle in combination with the Mehler-peroxidase reaction, which is the major pathway for scavenging potentially toxic intermediates of oxygen metabolism in photosynthesis, which at the same time enables down-regulation of electronflux (Polle 1996). Dehydroascorbate formed by oxidation of ascorbic acid for scavenging of H_2O_2 by means of ascorbate peroxidase is reduced again to ascorbate, taking electrons from reduced glutathione by means of dehydroascorbate reductase. The product of these reactions, glutathione disulphide (GSSG), is reduced by the activity of GR and consumption of NADPH. Plants have been shown to increase GR activity in response to stress (Edwards et al. 1994). Increments of GR activity in response to UV radiation have been described in *Arabidopsis* (Kubo et al. 1999; Rao et al. 1996). In Arctic macroalgae, it seemed to be a faster stimulation of GR activities after 24 h exposure, followed by incubation in darkness again for 24 h, indicating some kind of a dark-enhanced repair system after damage in light. Recovery from UV damage in low light or darkness has been extensively investigated in macroalgae, especially in studies on photoinhibition of photosynthesis (Hanelt 1996, 1998). Thus, stimulation of the biochemical system involved in the scavenging of reactive oxygen species generated in the photoinhibitory status, mediates this recovery in photosynthesis. The role of antioxidants in the partial recovery of photosynthetic performance has been studied in symbiotic cnidarians and their zooxanthellae (Lesser and Shick 1989). According to these authors, the fluxes of reduced oxygen intermediates cause damage to the photosynthetic apparatus. In contrast to GR activities, no UV effects on SOD activities were found in *Monostroma arcticum*, *Coccotylus truncatus* and *Phycodrys rubens* after this period of treatment. However, in *Palmaria palmata*, UV radiation seemed to directly affect the SOD activity and a decrease, just after the 1st day of culture, was observed in comparison to the PAR control. These results are comparable to those observed in the green microalga, *Chlorella vulgaris*, in which long-term effects of increasing UVB radiation resulted in a decrease in SOD activities (Malanga and Puntarulo 1997). The reason for this negative effect may be an inhibition of gene expression for this enzyme as observed by Strid (1993) in *Pisum sativum* or an unspecific effect on enzyme activity. In contrast, Lesser and Shick (1989) found a stimulation of SOD activities in the symbiotic zooxanthellae of *Aiptasia pallida* by UV radiation. In that species, an increase in the SOD activities was correlated with an increase in the CAT activity, while in the present work no significant differences were found in the red algae *Palmaria palmata* and *Devaleraea ramentacea* after 8 days treatment.

No effects of artificial UV radiation on GR and SOD have been observed in the green alga, *Acrosiphonia penicilliformis*. In contrast, a significant reduction in GR and SOD activities was observed when natural UV radiation was cut off by selective filter foils in the field. This means that survival of this species in the intertidal zone is mediated by an enhanced oxygen-reactive scavenging system, in combination with

morphological strategies, as reported by Aguilera et al. (1999a). While the apical region of this plant is mainly exposed to strong solar radiation, the basal cells are well protected due to self-shading. In the field, yellow-coloured tips were often observed, indicating lack of chlorophyll as a consequence of photobleaching of the apical parts, along with dark-green pigmented, healthy and unstressed basal parts.

In conclusion, different biochemical capabilities of the enzymatic defence systems against reactive-oxygen species were observed for several Arctic macroalgae of different taxa and habitats. The antioxidant enzyme activity is enhanced in species that grow in the upper part of the rocky shore, where they are exposed to drastic changes in environmental conditions, especially those related to rapid and drastic changes in the UV region of the solar spectrum.

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Publication II

Interactive effects of ultraviolet radiation and salinity on the ecophysiology of two Artic red algae from shallow waters

KARSTEN U[1], DUMMERMUTH AL[2], HOYER K[2], WIENCKE C[2]

[1] University of Rostock,
Institute of Aquatic Ecology,
Albert-Einstein-Str. 3
18059 Rostock, Germany
email: ulf.karsten@biologie.uni-rostock.de

[2] Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12
27570 Bremerhaven

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ORIGINAL PAPER

Ulf Karsten · Angelika Dummermuth · Kirsten Hoyer
Christian Wiencke

Interactive effects of ultraviolet radiation and salinity on the ecophysiology of two Arctic red algae from shallow waters

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Abstract In a comparative ecophysiological study, the abundant red macroalgae *Devaleraea ramentacea* (L.) Guiry and *Palmaria palmata* (L.) O. Kuntze from shallow waters of the Arctic Kongsfjord (Spitsbergen) were exposed to hyposaline and hypersaline media, in combination with and without artificial UV radiation, to evaluate the interactive effects of both environmental parameters on optimum quantum yield of photosynthesis, as well as on the physiological capability to synthesise and accumulate photoprotective mycosporine-like amino acids (MAAs). While *D. ramentacea* exhibited euryhaline features and acclimated well to the UV radiation applied, *P. palmata* can be characterised as a stenohaline plant because of its high mortality even under mild hyposaline conditions (15 PSU). In addition, the latter species showed a limited ability to acclimate to changing PAR/UV radiation, pointing to a relatively low physiological plasticity. Both species synthesised and accumulated MAAs after UV treatment. However, only in *D. ramentacea* was a correlation between increasing MAA concentration and decreasing photosynthetic sensitivity under UV observed. All ecophysiological data from the laboratory correlate well with field observations, where both red-algal species co-exist in the same shallow-water habitat of the Kongsfjord. However, while *P. palmata* becomes more often greenish, sometimes slightly bleached over the summer months, *D. ramentacea* appears much more healthy under the prevailing environmental conditions.

U. Karsten (✉)
Institute of Aquatic Ecology, University of Rostock,
Albert-Einstein-Strasse 3, 18051 Rostock, Germany
E-mail: ulf.karsten@biologie.uni-rostock.de
Fax: +49-381-4986072

A. Dummermuth · K. Hoyer · C. Wiencke
Alfred-Wegener-Institute for Polar and Marine Research,
Am Handelshafen 12, 27570 Bremerhaven, Germany

Introduction

The Arctic Kongsfjord on Spitsbergen is a marine coastal ecosystem that has been intensively studied over recent years as a model for global change (Hanelt et al. 2001; Hop et al. 2002, and references therein). A typical feature of the fjord is a well-structured phytobenthic community down to a depth of almost 40 m (Hop et al. 2002) which plays an important role in primary production, being a food source for herbivores and detrivores, as well as a nursery area and habitat for fish and invertebrates (Lippert et al. 2001). Marine macroalgae of such high latitudes are exposed to seasonally fluctuating environmental factors such as solar radiation and temperature, as well as to long periods of ice cover (Hanelt et al. 2001; Hop et al. 2002).

Compared to the "ozone hole" over Antarctica, which has been known since the 1970s (Smith et al. 1992), the increase in ozone depletion over the Arctic represents a more recent phenomenon (see references in Wängberg et al. 1996; Rex et al. 2000; Hanelt et al. 2001). As a consequence of ozone springtime reduction in the polar regions, the UVB-radiation waveband (280–320 nm) rises markedly. Although the biological consequences of changes towards higher doses of UV radiation in marine ecosystems are not fully understood, many phototrophic organisms living in the intertidal, as well as in the upper subtidal, zone of the coasts are strongly affected (Franklin and Forster 1997).

The macroalgal species *Devaleraea ramentacea* (L.) Guiry and *Palmaria palmata* (L.) O. Kuntze are the most abundant Rhodophyta in the upper sublittoral of the Kongsfjord. While the first species represents one of the few endemics of the Arctic region, the latter species occurs from temperate to cold waters of the Atlantic ocean, and exhibits on Spitsbergen its northern distribution limit. In spring/summer, both organisms are often exposed to high solar radiation, and hence their photophysiology and protecting strategies to avoid or counteract UV-induced damage have been studied in

great detail (Hanelt et al. 1997; Aguilera et al. 1999, 2002; Karsten and Wiencke 1999). From these studies, it could be concluded that *D. ramentacea* and *P. palmata* are capable of physiologically acclimating to diurnally changing solar radiation due to dynamic photoinhibition, i.e. the up-and-down regulation of photosynthesis in response to the respective prevailing low and high visible light, as well as UV conditions (Hanelt 1998). In addition, to prevent UV photodamage, these macroalgal species are biochemically capable of synthesising and accumulating UV-absorbing substances, the so-called mycosporine-like amino acids (MAAs) (Dunlap and Shick 1998; Karsten and Wiencke 1999; Karsten et al. 1999). As passive sunscreens, MAAs preferentially absorb UV photons in the spectral range of 310–360 nm, followed by dissipation of the absorbed radiation energy in the form of harmless heat and fluorescence without generating photochemical reactions (Bandaranayake 1998; Cockell and Knowland 1999), and thereby protecting, at least partially, photosynthesis and growth of phototrophic organisms (Garcia-Pichel et al. 1993; Neale et al. 1998).

The motivation for the present study was the field observation that, in shallow waters of the Kongsfjord during the summer season, thalli of *P. palmata* often looked rather greenish, and sometimes slightly bleached compared to the mainly, although not always, red-coloured *D. ramentacea* from similar locations. Although, intuitively, radiation stress seemed to be the responsible factor, earlier results indicated a relatively high photosynthetic tolerance of *P. palmata* under increasing natural PAR and UV doses (Hanelt et al. 1997; Karsten et al. 2001). Since the large discharge of melting water into the fjord can locally and temporarily decrease the seawater salinity down to 23 PSU (Hanelt et al. 2001), and because of the fact that subtidal red algae are generally stenohaline (Kain and Norton 1990), we assumed that this abiotic factor may act as an additional stressor on the macroalgal physiology. Therefore, in a comparative ecophysiological study, we exposed *D. ramentacea* and *P. palmata* under controlled conditions on Spitsbergen to hyposaline and hypersaline media, in combination with and without artificial UV radiation, to evaluate the interactive effects of both environmental parameters on photosynthetic performance, as well as on the ability to synthesise and accumulate MAAs.

Materials and methods

Algal material and study site

The red macroalgae, *D. ramentacea* (L.) Guiry and *P. palmata* (L.) O. Kuntze, preferentially grow in shallow waters at the study site in the Kongsfjord (Ny-Ålesund, Spitsbergen, 78°55.5'N; 11°56.0'E). Both species are typically attached to coarse gravel and single rocks on sandy sediments in the fjord or occur as epiphytes on rhizoids of kelps such as *Laminaria saccharina* (L.) Lamouroux. In the Kongsfjord, *D. ramentacea* typically grows in depths from 1 m down to 8 m, while *P. palmata* is found slightly deeper, from 2 m to 10 m. All algal samples were collected from healthy-looking, dark-red plants at 3–5 m by scuba-divers and kept in black bags to avoid exposure to higher solar irradiances prior to laboratory experiments, and used straight away within 1–2 h.

Radiation and salinity experiments

Thalli of both species were cut at 3–4 cm from the apical region, using a razor blade to get almost homogeneous pieces of the same age class for the exposure experiments. All plantlets were kept for 24–36 h in running seawater at 3–5°C and dim light conditions (< 5 μmol PAR $m^{-2}s^{-1}$) to minimise potential wound-healing responses. Afterwards, algae were treated with hypo- and hypersaline media, in combination with PAR and PAR + UV exposure, over a period of 4 days. Hypersaline media of 50 PSU were prepared by freezing-out fresh water from fully marine fjord water. The dilution of fjord water with MilliQ water resulted in a hyposaline solution of 15 PSU. Salinity was checked using a refractometer. All salinity treatments were carried out in 300-ml glass containers. For each salinity/radiation treatment and sampling date, two containers each with four to five thalli were used. These vessels were irradiated from the top with 30 μmol PAR m^{-2} s^{-1}, 6.7 W m^{-2} UV-A (320–400 nm) and 0.25 W m^{-2} UV-B (280–320 nm). Due to technical limitations on the field station, it was not possible to adjust the ratio between PAR, UV-A and UV-B to natural conditions. Therefore we accepted the application of almost realistic UV-A and UV-B radiation, in combination with much too low PAR as background irradiance. As radiation source, a combination of Philips daylight fluorescence tubes and Q-Panel UV-A-340 fluorescence tubes (Q-Panel Company, Cleveland, Ohio) were used. Radiation measurements were carried out with a Li-Cor LI-190-SB cosine-corrected sensor connected to a Li-Cor LI-1000 datalogger (Lambda Instruments, Lincoln, Neb.) for PAR, and with an RM-21 broad-band UV radiometer (Dr. Gröbel, Ettlingen, Germany). While half of the containers (15, 34 and 50 PSU) were exposed to the full radiation spectrum, the other half was kept under a specific filter foil to cut off UV-A + B (PAR treatment) (400 nm cut-off; Folex PR, Folex, Dreieich, Germany). Scattering effects were neglected. All thalli were exposed to 24 h PAR per day, thus reflecting Arctic summer conditions, while supplemented UV radiation was applied for only 10 h per day, resulting in a 10 h UV-treatment interval followed by a 14 h recovery period. Temperature was kept constant at approximately 5°C. As a parameter to check optimum physiological performance, photosynthesis of separate thalli, i.e. true replicates, was always measured 8 h after on-set, as well as 7 h after off-set, of UV radiation. After 1, 2 and 4 days treatment with the different salinity and radiation combinations, samples for MAA analysis were taken.

Photosynthesis

After sampling, algal thalli were kept for 10–15 min inside a light-tight box. Afterwards, photosynthetic activity was determined in this container by measuring variable chlorophyll-fluorescence of photosystem II using a portable pulse amplitude modulated fluorometer (Diving-PAM, Walz, Effeltrich, Germany). The main application of the Diving-PAM is the determination of effective PS II quantum yield by the saturation pulse method ($\Delta F/F_m'$ = effective quantum yield of an irradiated sample, $\Delta F = F_m' - F_t$, Genty et al. 1989). However, if determined in the dark, as undertaken in the present study, the effective quantum yield equals the optimum quantum yield, which was calculated as the ratio of variable to maximum fluorescence F_v/F_m. Minimal fluorescence (F_o) was measured with a pulsed measuring beam (approximately 0.3 μmol photons m^{-2} s^{-1}, 650 nm), followed by short pulses of saturating white light (0.4–0.8 s, 1,000–5,000 μmol photons m^{-2} s^{-1}) to record F_m ($F_v = F_m - F_o$) (Hanelt 1998). F_v/F_m values of both red algal species acclimated for 24–36 h to the dim light conditions in the laboratory were characteristic for photosynthetically non-inhibited plants, and consequently set to 100% (= control). While

D. ramentacea exhibited a maximum F_v/F_m value of 0.65 ± 0.02 (n = 6), *P. palmata* showed an F_v/F_m value of 0.59 ± 0.03 (n = 9). All data recorded are expressed in relation to the respective value.

MAA extraction and analysis

After sampling, plants were oven-dried at 50°C, and then stored in sealed plastic bags under dry and dark conditions until analysis. Samples (4–5 replicates) of about 10–20 mg dry weight (DW) were extracted for 1.5–2 h in screw-capped centrifuge vials filled with 1 ml 25% aqueous methanol (v/v), and incubated in a waterbath at 45°C. After centrifugation at 5,000 *g* for 5 min, 700 µl of the supernatants were evaporated to dryness under vacuum (Speed Vac Concentrator SVC 100H). Dried extracts were re-dissolved in 700 µl 100% methanol and vortexed for 30 s. After passing through a 0.2-µm membrane filter, samples were analysed with a Waters HPLC system according to the method of Karsten et al. (1998a), modified as follows. MAAs were separated on a stainless-steel Phenomenex Sphereclone RP-8 column (5 µm, 250×4 mm I.D.) protected with an RP-8 guard cartridge (20×4 mm I.D.). The mobile phase was 5% aqueous methanol (v/v) plus 0.1% acetic acid (v/v) in water, run isocratically at a flow rate of 0.7 ml min^{-1}. MAAs were detected online with a Waters photodiode array detector at 330 nm, and absorption spectra (290–400 nm) were recorded each second directly on the HPLC-separated peaks. Identification was done by spectra, retention time and by co-chromatography with standards extracted from the marine red macroalgae, *Chondrus crispus* Stackhouse (Karsten et al. 1998a) and *Porphyra umbilicalis* (L.) Kützing, as well as from ocular lenses of the coral trout, *Plectropomus leopardus* Lacepède, kindly sent by Dr. David Bellwood, James Cook University, Townsville, Australia. Quantification was done using the molar extinction coefficients given in Karsten et al. (1998b).

Statistics

Mean values and standard deviation per treatment were calculated. Statistical significance of differences in photoinhibitory response in plants kept under different salinities and radiation scenarios was tested by one-way analysis of variance (ANOVA), followed by a multi-range test using Fisher's protected least-significant difference (LSD) according to Sokal and Rohlf (1995). Calculations were done using the program InStat (GraphPad, San Diego, Calif.). To evaluate interactive effects of UV and salinity on photosynthesis, a 3-way ANOVA followed by a Tukey-Kramer posthoc test was applied using the program NCSS (Number Cruncher Statistical Systems, Kaysville, Utah).

Results

During the course of the experiment, the optimum quantum yield (F_v/F_m) of the control thalli of *D. ramentacea* (34 PSU, PAR) always remained high, exhibiting values between 86 and 98% of the maximum, i.e. of non-inhibited plants (Fig. 1). Algae treated with 15 PSU showed a slight, but significant, decrease in F_v/F_m ($P < 0.01$) down to 76% of the control over the first 39 h, followed by some recovery, resulting in values 87% of those of non-treated samples at the end of the experiment. In contrast, in plants kept at 50 PSU, F_v/F_m showed a stronger and continuous decline to 58% of the optimum after 39 h ($P > 0.01$) (Fig. 1). Afterwards, optimum quantum yield gradually increased up to 76% of the control.

F_v/F_m of *D. ramentacea* treated with salinity plus UV radiation was generally much more affected compared to the salinity-only experiment (Fig. 2). Thalli kept at 15 and 34 PSU showed, at the end of the first two UV-exposure intervals, a decline in optimum quantum yield down to 50–60% of the control. However, during each recovery period, F_v/F_m increased to 75–80% of the maximum. In contrast, under hypersaline conditions photosynthesis was more strongly inhibited under UV (45–50% of control; $P < 0.01$) and did not show marked recovery under PAR conditions within 48 h ($P < 0.01$). However, after the last interval of the UV treatment at 50 PSU, F_v/F_m in *D. ramentacea* was much less affected, resulting in 67% of the maximum. The final

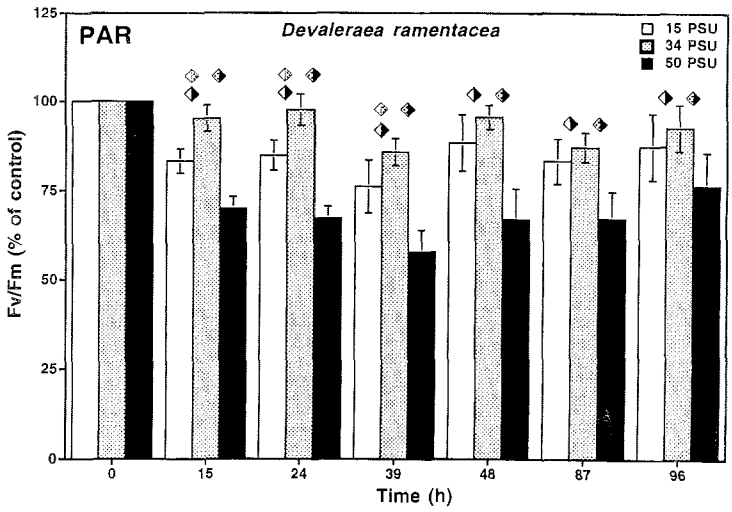

Fig. 1 Changes in photosynthetic optimum quantum yield (F_v/F_m) of *Devaleraea ramentacea* under various salinity conditions (15, 34, 50 PSU) and visible light (*PAR*) over the course of 96 h. F_v/F_m of non-inhibited plants was determined as 0.65 ± 0.02 and standardised to 100%. Given are the mean values ± SD (n = 10). Significant differences ($P < 0.01$) among samples under various salinity treatments are marked with *squares*: 15 PSU versus 34 PSU (*white-pointed triangle*), 15 PSU versus 50 PSU (*white-black triangle*), 34 PSU versus 50 PSU (*black-pointed triangle*)

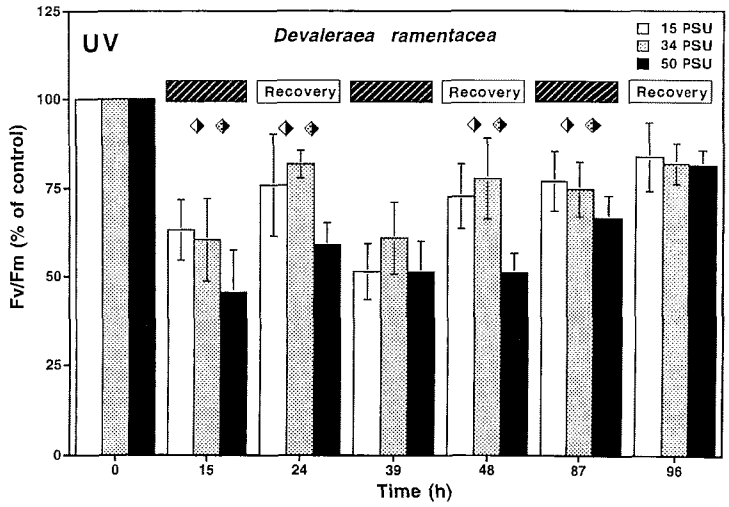

Fig. 2 Changes in photosynthetic optimum quantum yield (F_v/F_m) of *Devaleraea ramentacea* under various salinity conditions (15, 34, 50 PSU) and ultraviolet radiation (*UV*) over the course of 96 h. F_v/F_m of non-inhibited plants was determined as 0.65 ± 0.02 and standardised to 100%. Given are the mean values ± SD ($n = 10$). Significant differences ($P < 0.01$) among samples under various salinity treatments are marked with *squares*: 15 PSU versus 34 PSU (*white-pointed triangle*), 15 PSU versus 50 PSU (*white-black triangle*), 34 PSU versus 50 PSU (*black-pointed triangle*). The *black bars* indicate the 10-h UV treatment interval followed by the 14-h recovery period

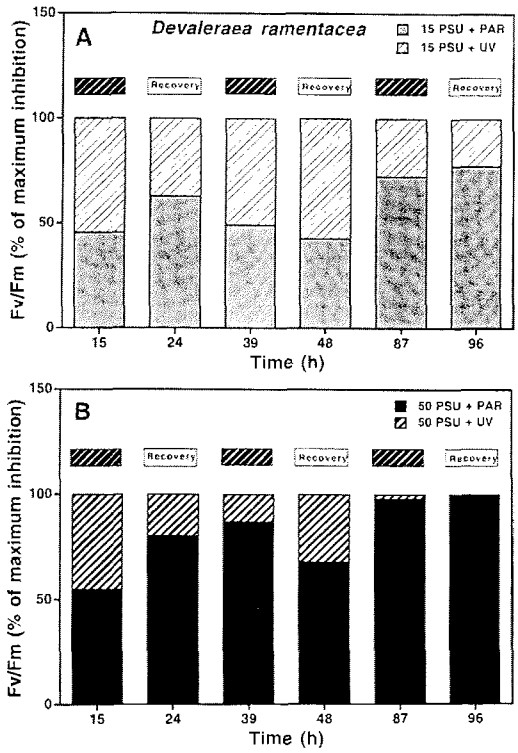

Fig. 3A, B The effect of salinity and UV treatment on the maximum decrease in the photosynthetic optimum quantum yield (F_v/F_m) of *Devaleraea ramentacea* over the course of 96 h. From the mean value data presented in Figs. 1 and 2, the proportional degree of photoinhibition due to both stress factors was calculated and expressed as percentage of maximum photoinhibition. **A** 15 PSU ± UV treatment; **B** 50 PSU ± UV treatment

measurement of recovery at the end of the experiment clearly indicated for all salinities identical optimum quantum yields >81% of the control (Fig. 2). Three-way ANOVA indicated strong interactive effects between UV and salinity in *D. ramentacea* ($P < 0.001$).

The proportional degree of photoinhibition (F_v/F_m) in *D. ramentacea* due to salinity and UV treatment is shown in Fig. 3. Under hyposaline conditions over the first 48 h, 15 PSU and UV led to nearly equal photoinhibitory responses (Fig. 3A). However, after 87 and 96 h exposure, the UV effect strongly decreased, resulting in only 23–28% of the total decline in optimum quantum yield. In contrast, under hypersaline conditions, the UV effect on F_v/F_m in *D. ramentacea* was generally much less pronounced, and continuously decreased over the course of the experiment (Fig. 3B). After 87 and 96 h treatment, the salinity factor only was responsible for the observed decline in optimum quantum yield.

Seven different MAAs were detected in *D. ramentacea*, namely, mycosporine-glycine, shinorine, porphyra-334, palythine, asterina-330, palythinol and palythene (data not shown). Porphyra-334 and palythine were the quantitatively dominating MAAs in all samples and were present in almost equimolar concentrations. Plants at the beginning of the experiment contained total MAAs of 1.2 mg g^{-1} dry weight (Fig. 4). After 1 day treatment with salinity and UV, both 15-PSU samples showed a decrease in total MAAs (0.8 mg g^{-1} DW) and both 50-PSU samples showed an increase in total MAAs (1.5–1.7 mg g^{-1} DW). MAAs in plants kept at 34 PSU with and without UV were unaffected. While after 2 days exposure, thalli at all 15-PSU and 34-PSU conditions showed unchanged total MAA concentrations, algae at both 50-PSU conditions exhibited a decrease in total MAAs (1.0–1.2 mg g^{-1} DW). A strong UV-induced increase in total MAAs (mainly due to

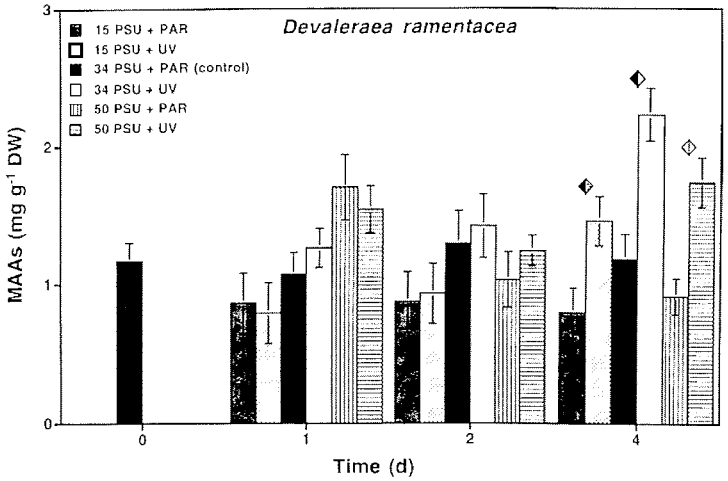

Fig. 4 The interactive effects of salinity and UV treatment on the total intracellular mycosporine-like amino acid contents (*MAAs*) in *Devaleraea ramentacea* over the course of 96 h. Given are the mean values ± SD ($n = 4$–5)

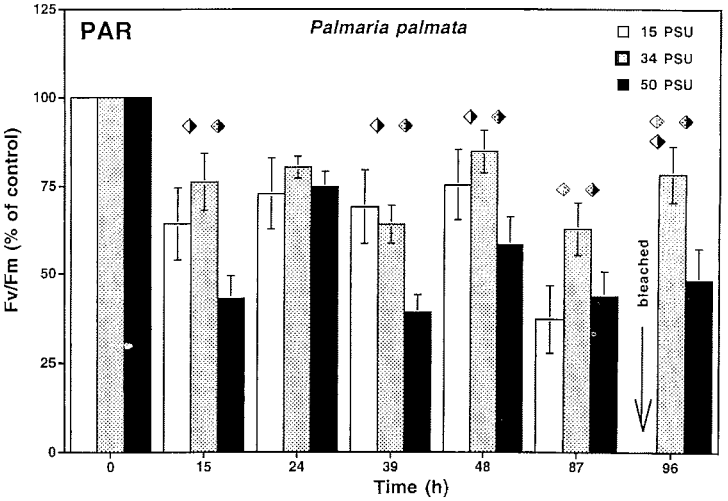

Fig. 5 Changes in photosynthetic optimum quantum yield (F_v/F_m) of *Palmaria palmata* under various salinity conditions (15, 34, 50 PSU) and visible light (*PAR*) over the course of 96 h. F_v/F_m of non-inhibited plants was determined as 0.59 ± 0.03 and standardised to 100%. Given are the mean values ± SD ($n = 10$). Significant differences ($P < 0.01$) among samples under various salinity treatments are marked with *squares*: 15 PSU versus 34 PSU (*white-pointed triangle*), 15 PSU versus 50 PSU (*white-black triangle*), 34 PSU versus 50 PSU (*black-pointed triangle*). *Arrow* indicates completely bleached (dead) thalli at 15 PSU

porphyra-334 and palythine) was observed at the end of the experiment. Under all salinities, UV exposure led to an almost doubling of the MAA contents. However, while at 15 PSU and 50 PSU, 1.5 mg and 1.7 mg MAAs g^{-1} DW, respectively, were measured in *D. ramentacea*, at 34 PSU the highest total MAA concentration of 2.2 mg g^{-1} DW was determined (Fig. 4).

While in *D. ramentacea*, F_v/F_m under the control conditions (34 PSU, PAR) remained unchanged over the course of the experiment, in *Palmaria palmata*, a small, but continuous decline of this parameter was observed, resulting in 75–80% of the maximum at the end of the experimental phase (Fig. 5). Compared to *D. ramentacea*, the optimum quantum yield of *Palmaria palmata* was much more strongly affected by salinity, particularly at 50 PSU over the first 48 h ($P < 0.01$) (Fig. 5). After that period, F_v/F_m in algae kept at 15 PSU also strongly declined, resulting in fully bleached and hence dead thalli at the end of the experiment. While after 96 h the 34-PSU samples exhibited F_v/F_m values 78% of the maximum, the 50-PSU plants showed values only 48% of the optimum (Fig. 5).

Under the salinity plus UV treatments, F_v/F_m in *Palmaria palmata* decreased even more, indicating strong interactive effects of both abiotic factors (Fig. 6). While the 34-PSU samples always showed declining optimum quantum yields (41–54% of the control) during on-set of UV radiation, marked recovery occurred (67–84% of the control) after switching off the UV source. Algae incubated at 15 PSU plus UV also died

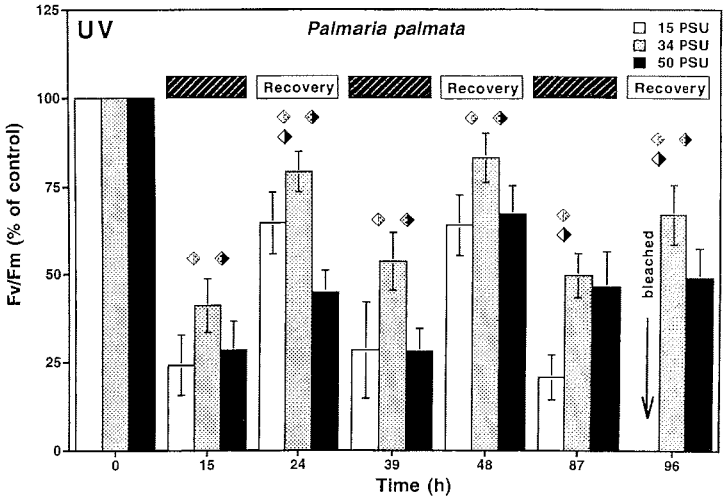

Fig. 6 Changes in photosynthetic optimum quantum yield (F_v/F_m) of *Palmaria palmata* under various salinity conditions (15, 34, 50 PSU) and ultraviolet radiation (*UV*) over the course of 96 h. F_v/F_m of non-inhibited plants was determined as 0.59 ± 0.03 and standardised to 100%. Given are the mean values ± SD ($n = 10$). Significant differences ($P < 0.01$) among samples under various salinity treatments are marked with *squares*: 15 PSU versus 34 PSU (*white-pointed triangle*), 15 PSU versus 50 PSU (*white-black triangle*), 34 PSU versus 50 PSU (*black-pointed triangle*). *Arrow* indicates completely bleached (dead) thalli at 15 PSU. The *black bars* indicate the 10-h UV treatment interval followed by the 14-h recovery period

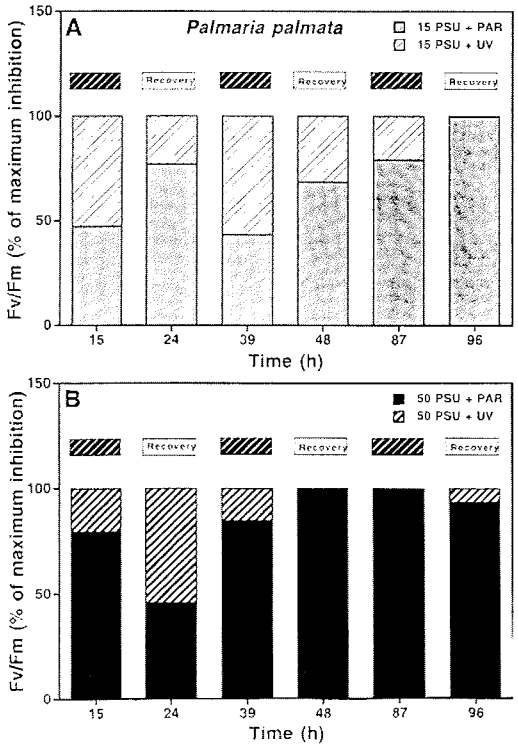

Fig. 7A, B The effect of salinity and UV treatment on the maximum decrease in the photosynthetic optimum quantum yield (F_v/F_m) of *Palmaria palmata* over the course of 96 h. From the mean value data presented in Figs. 1 and 2 the proportional degree of photoinhibition due to both stress factors was calculated and expressed as percentage of maximum photoinhibition. **A** 15 PSU ± UV treatment; **B** 50 PSU ± UV treatment

after 87 h treatment, as indicated by completely bleached tissue (Fig. 6). Thalli of *Palmaria palmata* at 50 PSU plus UV exhibited, over the course of the experiment, a similar photosynthetic response compared with plants at 50 PSU without UV. Three-way ANOVA indicated strong interactive effects between UV and salinity in *Palmaria palmata* ($P < 0.001$).

As in *D. ramentacea*, the proportional degree of photoinhibition (F_v/F_m) in *Palmaria palmata* due to both abiotic factors was, on average, mainly due to the salinity treatment (Fig. 7). Although at the beginning of the experiments, UV radiation also led to some decrease in optimum quantum yield, this effect got weaker after 48 h.

In *Palmaria palmata*, six different MAAs were detected, namely shinorine, porphyra-334, palythine, asterina-330, palythinol and palythene (data not shown). As in *D. ramentacea*, porphyra-334 and palythine contributed to >95% of total MAAs (data not shown). Plants at 34 PSU plus UV showed even after 24 h a small, but significant, increase in total MAA concentration ($P < 0.01$) (Fig. 8). These samples accumulated MAAs twofold over the course of the experiment. Although under hyposaline conditions, total MAAs decreased at the beginning, after 48 h a significant UV-induced formation could be observed ($P < 0.01$), followed by bleaching of the tissue. Under hypersaline treatment, thalli of *Palmaria palmata* exhibited after 48 h a strong accumulation of MAAs due to UV ($P < 0.01$), and after 96 h a decline from 2.4 to 1.9 mg MAAs g^{-1} DW (Fig. 8).

Discussion

On Spitsbergen, solar radiation, as the primary environmental factor for photosynthesis and productivity of macroalgae, is not only seasonally fluctuating, but also

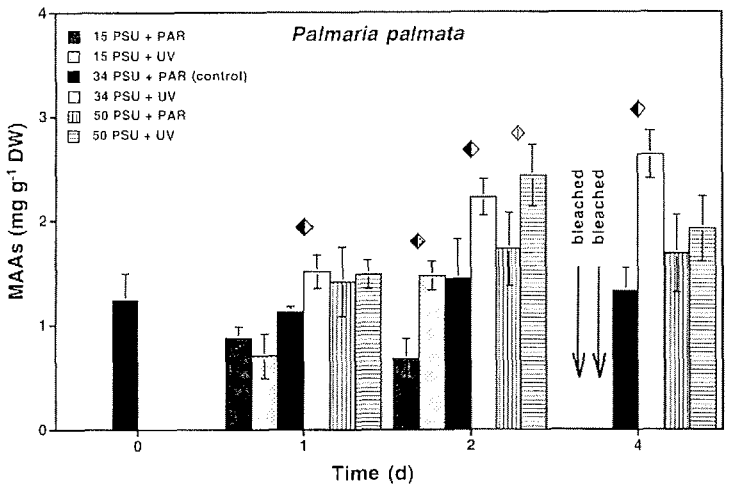

Fig. 8 The interactive effects of salinity and UV treatment on the total intracellular mycosporine-like amino acid contents (*MAAs*) in *Palmaria palmata* over the course of 96 h. Given are the mean values + SD ($n = 4$–5). *Arrows* indicate completely bleached (dead) thalli at 15 PSU

diurnally extremely variable at the earth's surface, due to rapidly changing weather conditions (Hanelt et al. 2001). In addition, during summer, the underwater light climate of the Kongsfjord is further affected by calving glaciers and strong melting-water influx, resulting in increasing turbidity due to suspended particles and, hence, a strong decrease of the water-column transmittance (Bischof et al. 1998a). The irradiance, particularly of the PAR range, to which *D. ramentacea* and *Palmaria palmata* were exposed in the laboratory, was much lower compared to nature. While, in the Arctic, typical summer insolation at the earth's surface may reach 1,300 μmol $m^{-2}s^{-1}$ PAR, 19 Wm^{-2} UV-A and 1.1 W m^{-2} UV-B (Bischof et al. 1998a), we used only 30 μmol m^{-2} s^{-1} PAR, 6.7 W m^{-2} UV-A and 0.25 W m^{-2} UV-B to simulate realistic underwater UV values. In spite of this experimental limitation, the data allow a comparative evaluation of photobiological responses in both species. In the water column, maximum transmittance for UV-B, as expressed by the 1% depth, ranges from 3.4 to 9 m (Bischof et al. 1998a). Consequently, both red-algal species may experience the UV-B irradiances applied in their natural habitat.

Macroalgae living under such fluctuating conditions need a broad physiological plasticity to acclimate to the wide range of incident solar radiation, to receive, on the one hand sufficient energy for photosynthesis, and on the other a flux low enough to avoid photodamage. Shallow-water and intertidal macroalgae are known to undergo dynamic photoinhibition when exposed to excessive sunlight that typically occurs at midday (Häder and Figueroa 1997; Hanelt 1998). Dynamic photoinhibition is considered as a photoprotective mechanism, which dissipates excessively absorbed energy as physiologically harmless thermal radiation (Osmond 1994). Previous studies have shown that the in vivo chlorophyll fluorescence of photosystem II, as used in the present investigation, is a suitable method for the evaluation of PAR- and UV-induced inhibition of photosynthesis in macroalgae (Häder and Figueroa 1997; Hanelt et al. 1997; Bischof et al. 1998b; Hanelt 1998). The optimum quantum yield (F_v/F_m) was demonstrated to be a sensitive parameter to evaluate the status of the photosynthetic apparatus (Cordi et al. 1997), and hence it represents a measure of physiological performance.

The experimental set-up was designed to comparatively test the photosynthetic performance of the shallow-water species *D. ramentacea* and *Palmaria palmata* in response to UV radiation and salinity. While in the former species the strongest photoinhibitory effect was measured under hypersaline conditions without UV (25% inhibition after 4 days), at 15 PSU only a small decrease in optimum quantum yield was observed. In strong contrast, *Palmaria palmata* did not survive hyposaline treatment over the course of the experiment, and also showed 50% inhibition in F_v/F_m at 50 PSU. Consequently, while *D. ramentacea* can be characterised as a euryhaline species, *Palmaria palmata* exhibits rather stenohaline features. From an ecological point of view, stenohalinity with respect to growth is typical for sublittoral red algae compared to the broad salinity tolerance of intertidal species (Kain and Norton 1990). In agreement with these authors, it should be mentioned that *Palmaria palmata* has its main distribution in temperate/cold-temperate waters of the Northern Atlantic where it grows sublittorally in depths down to 20 m, or protected as a typical understorey plant of kelp forests (Irvine 1983; Lüning 1990). These habitats are characterised by rather stable salinity conditions that support the development of stenohaline organisms. Consequently, the strong inhibition of photosynthesis and high mortality of the Arctic isolate of *Palmaria palmata* at 15 PSU can be explained by a limited physiological capacity to acclimate to external salinity fluc-

tuations. In addition, the occurrence of this species at the northern distribution limit on Spitsbergen, which is characterised by extremely low water temperatures, may contribute to the reduced photosynthetic tolerance. Although many algal species, particularly from polar regions, are physiologically able to acclimate to lower temperatures using various adaptive mechanisms (Raven and Geider 1988; Kirst and Wiencke 1995), for example, by increasing enzyme content to compensate for lower activity, this response depends on the degree of temperature decrease. Since the temperate *Palmaria palmata* grows at Spitsbergen at its distribution limit, it may be speculated that the primary metabolism under such, for this species, extreme cold conditions is just functioning and hence most probably slowed down according the Q10-rule. Consequently, all acclimation responses would be affected as well. This hypothesis is supported by the fact that temperature optima for photosynthesis and growth are only, in endemic Antarctic macroalgae, significantly lower compared to Arctic and cold-temperate species, which typically exhibit strong decline of both processes with decreasing temperatures (Healey 1972; Wiencke et al. 1993, 1994; Kirst and Wiencke 1995). While many Antarctic seaweeds seem to be relatively strongly adapted, Arctic and cold-temperate counterparts show a much weaker adaptation to low temperatures, and hence the general physiological performance may be species specifically more or less affected. Although *Palmaria palmata* is abundant in the Kongsfjord, the data presented, in combination with the observation of rather greenish, sometimes slightly bleached, thalli during summer in the field, indicate stressed plants. However, the interactive effects of salinity and temperature should still be experimentally evaluated.

Although salinities in the upper layers of the water column do not generally decline to values lower than 23 PSU (Hanelt et al. 2001), it should be mentioned that 15 PSU, as tested in the present study, represents a mild hyposaline stress for marine organisms (Kirst 1990). In contrast to *Palmaria palmata*, many other red macroalgae from intertidal, as well as sublittoral, habitats grow well or even preferentially, and photosynthesise at this salinity (Bird et al. 1979; Kirst 1990; Mostaert et al. 1995).

When UV radiation was applied on top of the salinity treatment, both species studied initially exhibited similar photosynthetic responses, i.e. a decline of the optimum quantum yield after on-set of UV followed by some degree of recovery after switching off the UV. While UV-induced photoinhibition compared to the control was relatively small in *D. ramentacea*, *Palmaria palmata* exhibited a much stronger response (Figs. 2, 6). In addition, the former species showed an increasing UV tolerance of photosynthesis over the course of the experiment, while the latter species seemed unable to photoacclimate. This confirms the data of Hanelt (1998) who showed that photosynthesis of *Palmaria palmata* collected along a depth profile in the Kongsfjord did not acclimate to the prevailing radiation gradient. Within other macroalgal species from the Arctic and Antarctica, the degree of photoinhibition is normally a function of the collecting depth, i.e. shallow-water isolates are more PAR/UV resistant than plants from deeper waters (Bischof et al. 1998a, 1998b). The difference in the acclimation potential of the photosynthetic performance between *D. ramentacea* and *Palmaria palmata* under UV is reflected by the vertical distribution in the Kongsfjord, since the former species grows in shallower waters. In addition, at more temperate locations, *Palmaria palmata* preferentially inhabits deeper waters than in the Arctic and hence the shallow-water growth habit in the Kongsfjord appears unusual. Due to incomplete osmotic adjustment, both algae may be better able to tolerate an increase in UV radiation as compared to salinity change.

In recent studies, the photobiological function of MAAs as a cellular defence system against the harmful effects of UV radiation on growth, photosynthesis and other processes has been reported for various marine phototrophic organisms (Garcia-Pichel et al. 1993; Dunlap and Shick 1998; Neale et al. 1998). In a convincing bio-assay, Adams and Shick (1996) documented experimentally that UV-treated, and subsequently fertilised, sea-urchin eggs typically show a UV-dose-dependent delay in the first cell division, compared to unirradiated eggs from the same batch. The determination of the cleavage delay in eggs having different MAA contents, produced by feeding adults different macroalgal diets (with high and low MAA levels) in the absence of UV, proved to be a perfect indicator for the sunscreen function of these compounds. The authors documented that the greater the MAA concentration in the eggs, the less they were affected by UV radiation. In the present study, both *D. ramentacea* and *Palmaria palmata* synthesised and accumulated MAAs over the course of the experiment in response to the UV treatment, except for those samples treated with 15 PSU. The highest MAA concentrations were usually measured at 34 PSU (Figs. 4, 8). The UV-induction data for MAAs are well supported by earlier experiments in the field, where both species were transplanted in the Kongsfjord from deeper waters to the surface, followed by exposure to natural full, as well as filtered, solar radiation (Karsten and Wiencke 1999; Karsten et al. 1999). However, the most striking fact is that, although both *D. ramentacea* and *Palmaria palmata* form MAAs in a similar manner and concentration, this increase correlates well with the rising photosynthetic tolerance under UV only in the former species. In *Palmaria palmata*, the optimum quantum yield under UV does not seem to benefit from higher MAA contents. These contradictionary results on the potential sunscreen function of MAAs in both red algae clearly indicate species-specific physiological advantages are not solely due to the synthesis and accumulation of UV-absorbing compounds.

In conclusion, while *D. ramentacea* is able to resist different environmental stress factors in the upper

sublittoral of the Arctic Kongsfjord, indicating a relatively high degree of physiological plasticity, *Palmaria palmata* exhibits a marked sensitivity against salinity and a limited capabilty to acclimate to changing PAR/UV radiation, pointing to a rather inflexible metabolism.

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Publication III

Responses of marine macroalgae to hydrogen-peroxide stress

DUMMERMUTH AL[1], KARSTEN U[2], FISCH K[3], WIENCKE C[1], KÖNIG G[3]

[1]Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12
27570 Bremerhaven
email: adummermuth@awi-bremerhaven.de

[2] University of Rostock,
Institute of Aquatic Ecology,
Albert-Einstein-Str. 3
18059 Rostock, Germany

[3] University of Bonn,
Institue for Pharmaceutical Biology,
Nussallee 6,
53115 Bonn, Germany

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Responses of marine macroalgae to hydrogen-peroxide stress

A.L. Dummermuth[a,*], U. Karsten[b], K.M. Fisch[c], G.M. König[c], C. Wiencke[a]

[a] *Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570, Bremerhaven, Germany*
[b] *University of Rostock, Institute of Aquatic Ecology, Albert-Einstein-Str. 3 D-18051, Rostock, Germany*
[c] *University of Bonn, Institute for Pharmaceutical Biology, Nussallee 6, D-53115, Bonn, Germany*

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Abstract

In this study, we determined the antioxidative potential of 15 marine macroalgae by measuring the photosynthetic efficiency under artificial oxidative stress after a 30-min exposure to a series of ascending H_2O_2 concentrations. Species exhibiting high maximum quantum yields (F_v/F_m values) were regarded as not susceptible towards H_2O_2 stress. In addition to the short-term stress experiments, the antioxidative defense systems (enzymatic and non-enzymatic) of selected algal species under longer exposure times to H_2O_2 were investigated.

Species with striking photosynthetic activity under H_2O_2 stress were *Chaetomorpha melagonium* (Chlorophyta), showing 40% reduced F_v/F_m as compared to the control after 8 days of exposure to 20 mM H_2O_2. In *Fucus distichus* (Phaeophyta) F_v/F_m decreased to 50% of the control under the same exposure conditions. *Polysiphonia arctica* (Rhodophyta) exhibited highest F_v/F_m values with a reduction of only 25%, therefore possessing the highest antioxidative potential of the investigated species.

Abbreviations: APX, ascorbate peroxidase; BHT, butylated hydroxytoluene; CAT, catalase; DCM, dichloromethane; DPPH, α,α-diphenyl-β-picrylhydrazyl radical; FGM1, degrees of freedom of model 1; FGM2, degrees of freedom of model 2; FGR2, degrees of freedom of residuals of model 2; GC–MS, gas chromatography mass spectrometry; GR, glutathione reductase; MDA, malondialdehyde; MSTFA, *N*-methyl-*N*-trimethylsilyltriflouracetamid; NADPH, nicotinamid adenine dinucleotide phosphate; ROS, reactive oxygen species; SAQM1, sum of squares of model 1; SAQM2, sum of squares of model 2; SAQR2, sum of squares of residuals of model 2; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; TMS, tetramethylsilan; TSP, total soluble protein.

* Corresponding author. Tel.: +49-471-4831-1540; fax: +49-47148311425.
E-mail address: adummermuth@awi-bremerhaven.de (A.L. Dummermuth).

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In *P. arctica* the activities of the antioxidative enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), as well as the pool size of the antioxidant ascorbic acid were investigated. When exposed to different H_2O_2 concentrations (0–2 mM) over 6 days, the intrinsic activities of SOD and GR were stimulated. In a kinetic study over 8 days, the activity of antioxidative enzymes APX and CAT as well as ascorbic acid content were recorded. APX activity was much higher in H_2O_2-treated thalli at the end of the experiment than in the control, also CAT activity increased significantly with increasing H_2O_2 stress. In parallel, ascorbic acid content was reduced under high H_2O_2 concentrations. Furthermore, by using GC–MS techniques in *P. arctica* bromophenolic compounds with antioxidative properties were identified.

This study shows that the measurement of the *in vivo* fluorescence of photosystem II is a suitable tool to determine the effect of oxidative stress on macroalgae. From these studies it is obvious that different algal species have varying strategies against oxidative stress which correlate with zonation on the shore.

Keywords: Antioxidants; Antioxidative potential; Ascorbic acid; Ascorbate peroxidase; Bromophenolic compounds; Catalase; Marine macroalgae

1. Introduction

Many metabolic responses in plants induce formation of reactive oxygen species (ROS), especially light-dependent processes such as photosynthesis. The photosynthetic electron transport system is the major source of ROS in plant tissues having the potential to generate singlet oxygen ($^1O_2$) and superoxide ($O_2^{\cdot-}$) (Asada, 1994a,b). Furthermore, in successive reduction of dioxygen (O_2), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) are formed. Hydrogen peroxide is also produced in the pseudocyclic photophosphorylation and Mehler reaction (Collen et al., 1995; Pedersen et al., 1995; Polle, 1996) and under stress in general (Fourcroy, 1999; Schreck et al., 1996). All these ROS, except hydrogen peroxide, are characterized by a short lifetime, as they interact rapidly with either water or cellular components (Asada, 1994a). Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but it is probably an intracellular precursor for more reactive oxidants as it passes quickly through membranes by diffusion (Apostol et al., 1989). It reduces photosynthesis (e.g. in spinach) (Kaiser, 1976) by inhibiting a number of photosynthetic enzymes (Elstner, 1982, 1987), such as fructose bisphosphatase, ribulose phosphate kinase and ribulose bisphosphate carboxylase/oxygenase (Rubisco) (Kaiser, 1979; Badger et al., 1980; Bischof et al., 2000; Tanaka et al., 1982) and other enzymes such as SOD (Asada et al., 1975; Forti and Gerola, 1977).

Production of ROS especially occurs under stress conditions during exposure to excessive light or UV radiation as well as during desiccation, under nutrient deficiency, exposure to heavy metals, high or low temperatures and temperature changes (McKersie and Lesham, 1994). If accumulation of ROS exceeds the capacity of enzymatic and non-enzymatic antioxidant systems the photosynthetic apparatus is damaged due to destruction of lipids, proteins and nucleic acids, finally leading to cell death (Asada and Takahashi, 1987; Fridovich, 1978; Halliwell and Gutteridge, 1989; Karpinski et al., 1999; Vacha,

1995). Cellular protection mechanisms against toxic oxygen species are essential for the maintenance of all metabolic processes including photosynthesis (Allen, 1977; Asada and Takahashi, 1987; Elstner, 1982; Halliwell, 1982). Higher plants are well equipped with enzymatic detoxification systems and antioxidants of different chemical groups diminishing oxidative stress by elimination and reduction of the ROS to less toxic and less reactive products (Larson, 1988). SOD catalyses the conversion of $O_2^{\cdot-}$ to H_2O_2 and oxygen. CAT reduces H_2O_2 to water and oxygen in two steps. Hydrogen peroxide is also reduced by ascorbate peroxidase (APX) via the ascorbate–glutathione cycle. Ascorbic acid is dehydrated to monodehydroascorbate and dehydroascorbate and recycled to ascorbic acid by monodehydroascorbate reductase and dehydroascorbate reductase. Both enzymes are dependent on $NADPH+H^+$, the latter enzyme coupled with GR converting oxidized glutathione to reduced glutathione (Elstner, 1982; Halliwell, 1982). APX shows a higher affinity to H_2O_2 than CAT and is located in the chloroplasts, CAT is located in peroxisomes (Halliwell and Gutteridge, 1989; Polle, 1996). In addition to proteins with antioxidative properties, phenolic compounds such as flavonoids, coumarins and tocopherols, nitrogen containing compounds including alkaloids, chlorophyll derivates, amino acids and amines as well as other compounds such as carotenoids, ascorbic acid, glutathione and uric acid are powerful antioxidants in plants (Fujimoto et al., 1985; Larson, 1988; Paya et al., 1992; Potterat, 1997).

Ascorbic acid, β-carotene and α-tocopherol are well known antioxidants in marine algae. The activities of antioxidative enzymes and the content of antioxidants in Arctic marine macroalgae was recently studied by (Aguilera et al., 2002a,b) with particular emphasis on photooxidative stress and the activity of these biochemical defense systems against high light and ultraviolet radiation. However, data on further properties of these antioxidative systems in Arctic species, particularly under direct H_2O_2 stress, are missing.

The aim of the present study was firstly to determine the antioxidative potential in macroalgae by using a fast assay originally developed by Collen and Pedersen (1996). Species with a high H_2O_2 tolerance might be a possible source for antioxidative substances for commercial or pharmaceutical purposes. Secondly, the ability of macroalgae to cope with hydrogen peroxide stress over a longer time period was investigated. Therefore, *P. arctica* was incubated in ascending hydrogen peroxide concentrations over 8 days and enzymatic detoxification systems (SOD, CAT, GR, APX) as well as the antioxidant ascorbic acid were determined. In addition, antioxidative phenolic compounds were isolated from this red alga and characterized by gas chromatography–mass spectrometry (GC–MS) in order to describe the chemical mechanisms responsible for the detected extraordinary high antioxidative potential.

2. Material and methods

2.1. Algal material and study site

Plants were collected by scuba diving in summer 1999 and summer 2000 at the study site in the Kongsfjord (Ny Ålesund, Spitsbergen, Norway 78°55.5′N; 11°56.0′E) from

depths between 0 and 20 m (Table 1). Algal samples were collected in black bags to avoid exposure to high irradiance and kept in seawater during transport. In the laboratory the plant material was kept at 2–5 °C for at least 24 h under dim white fluorescent lamps (Philips 58 W/950) adjusted to 25 μmol photons m^{-2} s^{-1} in running seawater pumped direetly from the fjord.

2.2. Assay for the detection of the antioxidative potential

To determine the antioxidative potential, 3- to 10-cm-long thallus parts were incubated for 30 min in 50-ml transparent plastic bottles in seawater at about 5 °C enriched with H_2O_2 in concentrations of 0–20 mM. During the last 5 min of the incubation, the algal samples were kept in darkness. Afterwards the photosynthetic capacity was determined by measuring the variable chlorophyll-fluorescence of photosystem II as described below.

Additionally, thalli of *P. arctica* were supplemented for 1 week in 2-l plastic bottles containing pure seawater (control) or seawater enriched with H_2O_2 to reach final concentrations of 0.5, 1, 2 and 5 mM. The medium was changed daily to keep H_2O_2 concentration constant. The algae were illuminated 24 h with white fluorescent lamps (Philips 58 W/950) at a photon fluence rate of 25 μmol m^{-2} s^{-1}. Samples for the detection of antioxidative activities were taken on days 0, 1, 2, 4, 6 and 8. Samples were put in liquid nitrogen, lyophylized and subsequently stored at −30 °C until analysis.

Table 1
Investigated species, their classification in the three main groups of macroalgae and their habitat

Species	Habitat
Chlorophyta	
Acrosiphonia sp.	Eulittoral–upper sublittoral
Monostroma aff. *arcticum* Wittrock	Upper–lower sublittoral
Chaetomorpha melagonium (F. Weber et Mohr) Kützing	Upper–lower sublittoral
Rhodophyta	
Coccotylus truncatus (Pallas) M.J. Wynne and J.N. Heine	Lower sublittoral
Devaleraea ramentacea (L.) Guiry	Eulittoral–lower sublittoral
Palmaria palmata (L.) Greville	Upper–lower sublittoral
Phycodrys rubens (L.) Batters	Lower sublittoral
Odonthalia dentata (L.) Lyngbye	Lower sublittoral
Polysiphonia arctica J. Agardh	Lower sublittoral
Ptilota gunneri P.C. Silva, Maggs and L.M. Irvine	Lower sublittoral
Phaeophyta	
Alaria esculenta (L.) Greville	Upper–lower sublittoral
Laminaria digitata (Huds.) Lamouroux	Upper–lower sublittoral
Laminaria solidungula J. Agardh	Lower sublittoral
Fucus distichus L.	Eulittoral–lower sublittoral
Saccorhiza dermatodea (de la Pylaie) J. Agardh	Upper–lower sublittoral

2.3. Photosynthesis measurements

Photosynthetic efficiency (F_v/F_m) was determined by measuring the variable chlorophyll-fluorescence of photosystem II (PSII) using a portable pulse amplitude modulated fluorometer (Diving-PAM, Walz, Effeltrich, Germany) as described by Hanelt (1998). F_v/F_m values of all plants acclimated for 24 h to dim light conditions in the laboratory were characteristic for photosynthetically non-inhibited plants and set to 100% (=control). All PAM data recorded are expressed in relation to this value. *P. arctica* exhibited a maximum F_v/F_m value of 0.67, which is typical for red algae.

2.4. Activities of antioxidative enzymes

Samples (0.010–0.012 g DW) were ground in liquid nitrogen and extracted with 1–1.5 ml 50 mM potassium phosphate buffer (pH 7.0) containing Complete™ protease inhibitor cocktail (Boehringer, Mannheim; 2 tablets in 100 ml buffer). Extracts were then centrifuged for 15 min at 15,000 rpm at 4° C. Enzymes were analysed in the supernatant according to Acbi (1984) for CAT, to Chen and Asada (1989) for APX, to Goldberg and Spooner (1983) for GR and McCord and Fridovich (1969) for SOD as described by Aguilera et al. (2002b) and modified for use of a microtiterplate spectrophotometer (Spectramax, Molecular Devices, Sunnyvale, CA 94089, USA). In order to get replicate measurements, the reaction mixture was scaled up to 2 ml and then dispersed with a multichannel pipette in six slots of the microtiterplate each containing 300 µl reaction mixture.

2.5. Determination of ascorbic acid

Ascorbic acid was measured according to Foyer et al. (1983), as described by Aguilera et al. (2002b), and also modified in the same way as for the enzymes for use of a microtiterplate spectrophotometer.

2.6. Protein assay

Total soluble protein (TSP) content in crude extracts was determined using a commercial Protein Assay (BioRad, Germany), based on the method described by Bradford (1976). Protein content was determined spectrophotometrically at 595 nm and concentrations were calculated compared to a calibration curve of bovine serum albumin (Sigma, Germany).

2.7. Determination of phenolic compounds

The sample of *P. arctica* was lyophylized (135.8 g DW) and extracted with DCM (3 × 0.2 l) and then with MeOH (3 × 0.2 l), yielding 2.3 g DCM extract and 12.9 g MeOH extract. The MeOH extract was again extracted with acetone, giving 1.0 g acetone soluble extract. The latter was fractionated on an RP18 vacuum liquid chromatography column (VLC), using ~ 100 ml solvent per fraction leading to nine fractions. Samples were derivatized with *N*-methyl-*N*-trimethylsilyltriflouracetamid (MSTFA). The analysis was

carried out on a Perkin-Elmer (Auto system XL) gas chromatograph coupled with a Perkin-Elmer Turbomass spectrometer using a 30 m × 0.32 mm N931-6023 Pe-1 (film thickness of 0.25 μm) capillary column. Conditions: injector 250 °C, split 1:20; temperature program 100–300 °C, 6 °C/min, helium flow 2 ml/min. Mass spectral scan range was 35–650 Da.

2.8. Test systems for antioxidative activities

2.8.1. Thiobarbituric acid reactive substances

The assay was modified after Wallin et al. (1993), and performed as previously described (Abdel-Lateff et al., 2002). Briefly, linolenic acid methyl ester was oxidized in 50 mM phosphate buffer (pH = 7.2), under $FeSO_4$ catalysis. Butylated hydroxytoluene (BHT) in ethanol was added to prevent further oxidation. Thiobarbituric acid reacting substances (TBARS) were determined using trichloroacetic acid and thiobarbituric acid (TBA) at 60 °C for 30 min. The absorbance was read at 532 nm less the background absorbance at 600 nm.

2.8.1.1. Calculations. The percentage of inhibition (% I) has been calculated from the absorbance readings and is expressed as the inhibition of lipid peroxidation of that sample compared to the not inhibited reaction in the control (Eq. (1)). A_{blank} = absorbance of the blank ($A_{532\ \text{nm}} - A_{600\ \text{nm}}$), A_{control} = absorbance of the control ($A_{532\ \text{nm}} - A_{600\ \text{nm}}$), A_{sample} = absorbance of the sample ($A_{532\ \text{nm}} - A_{600\ \text{nm}}$), $A_{\text{sample blank}}$ = absorbance of the sample blank ($A_{532\ \text{nm}} - A_{600\ \text{nm}}$).

$$\%I = 100 - \frac{(A_{\text{sample}} - A_{\text{sample blank}}) \times 100}{A_{\text{control}} - A_{\text{blank}}}. \quad (1)$$

2.8.2. α,α-Diphenyl-β-picrylhydrazyl radical (DPPH) assay

Assays were performed in flat bottom polystyrene 96-well microtiter plates using a modified previously established methodology (Amarowicz et al., 2000; Blois, 1958). To 100 μl of each sample (50 μg ml^{-1}–1 mg ml^{-1}) in EtOH, 25 μl DPPH (1 mM) in EtOH and 75 μl EtOH were added. The resultant mixture was briefly shaken and maintained at room temperature, in the dark for 30 min. At the end of this period, the absorbance of the mixture was read at 517 nm, using an SLT Spectral Rainbow microtiter plate reader.

2.8.2.1. Calculations. The percentage of scavenging of DPPH radical from a sample at a given concentration can be calculated from the absorbance readings as shown in Eq. (2).

$$\%\text{Scavenging} = 100 - \frac{(A_{\text{sample}} - A_{\text{sample blank}}) \times 100}{A_{\text{control}} - A_{\text{blank}}}. \quad (2)$$

2.9. Statistics

Mean values and standard deviations of 5–10 replicates per treatment were calculated. The statistical significance of differences in the assay for the detection of the antioxidative potential was tested via a one-way ANOVA followed by a least significant difference test

(Statistica; Statsoft, Tulsa, USA). Data for the enzyme kinetic were treated by non-linear regression (Statistica; Statsoft) of each concentration compared by an F-distribution. For the non-linear regressions polynomic formulae were used.

The following formula was used for the calculation of the F-value:

$$F = [(\mathrm{SQM2} - \mathrm{SQM1})/(\mathrm{FGM2} - \mathrm{FGM1})]/[(\mathrm{SQR2}/\mathrm{FGR2}]$$

where SQM1 = sum of squares of model 1; SQM2 = sum of squares of model 2; SQR2 = sum of squares of residuals of model 2; FGM1 = degree of freedom model 1; FGM2 = degree of freedom model 2; FGR2 = degree of freedom of residuals of model 2.

Model 1 is the sum of two polynomic regressions of the two concentrations to compare. Model 2 consists of the combined data of the two concentrations to compare, building one polynom, it is the big model, because of the higher number of degrees of freedom.

F-values higher than table values indicate significant differences. For comparison between the concentrations, each concentration was compared to the control and to the next higher concentration to reduce statistical expense.

3. Results

The photosynthetic capacity under H_2O_2 stress of the studied species was regarded as indicator for the antioxidative potential. In the group of red algae, *P. arctica* was the most resistant of all investigated species. There were no significant differences in F_v/F_m up to a concentration of 10 mM H_2O_2. At a concentration of 20 mM H_2O_2 the photosynthetic capacity was reduced significantly ($p < 0.0001$) by 20% (Fig. 1). In contrast, incubation in 5 mM H_2O_2 resulted in a significant reduction of the maximum quantum yield of 55% in

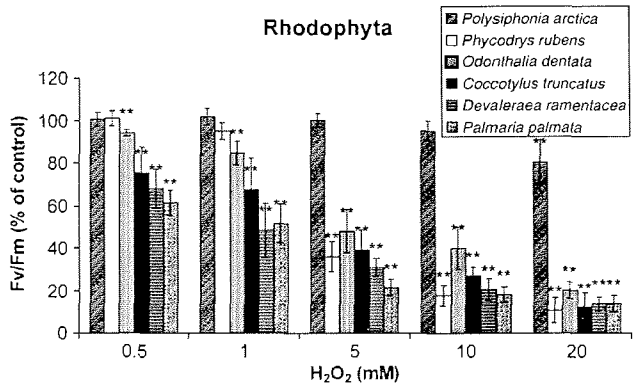


Fig. 1. The effect of increasing H_2O_2 concentrations on the maximum quantum yield of six red macroalgae from Kongsfjorden, Spitsbergen, mean values ± S.D., $n = 10$. F_v/F_m values of the control were between 0.54 and 0.57 for all the red algae. *, **: Significant differences to the control value at $p < 0.05$ and $p < 0.01$, respectively.

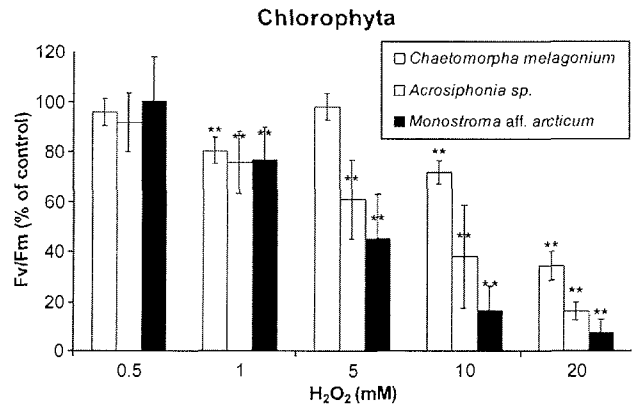


Fig. 2. The effect of increasing H_2O_2 concentrations on the maximum quantum yield of three green macroalgae from Kongsfjorden, Spitsbergen, mean values ± S.D., $n=10$. F_v/F_m values of the control were 0.61 for *C. melagonium*, 0.71 for *Acrosiphonia* sp. and 0.49 for *M. arcticum*. *,**: Significant differences to the control value at $p<0.05$ and $p<0.01$, respectively.

Phycodrys rubens ($p<0.0001$) and 64% in *Odonthalia dentata* ($p<0.0001$, Fig. 1). *Coccotylus truncatus*, *Devaleraea ramentacea* and *Palmaria palmata* were most sensitive to H_2O_2. After incubation in 1 mM H_2O_2 F_v/F_m, values decreased to 73% ($p<0.0001$), 47% ($p<0.0001$) and 50% ($p<0.0001$) in the three species, respectively (Fig. 1). Within the

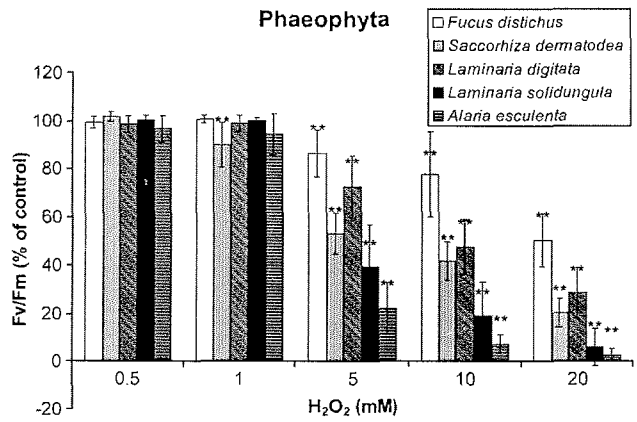


Fig. 3. The effect of increasing H_2O_2 concentrations on the maximum quantum yield of five brown macroalgae from Kongsfjorden, Spitsbergen, mean values ± S.D., $n=10$. F_v/F_m values of the control were between 0.70 and 0.77 for all brown algae. *,**: Significant differences to the control value at $p<0.05$ and $p<0.01$, respectively.

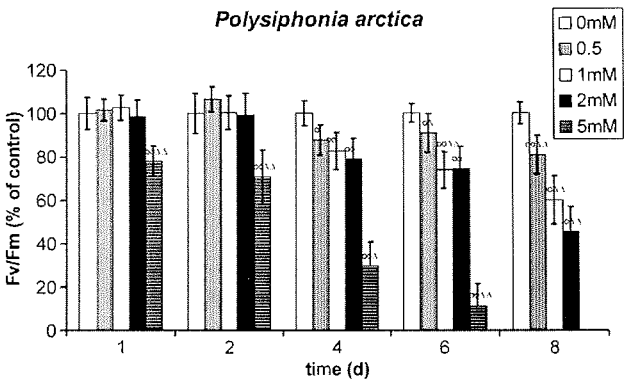


Fig. 4. F_v/F_m mean values ± S.D. (% of control) of *P. arctica* after incubation in a series of ascending hydrogen peroxide concentrations in relation to incubation time, $n = 10$. F_v/F_m values of the daily controls were between 0.54 (day 8) and 0.61 (day1). O,OO: Significant differences to the control at $p < 0.05$ and $p < 0.01$, respectively. △,△△: Significant differences to the preceding concentration at $p < 0.05$ and $p < 0.01$, respectively.

green algae, *C. melagonium* showed the highest resistance against H_2O_2 (Fig. 2). F_v/F_m decreased significantly ($p < 0.0001$) to 73% of the control after exposure to 10 mM H_2O_2 whereas *Acrosiphonia* sp. and *Monostroma* aff. *arcticum* were more susceptible to concentrations ≥ 5 mM. All brown algae tolerated up to 1 mM H_2O_2.. In *F. distichus* the F_v/F_m value was significantly reduced ($p < 0.0001$) but still above 50% in 20 mM H_2O_2 (Fig. 3). *Laminaria digitata* and *Sacchorhiza dermatodea* showed a 50% ($p < 0.0001$) reduction in quantum yield at 10 mM H_2O_2. In the most sensitive phaeophyte species, *Laminaria solidungula* and *Alaria esculenta*, incubation in 5 mM H_2O_2 resulted in a significant decline of F_v/F_m by 60% ($p < 0.0001$) and 80% ($p < 0.0001$), respectively (Fig. 3).

Table 2
Statistical evaluation of APX and CAT activities (U g DW^{-1}) in *P. arctica* in relation to time and H_2O_2 concentration (see Figs. 5 and 6) after non-linear regression resulting in *F* and p values

Comparison	APX		CAT	
	F	*p*	*F*	*p*
0/0.5	14.27	0.024*	21.40	0.013*
0/1	100.10	0.001**	60.34	0.003**
0/2	75.33	0.002**	33.70	0.007**
0/5	27.44	0.009**	53.53	0.003**
0.5/1	81.27	0.002**	30.74	0.008**
1/2	7.64	0.058	22.55	0.012*
2/5	18.23	0.017*	19.86	0.015*

Each concentration was compared to the control and the next higher concentration. For detailed description of the test, see Material and methods.

* Indicating statistical significance at $P < 0.05$.

** At $P < 0.01$.

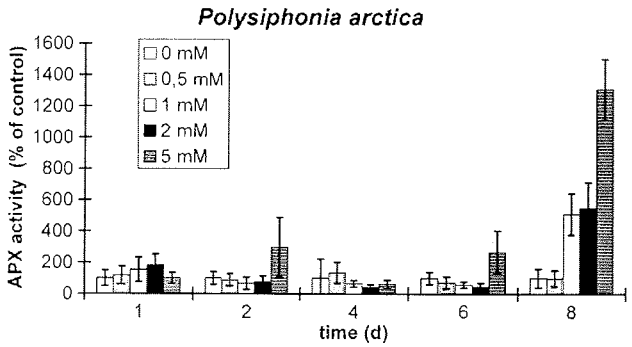


Fig. 5. Kinetic of APX activity under H_2O_2 stress in *P. arctica* (% of control, related to U mg TSP^{-1}), mean values ± S.D., $n=5$.

Maximum quantum yield of *P. arctica* was gradually reduced under H_2O_2 stress in dependency of exposure time and H_2O_2 concentration. In the first 24 h of exposure, F_v/F_m remained unchanged up to 2 mM H_2O_2 treatment (Fig. 4). Even a slight increase in F_v/F_m could be recorded at 0.5 and 1 mM in the first 24 h of exposure. At 5 mM H_2O_2, F_v/F_m was significantly reduced to 78% of the control ($p<0.0001$, Fig. 4). The same pattern was observed after 2 days of exposure. After 4 days of exposure, a drastic decrease in F_v/F_m was recorded for the 5 mM treatment differing significantly to the control as well as to all other treatments ($p<0.0001$, Fig. 4). The treatments of 0.5–2 mM showed also a gradual reduction of F_v/F_m values along the concentration gradient, each differing significantly to the control with $p<0.01$, as well as the 2-mM treatment to the 0.5-mM treatment with $p<0.02$. After 6 days of exposure, all treatments showed significantly reduced F_v/F_m values

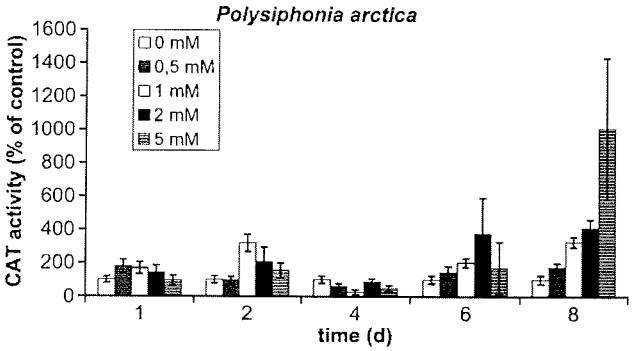


Fig. 6. Kinetic of CAT activity under H_2O_2 stress in *P. arctica* (% of control, related to U mg TSP^{-1}), mean values ± S.D., $n=5$.

($p<0.0$ for 1–5 mM and $p<0.015$ for 0.5 mM). Under the highest H_2O_2 concentration, F_v/F_m decreased to 11% of the control ($p<0.0001$) and the thalli of *P. arctica* started to bleach. In the 1- and 2-mM treatments the reduction went on, resulting in F_v/F_m values of 74% of the control, differing significantly to the control ($p<0.0001$) and to 0.5 mM ($p<0.0001$, Fig. 4). After 8 days of exposure, the gradual reduction of F_v/F_m was most obvious. Exposure to 0.5 mM H_2O_2 resulted in a decrease of F_v/F_m to 80% of the control ($p<0.0001$), in the 1-mM treatment a significant reduction to 60% of the control was recorded ($p<0.0001$) and 45% under 2 mM H_2O_2 ($p<0.0001$, Fig. 4). The bleaching process now also affected thalli exposed to 2 mM H_2O_2. Concomitantly the alga exhibited a much softer consistency. In the 5-mM treatment, no photosynthetic activity was found (Fig. 4).

APX activity in *P. arctica* showed an induction with increasing H_2O_2 stress. After 1 day of exposure, the gradual increase of APX activity is obvious up to concentrations of 2 mM

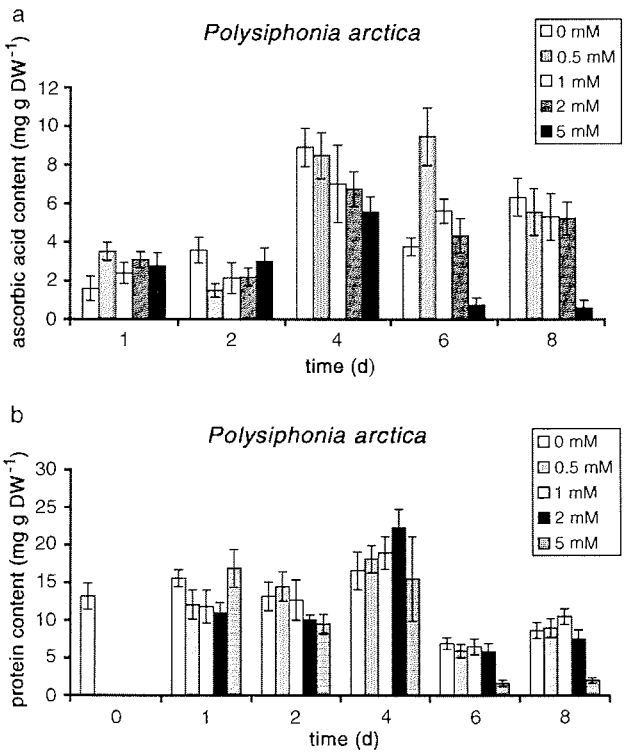


Fig. 7. (a) Ascorbic acid content (mg g DW^{-1}) and (b) protein content (mg g DW^{-1}) in *P. arctica* after incubation in a series of ascending hydrogen peroxide concentrations in relation to incubation time, $n=5$.

H_2O_2; the 5-mM treatment showed a delay, which was cached up with ongoing of the exposure time. Enzyme activities differed significantly to the control, as well as the treatments from each other except the 1- and 2-mM treatments, p-values are given in Table 2. Enzyme activity was up to 13-fold higher ($p < 0.009$) in 5 mM H_2O_2 in comparison to the control with 2.9 U mg TSP^{-1} (Fig. 5, Table 2). Maximum APX activity was recorded in 1–5 mM H_2O_2 on day 8 of the experiment (Fig. 5).

CAT activity in *P. arctica* was induced by H_2O_2 within the first 24 h of the experiment. After exposure to 0.5 mM H_2O_2, the CAT activity rose to 180% (Fig. 6) corresponding to 7 U mg TSP^{-1}. Exposure to 1 and 2 mM H_2O_2 lead to activities of 141% and 168% of the control, respectively (Fig. 6). After 4 days of exposure, the lowest CAT activities were

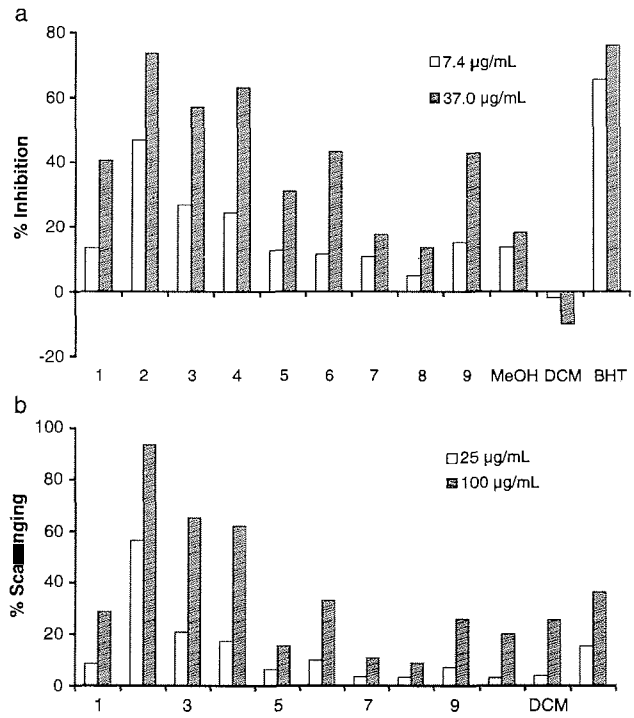


Fig. 8. (a) Inhibition of linolenic acid methyl ester oxidation by *P. arctica* extracts and acetone soluble fractions in the TBARS assay, positive control: butylated hydroxytoluene (BHT). %Inhibition = $100 - (A_{sample} - A_{sample\ blank}) \times 100/(A_{control} - A_{blank})$. (b) Scavenging activity of *P. arctica* extracts and acetone soluble fractions in the DPPH assay, positive control: butylated hydroxytoluene (BHT). %Scavenging = $100 - (A_{sample} - A_{sample\ blank}) \times 100/(A_{control} - A_{blank})$.

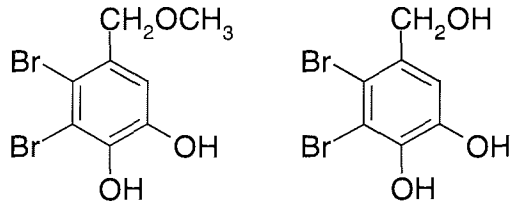
measured with 25–86% of the control. From day 6 onwards, CAT activity increased with increasing H_2O_2 concentration to a 10-fold higher value. Maximum CAT activity was found after 8 days in 5 mM H_2O_2 with 34 U mg TSP^{-1}. CAT activities differed significantly to the control, as well as the treatments from each other, regarding the different treatments each as one unit described by a polynomic curve. *p*-Values are given in Table 2.

In *P. arctica*, the ascorbic acid content increased during the first 24 h of exposure in all H_2O_2 concentrations. On day 2, ascorbic acid content decreased and on day 6 it increased strongly when exposed to 0.5 mM H_2O_2. Under high H_2O_2 stress (5 mM), the ascorbic acid content decreased from 2.75 mg g DW^{-1} at the beginning to 0.59 mg g DW^{-1} after 8 days of treatment (Fig. 7a).

The protein content of *P. arctica* varied between 11 and 18 mg g DW^{-1} within the first 4 days of exposure; in the following 4 days protein content fell below 10 mg g DW^{-1} (Fig. 7b). At the end of the experiment, the protein content of thalli exposed to 2 and 5 m H_2O_2 decreased below 2 mg g DW^{-1} (Fig. 7b).

3.1. Antioxidative potential as determined by TBARS and DPPH assay

In order to determine compounds that may influence the resistance of *P. arctica* to oxidative stress the alga was extracted with lipophilic and hydrophilic solvents. Subsequently the antioxidative activity of extracts and fractions, obtained after chromatographic separation, was assessed. *P. arctica* showed both prooxidative activity in the DCM extract and antioxidative activity in the MeOH extract as determined by the TBARS assay. Fractions of the MeOH extract from *P. arctica* were again tested in the TBARS assay at concentrations of 7 and 37 μg ml^{-1}. Fraction 2 showed the strongest inhibitory effect on linolenic acid methyl ester oxidation at both concentrations (Fig. 8a). At 37 μg ml^{-1}, the inhibition of fraction 2 was in the same range as that of BHT, whereas at 7 μg ml^{-1} inhibition was clearly less than that of the positive control. The MeOH extract and the fractions of the MeOH extract from *P. arctica* were also tested in the DPPH assay at concentrations of 25, 50, 100, 500 μg ml^{-1}. Results from concentrations at 25 and 100 μg ml^{-1} are given in Fig. 8b. In the DPPH assay, fraction



1 **2**

Fig. 9. Chemical structure of the bromophenolic compounds in *P. arctica*.

2 was also the most active one. The radical scavenging activity was much stronger than that of BHT at both concentrations.

All fractions tested for their antioxidative activity were analysed by GC–MS for their bromophenol content. Only in fraction 2 could bromophenols be identified. A chromatogram of fraction 2 contained as major metabolites compound **1** identified as the TMS derivative of 2,3-dibromo-4,5-dihydroxybenzyl methyl ether, and **2** the TMS derivative of 2,3-dibromo-4,5-dihydroxybenzyl alcohol. Formulas are given in Fig. 9.

4. Discussion

The present study gives an insight into the antioxidative properties of Arctic marine macroalgae under artificial H_2O_2 stress. Using PS II fluorescence as indicator we identified species with a high antioxidative potential in short-term stress experiments. The plants with high photosynthetic activity under oxidative stress, were *C. melagonium* as representative of the green algae, *F. distichus* belonging to the brown algae and *P. arctica* within the red algae. Since the latter species exhibited the highest tolerance against H_2O_2 among all the algae tested, long-term exposure of *P. arctica* to H_2O_2 was investigated as well. The underlying mechanisms of protection and defense against oxidative stress were studied in detail.

The maximum quantum yield of photosynthesis is a common parameter in plant ecophysiology and stress research and expressed as the ratio of variable to maximum chlorophyll fluorescence (F_v/F_m). The method of PAM fluorometry offers the advantage that the plant is not stressed or damaged by the technique itself (Bilger et al., 1995) and it allows fast assessment of photosynthetic activity. As oxidative stress directly intervenes in the photosynthetic process we found that this method can also be used to determine the relative antioxidative properties of macroalgae. Collen and Pedersen (1996) already tested the effects of H_2O_2 on variable fluorescence of the green alga *Ulva rigida*. In this species the variable fluorescence was not influenced by exposure to concentrations lower than 1 mM, however was totally inhibited at 100 mM H_2O_2. This is in agreement with our data, at least for short-term treatments. Therefore, PAM fluorometry is a suitable technique to rapidly screen for the antioxidative potential in comparative studies in macroalgae. For longer exposure times, as performed for *P. arctica* in the present study, F_v/F_m is also affected at concentrations lower than 1 mM, indicating that concentration as well as exposure time are responsible for the observed toxic effects.

The species with the highest antioxidative potential, *C. melagonium*, *F. distichus* and *P. arctica*, are also described by Aguilera et al. (2002b) as macroalgae with high antioxidative properties. *C. melagonium* possesses high enzyme activities of SOD and CAT, which are typical for green macroalgae and explaining the high capability of resistance of these algae against H_2O_2 stress (Aguilera et al., 2002b). The high antioxidative properties of *F. distichus* can be explained by enzyme and non-enzymatic mechanisms such as SOD activity of 151 U mg TSP^{-1}, 3.6 U mg TSP^{-1} CAT and 0.29 mg g FW^{-1} of ascorbic acid (Aguilera, 2002b). As representative of brown seaweeds, *F. distichus* also possesses phenolic compounds which can act as antioxidants. *P. arctica* showed high APX activity with 0.43–1.14 U mg TSP^{-1}, which was up to 50-fold higher for H_2O_2-treated thalli, in comparison to data on untreated thalli presented by Aguilera et al. (2002b) for this species.

The big discrepancy between these data could have resulted from strong seasonal differences in enzyme activities as shown in Aguilera et al. (2002a) for SOD, CAT and GR for several macroalgal species from the Arctic. The activities of antioxidant enzymes such as APX, catalases and SODs are up-regulated in response to several abiotic stresses such as drought (Smirnoff and Colombe, 1988), low temperatures (Schöner and Krause, 1990), high light intensities (Camak and Marschner, 1992), ozone, SO_2, UV-B (Willekens et al., 1994) and salinity (Lopez et al., 1996).

In this study, this general statement is also true for *P. arctica* which had enhanced APX and CAT activities with increasing H_2O_2 concentrations on a basis of TSP but not for dry weight normalized data. Also GR activity was enhanced with increasing H_2O_2 concentrations from 0.02 to 0.05 U mg TSP^{-1} (data not shown) as well as SOD maintained high activities, varying between 48 and 97 U mg TSP^{-1}, throughout the experiment (data not shown).

But there are also several examples showing decrease of antioxidative enzymes under stress. Schrick (2000) for example measured decreased GR activities in the diatoms *Entemoneis kufferathii* and *Chaetoceros* sp. with increasing temperature and light intensities. Aguilera et al. (2002b) showed decreased SOD activities in *P. palmata* and decreased GR activities in *Monostroma* aff. *arcticum* under UV radiation. In the case of UV, this could be a direct radiation damage to the enzyme as discussed for catalase in higher plants (Foyer and Mullineaux, 1994), because many proteins absorb short wavelengths of UVB and thereby may break disulfide bridges that are essential for native structure and function. High levels of H_2O_2 (3 mM and above) caused oxidative stress in *U. rigida* (Collen and Pedersen, 1996) and finally led to cell death due to enzyme damage. This seems also the case in our study for *P. arctica* treated with 2 and 5 mM H_2O_2. The bleaching of the algal thalli after 6 days of incubation fortify this thesis.

The content of TSP in *P. arctica* was negatively affected by H_2O_2 stress, particularly after 8 days of treatment. The bleaching of the algal thalli after 4 days explained the drastic decline in protein content most probably due to the degradation of phycobiliproteins. At the same time, photosynthetic efficiency was reduced drastically under high H_2O_2 stress as a result of fewer pigments working for photosynthesis. If the protein content is considerably lower than the control value, a much higher protein-based enzyme activity would result from the calculation. Negatively effected protein content after H_2O_2 stress was also described by Pastori and Trippi (1993), whereas the enzyme activities of APX and SOD were increased—which support the data presented here for APX and CAT. The interpretation of the enzyme activity data should be made carefully. Ascorbic acid content in *P. arctica* was reduced under high H_2O_2 concentrations and after longer exposure times to H_2O_2 only. This decline of ascorbic acid correlates well with an obvious stimulation in APX and CAT activity. This could be explained by ascorbic acid acting as primary protection mechanisms against H_2O_2 and the activity of antioxidative enzymes as secondary protection system. From an energetic standpoint the synthesis of ascorbic acid is not as costly for the plant as the de novo synthesis of protective enzymes. In spite of strongly reduced photosynthesis after 8 days of exposure to H_2O_2 concentrations <2 mM, other metabolic reactions such as the antioxidative enzymes could be recorded, indicating that the enhancement of antioxidative enzymes was not sufficient to sustain photosynthesis.

In comparison to other marine macroalgae such as *Fucus* species (Collen and Davison, 1999a), APX activity in *P. arctica* was 1- to 3-fold lower, assuming a DW/FW ratio of 1:10.

Despite the fact that it showed rather moderate antioxidative enzyme activities and ascorbic acid concentrations, *P. arctica* was extremely resistant against H_2O_2 in our assay, indicating the presence of other antioxidative compounds and mechanisms, respectively.

Further investigations of extracts from *P. arctica* evince the existence of bromophenolic compounds with antioxidative properties. The bromophenolic compounds were identified by GC–MS and their antioxidative abilities tested by TBARS and DPPH assays. The identified compounds resemble bromophenolic substances earlier described for several *Polysiphonia* species (Glombitza et al., 1974; Kurata and Amiya, 1980). The correlation of bromophenol content with high antioxidative activity in fraction 2 indicates that *P. arctica* contains bromophenols as low molecular weight antioxidants. Since fractions 3 and 4 also show notable antioxidant activity in the TBARS assay, other low molecular weight antioxidants could also be present in this alga. The extraction of the algae was done with MeOH, therefore, the 2,3-dibromo-4,5-dihydroxybenzyl methyl ether (**1**) could be an isolation artefact of (**2**). Glombitza et al. (1974) mainly found dibromophenols among the investigated *Polysiphonia* species. Only *Polysiphonia urceolata* contained monobromophenols as main bromophenols. In the GC–MS analysis of *P. arctica*, no monobromophenols were found.

In general, macroalgae from eulittoral and upper sublittoral have to cope with changing environmental conditions such as light (photosynthetic active radiation and ultraviolet radiation), temperature, salinity and desiccation. For this reason, it is obvious that species inhabiting the eulittoral and upper sublittoral exhibit higher enzyme activities and antioxidant concentrations as species inhabiting deeper waters. This relation between antioxidant capabilities and depth distribution has been suggested by Aguilera et al. (2002a,b) and accounts for the high resistibility to oxidative H_2O_2 stress of the *Chaetomorpha* species and *F. distichus* living in the eulitoral and upper sublitoral. Collen and Davison (1999b) also explain the increase of activities of reactive oxygen scavenging enzymes with increased environmental stress in the higher intertidal for *Mastocarpus stellatus*. High tolerance to various stresses of species from the uppermost sublittoral zone is also documented by Davison and Pearson (1996). For the *Chaetomorpha* species and *F. distichus*, this relation between tidal height and antioxidative properties is a possible explanation but not for *P. arctica* as a deep waters species living from 12 to 30 m where light stress (UV and high light) and light-induced oxidative stress do not occur. As *P. arctica* is occupied by a dense population of epiphytic diatoms which produce photosynthetic oxygen, this could be a possible explanation why a deepwater species possesses such a high antioxidative potential.

The different plant species show different strategies against oxidative stress. The responses in this study are time- and dose-dependent. As Collen and Davison (1999a) pointed out, the key element in reactive oxygen metabolism might be the balance between production and protection in individual compartments, such as chloroplasts rather than protection integrated over the entire cell. Our study supports this theory.

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Publication IV

Antioxidative properties of three Arctic green macroalgae

DUMMERMUTH AL[1], KARSTEN U[2], WIENCKE C [1]

[1] Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12
27570 Bremerhaven
email: adummermuth@awi-bremerhaven.de

[2] University of Rostock,
Institute of Aquatic Ecology,
Albert-Einstein-Str. 3
18059 Rostock, Germany

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Antioxidative properties of three Arctic green macroalgae

Angelika Luise Dummermuth*[1], Ulf Karsten [2], Christian Wiencke [1]

[1]Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany

[2] University of Rostock, Institute of Aquatic Ecology, D-18051 Rostock

* Author for correspondence:
Angelika L. Dummermuth
Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12, D-27570 Bremerhaven, Germany
Tel.: +49-471-4831-1540; Fax: +49-471-4831-1425,
Email: adummermuth@awi-bremerhaven.de

SUMMARY

The antioxidative properties of the three Arctic green macroalgae *Acrosiphonia* sp., *Chaetomorpha melagonium* and *Chaetomorpha linum* were investigated after exposure to increasing concentrations of H_2O_2. The biochemical defense systems against oxidative stress as there are the enzymes superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione reductase (GR) as well as the antioxidant ascorbic acid were measured after one week of exposure to different H_2O_2 concentrations. As control of the general physiological performance the photosynthetic efficiency, the ratio of variable to maximum chlorophyll fluorescence of photosystem II (Fv/Fm), was recorded. While Fv/Fm decreased significantly ($p<0.0001$) to 50% of the control in *Acrosiphonia* sp. under H_2O_2 concentration >1mM, photosynthetic efficiency in *Chaetomorpha melagonium* was only reduced to 95% of the control, even at a concentration of 2 mmol H_2O_2. Photosynthesis of *C. linum* was completely inhibited under the 2mM treatment ($p<0.0001$). In *Acrosiphonia* sp. activities of SOD were high, APX and GR were very low and intermediate in CAT. Activities of SOD, APX and CAT increased slightly under H_2O_2 treatment, whereas GR activity was not affected. The ascorbic acid content was low but not completely depleted under H_2O_2 stress. In *C. linum*, activities of CAT were low. APX activities, in contrast, were high and increased up to 5 fold when treated with H_2O_2. SOD activity was high and remained active under low H_2O_2 concentration but decreased to zero at >1 mM H_2O_2. GR activity was below the detection limit. The ascorbic acid content showed high values and decreased significantly during exposure to H_2O_2 but remained at a still relatively high level after H_2O_2 treatment. In *C. melagonium* SOD activity was high and slightly increased under oxidative stress. APX activity was high but not affected by H_2O_2 stress. CAT activity was extraordinarily high at the beginning of the experiment and decreased gradually after exposure to H_2O_2. GR activity was significantly ($p<0.0001$) increased under all H_2O_2 treatments in comparison to the control. In addition the ascorbic acid content of this species was high at the beginning of the experiment and remained at a lower but still relatively high level after H_2O_2 stress. In conclusion, the different tolerance of the three macroalgal species to H_2O_2 stress is species specific and clearly dependent on the different antioxidative properties. The antioxidative properties of the three species can be related to their specific growth habitats in different depths and light conditions.

Key words: Antioxidants, oxidative stress, superoxide dismutase, ascorbate peroxidase, catalase, glutathione reductase, ascorbic acid, macroalgae, Arctic

Abbreviations

APX = ascorbate peroxidase
CAT = catalase
GR = glutathione reductase
H_2O_2 = hydrogen peroxide
$^1O_2$ = singlet oxygen
$O_2^{\cdot-}$ = superoxide
$OH^{\cdot}$ = hydroxyl radical
NADPH = nicotinamid adenine dinucleotide phosphate
ROS = reactive oxygen species
SOD = superoxide dismutase
TSP = total soluble protein

INTRODUCTION

Oxygen is essential for the metabolism of aerobic organisms, however its participation in cellular metabolism results in the appearance of ever-present toxic reactive oxygen species (ROS): superoxide anion radical ($O_2^{\bullet-}$), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2). Production of ROS occurs especially under various stress conditions as, for example, during exposure to excessive light or UV radiation (McKersie and Lesham, 1994; Collen and Davison, 2001; He and Häder, 2002). If accumulation of ROS exceeds the capacity of any protective systems, lipids, proteins and nucleic acids are destroyed leading to damage of the photosynthetic apparatus and finally to cell death (Asada and Takahashi, 1987; Halliwell and Gutteridge, 1989; Karsten et al., 2002; Vacha, 1995).

ROS are characterized by a short lifetime except hydrogen peroxide and interact rapidly with water or cellular components (Asada, 1994). Hydrogen peroxide itself is not particularly reactive with most biologically important molecules, but is an intracellular precursor for more reactive oxidants as it passes quickly through membranes.

Therefore cellular protection mechanisms are essential (Asada and Takahashi, 1987; Halliwell, 1982). Enzymatic detoxifying systems and antioxidants of different chemical groups are known in higher plants to diminish oxidative stress by elimination and reduction of ROS to less toxic and less reactive products (Pedersen et al., 1996). Common powerful detoxifying systems are the enzymes SOD, APX, CAT and GR, as well as phenolic compounds such as flavonoids, coumarins and tocopherols, nitrogen containing compounds including alkaloids, chlorophyll derivates, amino acids and amines and other compounds such as carotenoids, ascorbic acid, glutathione and uric acid (Fujimoto et al., 1985; Larson, 1988; Paya et al., 1992; Potterat, 1997). In marine algae ascorbic acid, β-carotene and α-tocopherol are well described antioxidants (Aguilera et al., 2002b; Castillo et al., 1986; Collen and Davison, 1999b; Collen and Davison, 1999b; Honya et al., 1994; Jayasree et al., 1985; Nakamura et al., 1994; Potterat, 1997). In addition, the presence of enzymatic defense systems was recently reported in marine macroalgae (Aguilera et al., 2002a, b; Potterat, 1997) with particular emphasis on photooxidative stress and discussed in respect to the respective habitat. However, data on further properties of these antioxidants in Arctic species, particularly under direct H_2O_2 stress, are missing.

In aquatic environments, H_2O_2 predominantly derives from UV-driven photoactivation of dissolved organic material (DOM) (Cooper and Zika, 1983; Zika et al., 1985). H_2O_2 concentrations in seawater normally range between 20 and 300nM (Pamatmat, 1990; Price et al., 1992; Szymczak and Waite, 1988; Zika et al., 1985). However, during low tide in summer, H_2O_2 was found to accumulate to micromolar (<5 µmol L-1) concentrations in shallow intertidal pools on the German Wadden Sea coast (Abele-Oeschger et al., 1997). But also in polar regions high H_2O_2 concentrations in surface or tidal pool water < 2µmol L^{-1} were measured in Antarctica (Abele et al., 1998; Abele et al., 1999), here deriving from wet deposition in form of snow, wherein H_2O_2 levels amounted to 13µmol L^{-1} (Abele et al., 1999). The stratospheric ozone and the resulting increase in UVB radiation lead to an enhanced photochemical H_2O_2 production. A 10 % ozone reduction leads to a doubling of UVB surface irradiance at 300 nm, which entails a 40% increase of the apparent intertidal H_2O_2 concentrations (Abele-Oeschger et al., 1997).

The aim of the present study was to investigate the antioxidative reaction patterns of green macroalgae from an Arctic fjord under artificial hydrogen peroxide stress. Over

a period of 1 week *Chaetomorpha linum*, *Chaetomorpha melagonium* and *Acrosiphonia* sp. were incubated in seawater enriched with a series of ascending H_2O_2 concentrations. Extremely high concentrations (0-2 mM H_2O_2) were used to investigate the tolerance width of these green algal species to high H_2O_2 stress, as the physiological reaction patterns tolerated natural H_2O_2 concentrations in seawater without problems. As control for the physiological status of the algae under stress photosynthetic efficiency was measured. The antioxidative detoxifying systems were characterized and the resulting data correlated with the species-specific habitat.

MATERIAL AND METHODS

Algal material and study site

Algal material was collected by SCUBA diving in summer 1999 and 2000 at the study site in the Kongsfjord (Ny Ålesund, Spitsbergen, Norway 78°55,5'N; 11°56,0'E) from depths between 0 and 10 m (Table 1, Fig. 1). Algal samples were collected in black bags to avoid exposure to high irradiance and kept in seawater during transport. After sampling, the algal material was kept in running seawater pumped directly from the fjord at 3-5°C under dim white fluorescent lamps (Philips) (25µmol m^{-2} s^{-1}) for at least 24 hours.

Algal thalli with a fresh weight of ca. 4g were incubated in 1-2 L glass vessels containing pure seawater (control) or seawater enriched with 0.5 to 2 mM H_2O_2. Media were changed daily to keep H_2O_2 concentration constantly high. The algae were illuminated with white fluorescent lamps (Philips) covered with gray filter foil to reduce photosynthetic active radiation to 25 µmol m^{-2} s^{-1}. Samples were taken after 7 days of exposure and in case of *Chaetomorpha linum* after 6 days of exposure, deep frozen in liquid nitrogen and stored at -30°C or in case of *C. linum* lyophilized and subsequently stored at -30°C until analysis.

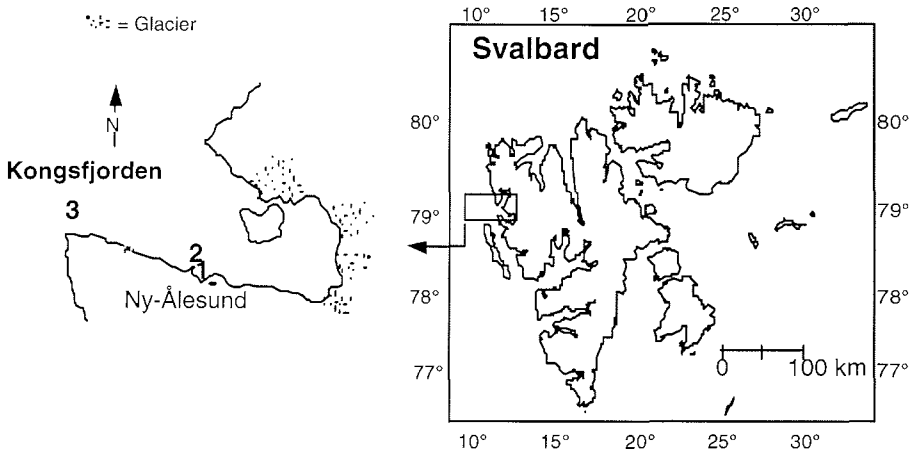

Fig. 1: Map of Svalbard archipelago, indicating the collection sites (1 Harbour; 2 Brandal; 3 Kvadehuken) in the Kongsfjord on Spitsbergen, the largest island of the archipelago

Table 1: Investigated macroalgal species from the Arctic Kongsfjord (Svalbard, Norway)

Species	Distribution	Sampling site/depth
Acrosiphonia sp.	0-10m	Brandal 7m
Chaetomorpha melagonium (F. Weber et Mohr) Kützing	1.5-5m	Kvadehuken 2m
Chaetomorpha linum (Müller) Kützing	5-7m	Harbour 6m

Photosynthesis

Photosynthetic efficiency was determined as ratio of variable to maximum chlorophyll-fluorescence (Fv/Fm) of photosystem II (PSII) using a portable pulse amplitude modulated fluorometer (Diving-PAM, Walz, Effeltrich, Germany). After 5-10 min in darkness minimal fluorescence (F_0) was measured with a pulsed measuring beam (approximately 0.3 µmol photons m^{-2} s^{-1}, 650 nm), followed by short pulses of saturating white light (0.4-0.8 s, 1000-5000 µmol photons m^{-2} s^{-1}) to record maximal fluorescence (Fm). The variable fluorescence results from the difference between maximal and minimal fluorescence ($F_v = F_m - F_0$) (Hanelt 1998). F_v/F_m values of all algae acclimated for 24 h to dim light conditions in the laboratory were characteristic for photosynthetically non-inhibited algae and consequently set to 100% (=control). All data recorded are expressed in relation to this value.

Activities of antioxidative enzymes

Samples (0.25-0.35 g FW or 0.010-0.012 g DW) were ground in liquid nitrogen and extracted with 1-1.5 mL 50 mM potassium phosphate buffer (pH 7.0) containing Complete^{TM} protease inhibitor cocktail (Boehringer, Mannheim, 2 tablets in 100 mL buffer). Extracts were then centrifuged for 15 min at 15,000 r.p.m. at 4° C. CAT was analyzed in the supernatant according to (Aebi, 1984) as described by (Aguilera et al., 2002b); modified for use of a microtiterplate spectrophotometer (Spectramax, Molecular Devices, Sunnyvale CA94089, USA). In order to get replicate measurements the reaction mixture was scaled up to 2 mL and then dispersed with a multichannel pipette in 6 slots of the microtiterplate each containing 300µL reaction mixture. CAT activity was calculated by subtracting the non-enzymatic reaction and using an extinction coefficient of 0.0398 mM^{-1} cm^{-1}. The other enzymes were also measured in the microtiterplate spectrophotometer and the methods adapted as described for CAT. GR was assayed according to Goldberg and Spooner (1983); as described by (Aguilera et al., 2002b) and calculated by subtracting the non-enzymatic reaction using an extinction coefficient of 6.22 mM^{-1} cm^{-1}. In samples for ascorbate peroxidase activities 0.5 mM of ascorbic acid were added to the extraction buffer for stabilization of the APX (Chen and Asada, 1989). APX activity was calculated by subtracting the non-enzymatic reaction using an extinction coefficient of 2.8 mM^{-1} cm^{-1}. Results for CAT, GR and APX are expressed as units (U) of enzyme activity per mg of total soluble protein [1 U= 1 µmol substratum (H_2O_2, NADPH and ascorbic acid respectively) converted min^{-1}]. SOD was measured using the xanthine oxidase-cytochrome *c* reduction method (McCord and Fridovich, 1969) as modified by (Aguilera et al., 2002b). 1 unit of SOD was defined as the amount of enzyme required inhibiting the rate of cytochrome *c* reduction by 50%.

Ascorbic acid

Ascorbic acid was measured according to (Foyer et al., 1983) as described by (Aguilera et al., 2002b) and also adapted for use in the microtiterplate spectrophotometer. The ascorbic acid content was determined by the disappearance of absorbance at 265 nm after addition of 10 U mL^{-1} ascorbate oxidase and 10-50 µL sample to 1925-1965 µL sodium phosphate buffer (100 mM, pH 5.6). Amounts were

quantified using a standard curve with 1.25-12.5 µM of pure ascorbic acid in the reaction mixture.

Protein Assay

Total soluble protein (TSP) content in crude extracts was determined using a commercial Protein Assay (BioRad, Germany), based on the method described by (Bradford, 1976). Protein content was determined spectrophotometrically at 595 nm and concentrations were calculated compared to a calibration curve of bovine serum albumin (SIGMA, Germany).

Statistics

Mean values and standard deviations of 5 replicates per treatment were calculated. Data were treated by one-way ANOVA followed by a Least Significant Difference-Test (LSD-Test). In case homogeneity of variance was not obtained, a Students t-test was assessed. The calculations were performed with the program Statistica (Statsoft Inc., Tulsa, USA) and significances occurred when probability was at $p< 0.05$.

RESULTS

The photosynthetic capacity of the three green algae decreased under increasing H_2O_2 concentrations. Treatment with 0.5 mM H_2O_2 did not affect Fv/Fm in *Acrosiphonia* sp.. Fv/Fm decreased significantly ($p<0.01$) to 80% of the control after 6 days of exposure to 1 mM H_2O_2 in this species. H_2O_2 concentration of 2 mM reduced Fv/Fm values drastically to 59 % of the control ($p<0.01$) (Fig. 2). In *Chaetomorpha melagonium* exposure to all H_2O_2 treatments resulted in a decrease of Fv/Fm to only 95-97 % of the control (Fig. 2). Exposure to 0.5 mM H_2O_2 slightly enhanced Fv/Fm in *Chaetomorpha linum* during the first 24 h to 108% of the control (data not shown). After 6 days of exposure an Fv/Fm value of 90% of the control was recorded at >1 mM H_2O_2. However, H_2O_2 concentrations >1 mM resulted in a drastic reduction of Fv/Fm to 9 % of the control (Fig. 2). After 4 days of exposure in 2 and 5 mM H_2O_2 the thalli of *C. linum* started to bleach, concomitantly the alga exhibited a much softer consistence. After 8 days under such extreme H_2O_2 concentrations no photosynthetic activity could be recorded (data not shown).

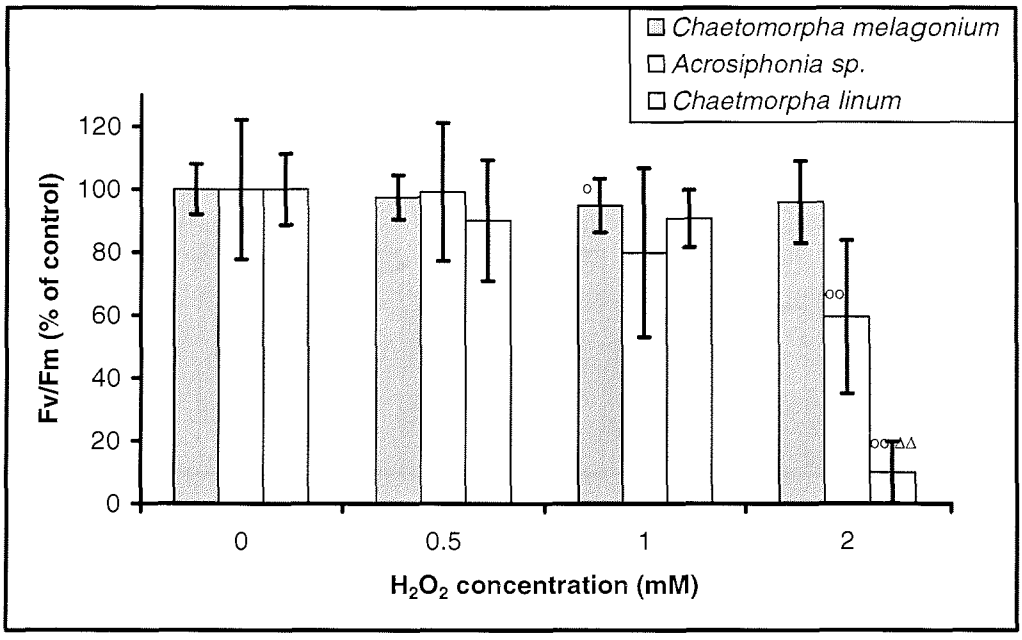

Fig. 2: Maximum quantum yield mean values ± SD of *Chaetomorpha melagonium, C. linum* and *Acrosiphonia* sp. after 6 days of incubation in a series of ascending hydrogen peroxide concentrations, n=12. Mean values marked with o significantly differ to the control at $p<0.05$ and oo with $p<0.01$ control as well as Δ differ significantly ($p<0.05$ and ΔΔ to $p<0.01$) to the preceding lower H_2O_2 concentration.

Enzyme activities in *Acrosiphonia* sp. and *C. melagonium* were affected in different ways. SOD activity in *Acrosiphonia* sp. rose from 179 to 266 U mg TSP^{-1} with increasing H_2O_2 concentration and varied in *C. melagonium* between 176 and 243 U mg TSP^{-1} without showing any correlation to H_2O_2 concentration (Fig. 3a). In *C. linum* SOD activity decreased slightly under exposure to 0.5 and 1 mM H_2O_2, while exposure to 2mM H_2O_2 was accompanied by a total loss of activity (Fig. 3a).

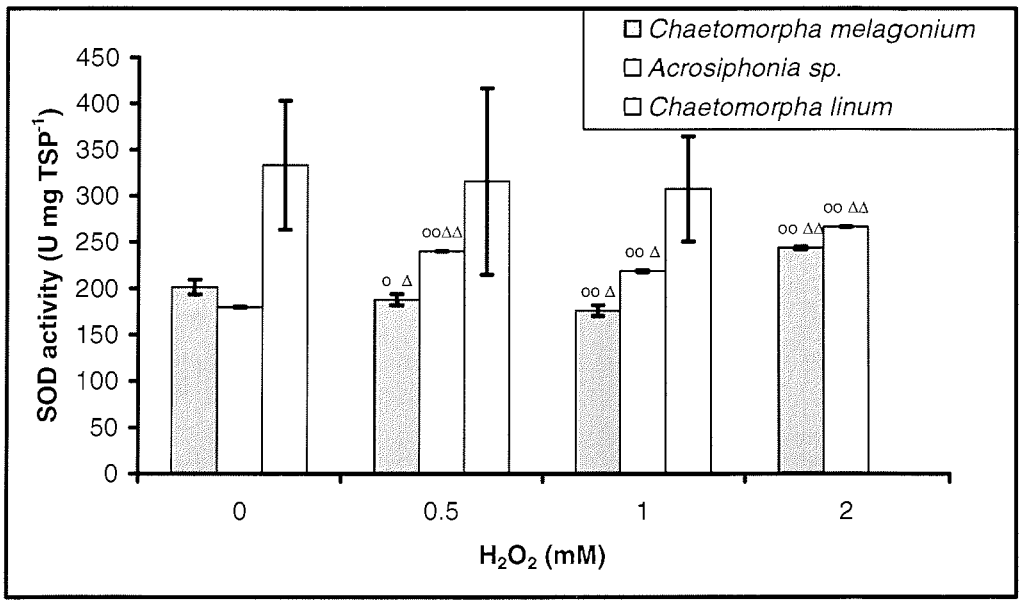

Fig. 3a: Superoxide dismutase activity after 6 days of exposure to 0-2 mM H_2O_2 in *Chaetomorpha melagonium* and *Acrosiphonia* sp. (U g TSP^{-1}) and after 7 days of exposure in *C. linum*, mean values SD, n=5. o, oo, Δ, ΔΔ as in Fig. 1

APX activity in the two *Chaetomorpha* species was high. For *Chaetomorpha melagonium* no clear correlation of APX activity to H_2O_2 concentration was found varying between 0.39 and 0.57 U mg TSP^{-1} and being 16 fold higher than in *Acrosiphonia* sp.. APX activity in *Acrosiphonia* sp. was extremely low varying between 0.007 and 0.034 U mg^{-1} TSP and showed a slight increase under higher oxidative stress. In *C. linum* APX activity rose significantly ($p<0.01$) from originally 0.54 U mg^{-1} TSP to 1.6 U mg^{-1} TSP and 2.2 U mg^{-1} TSP when exposed to 1 and 2 mM H_2O_2, respectively (Fig. 3b). Highest APX activities were measured at 2 mM H_2O_2 after 6 days with 2.2 U mg^{-1} TSP (Fig. 3b) but longer exposure time and higher H_2O_2 concentrations led to increasing mortality (data not shown).

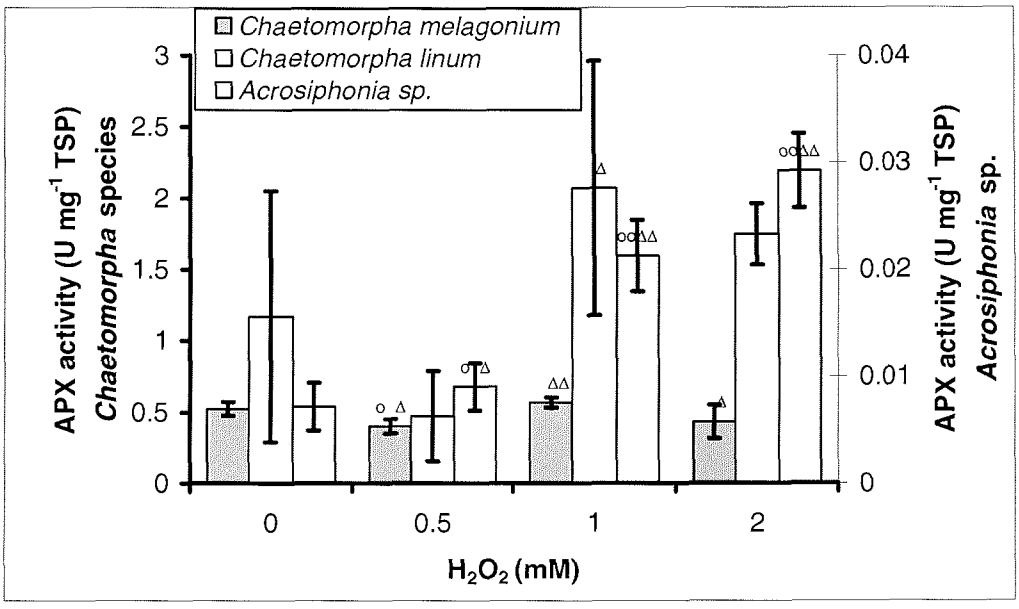

Fig. 3b: Ascorbate peroxidase (APX) activity after 6 days of exposure to 0-2 mM H_2O_2 in *Chaetomorpha melagonium* and *Acrosiphonia* sp. (U g TSP^{-1}) and after 7 days of exposure in *C. linum*, mean values SD, n=5. o, oo, Δ, ΔΔ as in Fig. 1

CAT activity increased with rising H_2O_2 stress in *Chaetomorpha linum* from 0.53 to 1.52 U mg^{-1} TSP and in *Acrosiphonia* sp. from 3.4 to 6.8 U mg^{-1} TSP, whereas in *C. melagonium* CAT activity decreased with increasing oxidative stress from 30 to 16 U mg^{-1} TSP (Fig. 3c). GR activity varied between 0.15 and 0.33 U mg^{-1} TSP in *C. melagonium* indicating a trend of increasing GR activity with increasing oxidative stress (Fig. 3d). *Acrosiphonia* sp. showed less variation within a range of 0.09 and 0.13 U mg^{-1} TSP whereas in *C. linum* no GR activity was detected.

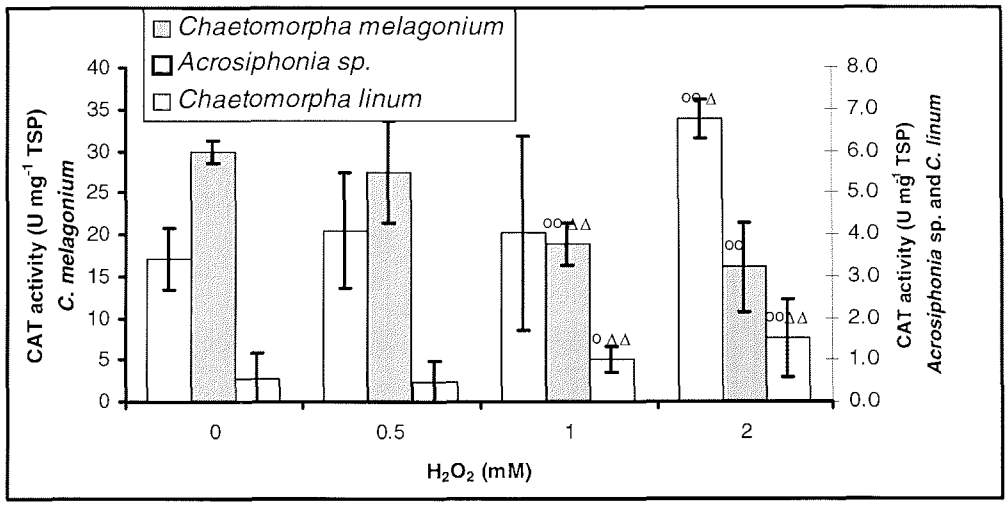

Fig. 3c: Catalase (CAT) activity after 6 days of exposure to 0-2 mM H_2O_2 in *Chaetomorpha melagonium* and *Acrosiphonia* sp. (U g TSP^{-1}) and after 7 days of exposure in *C. linum*, mean values SD, n=5. o, oo, Δ, ΔΔ as in Fig. 1

The content of the antioxidant ascorbic acid decreased continuously in all three species with increasing H_2O_2 concentrations (Table 2). The two *Chaetomorpha* species exhibited in contrast to *Acrosiphonia* sp. high ascorbic acid values. *Chaetomorpha linum* showed the highest concentrations of the three species with 1.74 mg g FW^{-1} at the beginning of the experiment, followed by a gradual decrease to 0.52 mg g^{-1} FW after H_2O_2 treatment. At H_2O_2 concentrations >2 mM and longer exposure times ascorbic acid content was fully depleted (data not shown). *Chaetomorpha melagonium* showed the same pattern as *C. linum* starting with a control value of 1.4 mg g^{-1} FW and decreasing to 0.44 mg g^{-1} FW after exposure to 2mM H_2O_2.

Table 2: Ascorbic acid content (mg g^{-1} FW) of *Chaetomorpha melagonium* and *Acrosiphonia* sp. after 7 days of exposure to 0.5 to 2mM H_2O_2 and after 6 days of exposure in *C. linum*, n=5.

mM H_2O_2	*Chaetomorpha melagonium*	*Acrosiphonia p.*	*Chaetomorpha linum*
0	1.397 ± 0.051	0.192 ± 0.014	1.74 ± 0.33
0.5	0.913 ± 0.115	0.121 ± 0.003	1.50 ± 0.28
1	0.741 ± 0.076	0.064 ± 0.010	1.47 ± 0.14
2	0.438 ± 0.078	0.073 ± 0.019	0.52 ± 0.19

DISCUSSION

The antioxidative properties of the three green macroalgae *Acrosiphonia* sp., *Chaetomorpha melagonium* and *Chaetomorpha linum* from the Arctic were investigated under artificial oxidative stress generated by H_2O_2. The outstanding result of this study is that H_2O_2 stress up to 1 mM was generally tolerated by all species while concentrations higher than 1 mM reduced the photosynthetic efficiency significantly in *Acrosiphonia* sp. and *C. linum* but not in *C. melagonium*. The sensitivity of the investigated green algal species to H_2O_2 can be rated as high for *C. linum*, intermediate for *Acrosiphonia* sp. and low for *C. melagonium*. This is consistent with the depth distribution and habitat of the three species assuming that H_2O_2 stress in first order derives from photochemical H_2O_2 accumulation driven by ultraviolet radiation (UVR) as well as the fact that the H_2O_2 concentration in the surface layer is higher than in deeper waters. Although measurements in H_2O_2 data from the Arctic Kongsfjord are missing, this hypothesis is well supported by Antarctic coastal waters (Abele et al., 1999) as well as in other coastal and estuarine areas (Fujiwara et al., 1993; Johnson et al., 1989; Price et al., 1992; Zika et al., 1985). Water column depth profiles typically show decreasing H_2O_2 concentrations, reflecting downward mixing below UV penetration depth (Cooper and Lean, 1989).

The intermediate sensitivity of *Acrosiphonia* sp., collected here at 7 m depth, can be explained by the broader distribution range of this alga. It is expected that thalli from the eulittoral would show lower sensitivity because the basic level of antioxidant defense systems should be higher due to preadaptation to the environmental conditions in the upper zone. A similar physiological classification in relation to UVR tolerance was presented by Hanelt et al. (2001) also classifying *Acrosiphonia* species with an intermediate sensitivity. The low sensitivity of *C. melagonium* can be explained by the high antioxidative properties of this alga with high enzyme activities as well as a high ascorbic acid content all of which is reflected by a shallow water growth habitat. In comparison to *Acrosiphonia* sp. and *C. linum, C. melagonium* exhibited highest activities of CAT and GR, whereas *C. linum* showed extremely low activities in CAT and GR but higher SOD activities up to 1mM H_2O_2. Enzyme activities in *Acrosiphonia sp.* were intermediate except for APX with extremely low values, reflecting the intermediate sensitivity.

High activities of the antioxidant enzymes found in the three species studied are similar to those of higher plants, micro- and macroalgae (Aguilera et al., 2002a;

Aguilera et al., 2002b) . In comparison with other algae APX activities of the two *Chaetomorpha* species are comparable to those of the symbiotic zooxanthellae of a sea anemone, *Aiptasia pallida* (Lesser and Shick, 1989) while CAT and SOD activities are much higher. Enzyme activities measured in this work are in the same range as to data given by Aguilera et al. (2002b) who carried out a general screening for antioxidative enzymes in Arctic marine macroalgae as well as a seasonal study with respect to changes in biochemical defense systems against radiation stress (Aguilera et al., 2002a). Compared to macroalgae from temperate waters enzyme activities detected in the three Arctic green algae are in the same range as in *Fucus* species (Phaeophyceae), the two intertidal red algae *Chondrus crispus* and *Mastocarpus stellatus* and the green alga *Ulva rigida* (Collen and Davison, 1999a; Collen and Davison, 1999c; Collen and Pedersen, 1996). Ascorbic acid content in the three species tested is similar to data from Aguilera et al. (2002b), Collen and Davison (1999a, 2001) and Sarojini and Sarma (1999).

The decrease in SOD activity after exposure to H_2O_2 concentrations >1 mM correlates to Fv/Fm data in *C. linum* and can be explained by a direct toxicity effect as observed by Collen and Pedersen (1996) in similar experiments with the green alga *Ulva rigida*. Exposure to high levels of H_2O_2 (3 mM and higher) in this species caused intolerable oxidative stress accompanied with full mortality as in *C. linum* after 8 days of exposure to 2 and 5 mM H_2O_2 (data not shown).

The stimulation of GR activity in *C. melagonium* indicates an active scavenging of H_2O_2 by means of the ascorbate-glutathione cycle. It has been shown that plants increase GR activity in response to stress. For example, in *Arabidopsis* GR activity was enhanced under UV radiation (Kubo et al., 1999) as well as in several Arctic macroalgae under artificial and natural UV stress (Aguilera et al., 2002b).

A small incremental decrease in SOD activity was found in *C. melagonium* and *Acrosiphonia* sp. after H_2O_2 treatment confirming earlier investigations of Kubo et al. (1999) and Aguilera et al. (2002b). In *C. linum*, however, H_2O_2 seemed to directly affect SOD activity and a decrease, especially in the first 24 h, was recorded for all treatments (data not shown). H_2O_2 concentrations > 1 mM fully inhibited SOD indicating the toxicity effect discussed earlier. These results are comparable to those observed in *Palmaria palmata* where SOD activity was directly affected after 1 day of exposure to UV radiation (Aguilera et al., 2002b) as well as in the green microalga *Chlorella vulgaris*, which showed a decrease of SOD activity after long-term exposure

to UV radiation inducing oxidative stress (Malanga and Puntarulo, 1995). Inhibition of gene expression for this enzyme may be the reason for this negative effect as observed by Strid (1993) in *Pisum sativum*. *Zea mays* leaves, in contrast, showed increased APX and SOD activity after12 h incubation in 1 mM H_2O_2 (Pastori and Trippi, 1993) which is in agreement to the increased APX activity in *Acrosiphonia* sp. and *Chaetomorpha linum*.

Low CAT activity in combination with enhanced APX activity after H_2O_2 treatment, as is the case in *C. linum* in our study, is consistent with low levels of CAT found in *Euglena gracilis*, which primarily uses APX to reduce H_2O_2 in the cell (Shigeoka et al., 1980). The advantage of using APX rather than CAT is, besides the H_2O_2 reduction, that NADPH-dependent ATP-production is caused, which functions as an additive sink for energy. *Acrosiphonia* sp., in contrast showed extremely low APX activity and intermediate CAT activity slightly increasing under H_2O_2 stress. This would suggest that *Acrosiphonia* sp. rather uses CAT as scavenger for H_2O_2 in case of oxidative stress. Alternatively (Aguilera et al., 1999) suggested morphological strategies as protection against UV radiation. As *Acrosiphonia* sp. is living in the upper eulittoral, it can be fully emergent at low tide, and hence, exposed to high radiation. The apical part of the *Acrosiphonia* bunches suffer chlorophyll bleaching by photooxidation, whereas the basal part of this alga is effectively protected due to self-shading. Increase in APX activity in *C. linum* correlates to the decrease in ascorbic acid content indicating a switch in antioxidant strategy with increasing H_2O_2 concentration changing from the scavenger ascorbic acid to the enzymatic defense system of APX.

The two *Chaetomorpha* species have a smaller depth distribution on Spitsbergen than *Acrosiphonia* sp.. *C. linum* prefers 5-7 m depth and sheltered areas, *C. melagonium* is found in clear shallow waters with high frequency of water exchange between 1.5 and 5 m. The different habitats of the three green algal species are well reflected in the responses of the different enzymatic defense systems against H_2O_2 stress.

In general, high antioxidant enzyme activities and ascorbic acid contents are typically measured in green algae (Aguilera et al., 2002b) because they occupy the upper part of the rocky shore, where they are exposed to high amplitudes in environmental conditions, especially those related to rapid and drastic changes in UVR causing oxidative stress. Comparing the three investigated Arctic green algal species to each

other, they have different biochemical capabilities of the enzymatic defense systems against H_2O_2 that are well reflected by the prevailing light conditions at different water depths. In conclusion the different tolerance of the three species to H_2O_2 stress clearly depends on the different antioxidative properties.

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Publication V

Seasonal variation in ecophysiological patterns of two red macroalgae from the Arctic Kongsfjord (Spitsbergen, Norway): A long-term culture study with emphasis on UV- protective mechanisms; I: *Palmaria palmata* (L.) Greville

DUMMERMUTH AL[1], HOYER K[2], KARSTEN U[3], WIENCKE C [1]

[1] Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12
27570 Bremerhaven
email: adummermuth@awi-bremerhaven.de

[2] Present address:
NMR Laboratory for Physiological Chemistry
Brigham and Women's Hospital, Harvard Medical School
221 Longwood Ave., BLI 247
Boston, MA 02115 USA

[3] University of Rostock,
Institute of Aquatic Ecology,
Albert-Einstein-Str. 3
18059 Rostock, Germany

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- Seasonal ecophysiology of *Palmaria palmata* -

Angelika Dummermuth[1*], Kirsten Hoyer[2], Ulf Karsten[3] Christian Wiencke[1]

[1]Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany

[2] present address:
NMR Laboratory for Physiological Chemistry, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Ave., BLI 247, Boston, MA 02115 USA

[3] University of Rostock, Institute of Aquatic Ecology, Albert-Einstein-Str. 3, D-18051 Rostock, Germany

* corresponding author:
Angelika Dummermuth
Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12, D-27570 Bremerhaven, Germany
Phone: ++49-471-4831-1540
Fax: ++49-471-4831-1540
Email: adummermuth@awi-bremerhaven.de

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ABSTRACT

Marine macroalgae inhabiting Arctic coastal ecosystems are exposed to pronounced seasonal variations in radiation regime, including harmful UV radiation. This long-term culture study presents data on the seasonal changes in growth, pigment concentrations and UV protecting mechanisms. The red alga *Palmaria palmata* was cultured at 0°C and constant high nutrient concentrations at fluctuating daylengths adjusted weekly according to the seasonal variation in daylength at the collection site (Spitsbergen, Norway 78°55,5'N; 11°56,0'E). In a second treatment, thalli were additionally exposed to 10 h UV radiation as soon as daylength exceeded 10 h in cultures. In winter low growth rates were recorded. Maximum growth was recorded in spring accompanied by maximum chl *a* concentrations. Later in summer, growth was inhibited under permanent light, as well as in thalli exposed to UVR. Phycobiliproteins showed reduced concentrations in late winter, before any exposure to light indicating a photoperiodic control. In spring and autumn, maximum concentrations of phycobiliproteins were measured. Protection against direct and indirect UV stress was provided by a high ascorbic acid content throughout the entire year, as well as by mycosporine-like amino acids (MAAs) doubling their concentrations in spring. Antioxidative enzymes showed high activities in winter as a kind of preparation for the situation in spring presumably controlled by photoperiod. A second species, *Devlaeraea ramentacea*, has been studied in the same way (see forthcoming paper).

ABBREVIATIONS

APX = Ascorbate peroxidase
CAT = Catalase
GR = Glutathione reductase
MAAs = mycosporine-like amino acids
PAR = Photosynthetic active radiation
ROS = Reactive oxygen species
SOD = Superoxide dismutase
UVR = ultraviolet radiation

INTRODUCTION

Polar regions are characterised by strong seasonal variations in environmental conditions. In the Arctic, macrophytes endure in winter at least 4 months of complete darkness and even longer when ice cover prevents or reduces light penetration into the water column (Gerland et al. 1999; Hanelt et al. 2001). In springtime, rapid increasing daylength and irradiance is also reflected in the underwater radiation regime, especially due to the high water transparency at that time (Hanelt et al. 2001). Then, the phytobenthic communities have suddenly to cope with high irradiances of photosynthetically active radiation (PAR) as well as ultraviolet radiation (UVR).

Many harmful UVR effects on marine macrophytes, phytoplankton and picoplankton are well documented, e.g. direct effects on the cellular level, damage and transformation of biomolecules such as proteins, nucleic acids and lipids by absorption of UVR, resulting in disturbance or even elimination of their biological functions (Karentz et al. 1991; Franklin & Forster 1997; Aguilera et al. 1999b; Bischof et al. 2000; Boelen et al. 2000; Vincent &Neale 2000). Indirect effects are caused by reactive oxygen species (ROS) as a consequence of radiative stress. ROS are inevitably produced by photosynthesis and absorption of UVR (Lesser 1996) and may lead to oxidative damage of cellular components, increased membrane permeability, lipid peroxidation, and damage to key enzymes, especially those regulated via redox state, which finally can be lethal for the cell (Aguilera et al. 2002a). The repair and protective mechanisms are manifold such as dynamic photoinhibition (Hanelt &Nultsch 2003), DNA repair via photolyases and excision enzymes (Mitchell &Karentz 1993; Pakker et al. 2000; van de Poll et al. 2002), synthesis of UV absorbing compounds such as the MAAs (Karsten et al. 1998), which are widely distributed in aquatic organisms (Karentz 2001; Hoyer et al. 2002b) and the expression and activation of detoxifying enzymes and antioxidants (Dunlap & Yamamoto 1995 ; Aguilera et al. 2002b). If these mechanisms are lacking or not properly functioning, the final result may be an inhibition in growth, reproduction and productivity (Dring et al. 1996; Aguilera et al. 1999b; Wiencke et al. 2000), genetic damage (Kulunscics et al. 1999; Vincent &Neale 2000), depression of photosynthesis (Gómez et al. 2001; Hanelt et al. 1997; Hanelt &Nultsch 2003; Bischof et al. 2000; 2002) or even an altered community structure (Madronich et al. 1995).

Polar macroalgae exhibit a high potential to acclimate to the rapid changes in irradiance as well as in spectral composition (Hoyer et al. 2001; Lüder et al. 2001) . Seasonal changes in solar and underwater light conditions control growth rates of macroalgae (Aguilera et al. 1999b). A significant inhibitory effect of UVA and UVB on growth of species from deeper water could be shown after transplantation to shallow waters. The ability to acclimate and adjust photosynthesis and growth to rapid changes in light climate is therefore regarded as one prerequisite for macroalgae to prevail under the strong seasonal changes in the Arctic.

In the context of a future depletion of stratospheric ozone and consequently an increase in UVR on Earth's surface, long-term experiments focusing on the integrative physiological parameters growth and reproduction are needed. Such experiments will show the balance between the damaging effects of UVR and protective and repair mechanisms. Therefore, growth is a useful indicator to assess UV effects in long-term experiments and should be used more often in combination with other parameters.

On Spitsbergen UV penetrates deep into the water column only for a short period of time right after sea ice break up. Later turbid melt water inflow decreases transparency (Hanelt et al. 2001). Phosphate and nitrate levels are low in summer and higher in winter. Water temperatures rise to about 5 °C in summer and are around –1.8 °C in winter (Aguilera et al. 2002a; Hanelt et al. 2001; Svendsen et al. 2002). All these changes in abiotic parameters hamper the interpretation of physiological reactions. To improve the knowledge on the seasonal variation of UV protection mechanisms and accompanied antioxidative defence systems we set up a long-term culture experiment under seasonally fluctuating daylength conditions and controlled nutrient concentration and temperature. This experimental approach has been applied successfully to Antarctic macroalgal species to understand their seasonal physiological activity (Gomez & Wiencke C. 1996; Weykam et al. 1997; Wiencke 1990). In the present study however, the algae were additionally exposed to UVR. The objective of the present study was, to evaluate the capacities of various protection mechanisms in *Palmaria palmata* to respond to direct and indirect UV effects in the course of the year. As indicator for physiological performance the integrative parameter growth (Altamirano et al. 2000) was chosen. In a second study the same experimental approach was performed with the co-existing red alga *Devaleraea ramentacea* (Dummermuth et al. 2003).

MATERIAL AND METHODS

Tetrasporophytes of *Palmaria palmata* were isolated in the Kongsfjord on Spitsbergen (Norway 78°55,5'N; 11°56,0'E) and established as an unialgal culture. Thalli were cultivated in membrane-filtered seawater (Sartorius Sartobran II, pore size 0,2µm) from the North Sea enriched with nutrients after Provasoli (Stein 1973). The medium was changed fortnightly to maintain nitrogen and phosphate levels above 0.6 and 0.025 moles m^{-3}, respectively. The culture experiment was started using thallus pieces of < 2 cm with a minimum fresh weight of 50 mg.

For the experiments, the algal thalli were cultivated in climate chambers at a temperature of 0±1°C and transferred to two different radiation conditions starting in summer under permanent light after 2 weeks of acclimation. As a control, algal thalli were exposed to photosynthetically active radiation (400 to 700 nm) with a fluence rate of 25 µmol photons m^{-2} s^{-1}. The second treatment was conducted using supplemental UVR, including UVA and UVB (295 to 400 nm) with irradiances of averagely 2.67 W m^{-2} UVA and 0.23 W m^{-2} UVB. Osram daylight fluorescent tubes (L58/W12) in combination with Q-Panel UVA-340 fluorescent tubes (Q-panel-Company, Cleveland, Ohio, USA), emitting a spectrum similar to solar radiation in the range between 280 and 340 nm, were used. Radiation was measured using a Spectro 320 D spectroradiometer (Instrument Systems, Germany). Daylength was adjusted weekly according to the seasonal variation in daylength at the sampling site. UVR was applied for 10 h per day as soon as daylength exceeded 10 h. The experiment was started in August under summer conditions with 24 h light and additional UVR from 10 am to 8 pm. First sampling took place in late September (for several parameters not before November) to allow acclimation to culture conditions. For this reason, samples taken from the UV treatments were exposed until October and then received the same spectrum as the PAR treatment but were still named as UV treated thalli to see possible differences in the measured parameters as consequence of the preceding UV exposure.

Growth measurements

Growth rates were determined by recording fresh weight of algae after dry blotting with paper tissue using the following equation:

$$\text{specific growth rate (\% day}^{-1}) = \frac{100 \ln W_t W_0^{-1}}{t}$$

where W_0 = initial fresh weight, W_t = fresh weight on day t, and t = time interval (Wiencke & tom Dieck 1989). Five individuals per treatment were used for the growth measurements and mean values with standard deviation were calculated.

Activities of antioxidative enzymes

Samples (0.25-0.35 g FW or 0.010-0.012 g DW) were ground in liquid nitrogen and extracted with 1-1.5 mL 50 mM potassium phosphate buffer (pH 7.0) containing Complete™ protease inhibitor cocktail (Boehringer, Mannheim, 2 tablets in 100 mL buffer). After centrifugation for 15 min at 15,000 r.p.m. and 4 °C, the enzyme activities were measured in the supernatant. Catalase was analysed according to Aebi (1984) as described by Aguilera et al. (2002b) modified for use of a microtiter plate spectrophotometer (Spectramax, Molecular Devices, Sunnyvale, USA). The reaction mixture was scaled up to 2 mL in order to get pseudoreplicate measurements and then dispersed with a multichannel pipette in 6 slots of the microtiterplate each containing 300µL reaction mixture. Enzyme activity was calculated by subtracting the non-enzymatic background reaction and using an extinction coefficient of 0.0398 mM^{-1} cm^{-1} for H_2O_2. Glutathione reductase was assayed according to Goldberg &Spooner (1983) as modified by Aguilera et al. (2002a; b). GR activity was calculated by subtracting the non-enzymatic reaction and using an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADPH. The reaction mixture for the measurement of ascorbate peroxidase activities contained 0.5 mM of ascorbic acid for stabilisation of the APX (Chen & Asada 1989). APX activity was calculated by subtracting the non-enzymatic reaction and using an extinction coefficient of 2.8 mM^{-1} cm^{-1} for ascorbic acid. Results for CAT, GR and APX are expressed as units (U) of enzyme activity per mg of total soluble protein [1 U= 1 µmol substrate (H_2O_2, NADPH and ascorbic acid, respectively) converted min^{-1}]. SOD activity was measured using the xanthine oxidase-cytochrome *c* reduction method (McCord &Fridovich 1969), as modified by Aguilera et al. (2002a). 1 unit of SOD was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50%.

Determination of ascorbic acid

The measurement of ascorbic acid followed the method by Foyer et al. (1983) as modified by Aguilera et al. (2002b), and was recorded as decreasing absorbance at 265 nm after addition of 10 U mL^{-1} ascorbate oxidase and 10-50 µL sample to 1925-1965 µL sodium phosphate buffer (100 mM, pH 5.6). Quantification was according to a calibration curve with 1.25-12.5 µM of pure ascorbic acid in the reaction mixture.

Determination of photosynthetic pigments

Samples were extracted in phosphate buffer 0.1 M, pH 6.8 at 4 °C containing 10 mM Na_2-EDTA after grinding the algal material in liquid nitrogen and quarz sand. After centrifugation, (15,000 g, 20 min) the supernatant was used for phycoerythrin (PE) and phycocyanin (PC) determination after Beer & Eshel (1985). For chlorophyll measurements, 1 mL 100% dimethylformamide (DMF) was added to the samples, which were then incubated in darkness for 24 h for extraction. Extracts were centrifuged at 10,000 g for 10 min and Chl *a* concentration in the supernatant was determined spectrophotometrically after Inskeep & Bloom (1985).

Protein assay

Total soluble protein (TSP) content in the crude enzyme extracts was determined using a commercial Protein Assay (BioRad, Germany), based on the method described by Bradford (1976). Protein content was determined spectrophotometrically at 595 nm and concentrations were calculated according to a calibration curve prepared with bovine serum albumin (SIGMA, Germany).

MAA extraction and analysis

A 25% aqueous methanol (v/v) extraction was made from 10-20 mg dry weight (DW) of each algal sample. After evaporating to dryness under vacuum (Speed Vac Concentrator SVC 100H) dried extracts were re-dissolved in 100% methanol. Samples were analysed using a Waters high-performance liquid chromatography (HPLC) system according to Hoyer et al. (2001). Total MAA concentrations are given as means of 5 replicates (±SD) expressed on a dry weight basis (mg g^{-1} DW).

Statistics

Mean values and standard deviations were calculated from four to five independent replicates per treatment. Statistical significance was tested with a model 2 two-way ANOVA (Statistica) followed by a Fishers protected least significant difference test (LSD). Significance level was $p<0.05$.

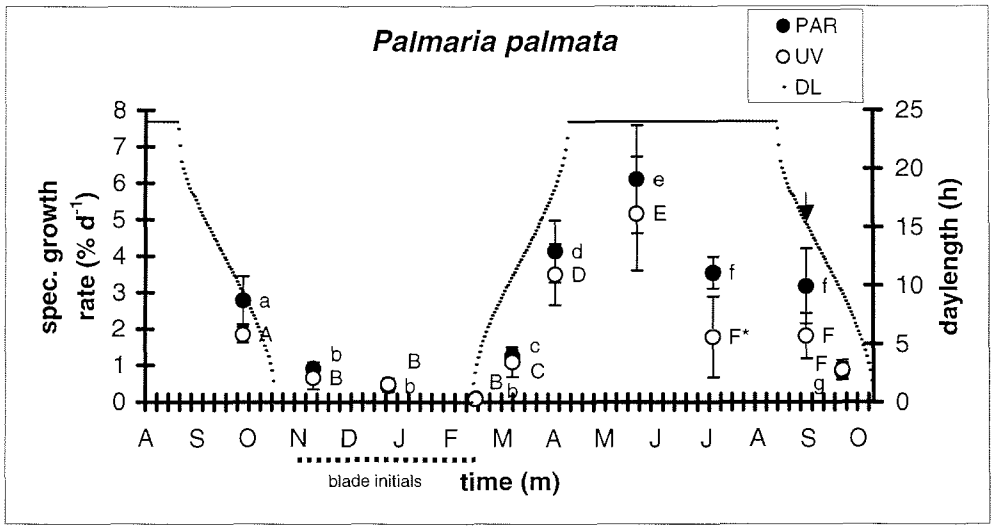

Figure 1: Specific growth rate (% d^{-1}) in the course of the season of *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment, line the day length and grey banner the time of UV exposure (10h d^{-1}). Date of sporulation is given by a black arrow and formation of new blades as a dotted black line. Mean values ±SD of 4 parallels. Statistical significance with $p<0.05$ between the treatments is indicated by *, as well as between the sampling in seasonal succession by alphabetic letters in capitals for UV treated thalli and in small letters for the PAR treated thalli.

RESULTS

Seasonal variation in growth

Specific growth rate in *Palmaria palmata* were high in spring, intermediate in summer and low in winter. Specific growth rates of UV treated thalli always tend to be lower than of those exposed to PAR, but significant differences were only observed in July ($p< 0.003$, Fig.1). At the beginning of the experiment in October specific growth rate under PAR exhibited nearly 3 % d^{-1} whereas under UV treatment just 2 % d^{-1} were reached

(Fig. 1). In darkness, only low growth rates were recorded. With increasing daylength in spring, growth rates increased significantly from February to March ($p<0.041$ for PAR and $p< 0.049$ for UV, Fig. 1). In the PAR treatment growth rates reached slightly higher values with 1.3 % d^{-1} than thalli under UV with 1 % d^{-1}. From March to April specific growth rates rose significantly up to 4.1 % d^{-1} under PAR ($p<0.0001$) and 3.5 % d^{-1} under UV ($p<0.001$, Fig. 1). In April daylength reached 24 h. From April to May specific growth rates still increased significantly to its seasonal optimum of 6.1 % d^{-1} ($p< 0.001$) and 5.2 % d^{-1} ($p<0.005$), respectively (Fig. 1). In July specific growth rates were significantly lower under both conditions, with the PAR-value being twice as high as the UV value (3.5 % d^{-1} ($p<0.0004$) and 1.8 % d^{-1} ($p<0.001$), respectively (Fig. 1)). Until September growth rates remained on the same level as in July. After UV was turned off and daylength declined drastically in September, specific growth rates decreased further to 0.8 % d^{-1} under PAR ($p<0.0001$) and 0.9 % d^{-1} under UV (Fig. 1). From November to February formation of blade initials was observed; tetraspores were formed in September after one year of culture under both radiation regimes.

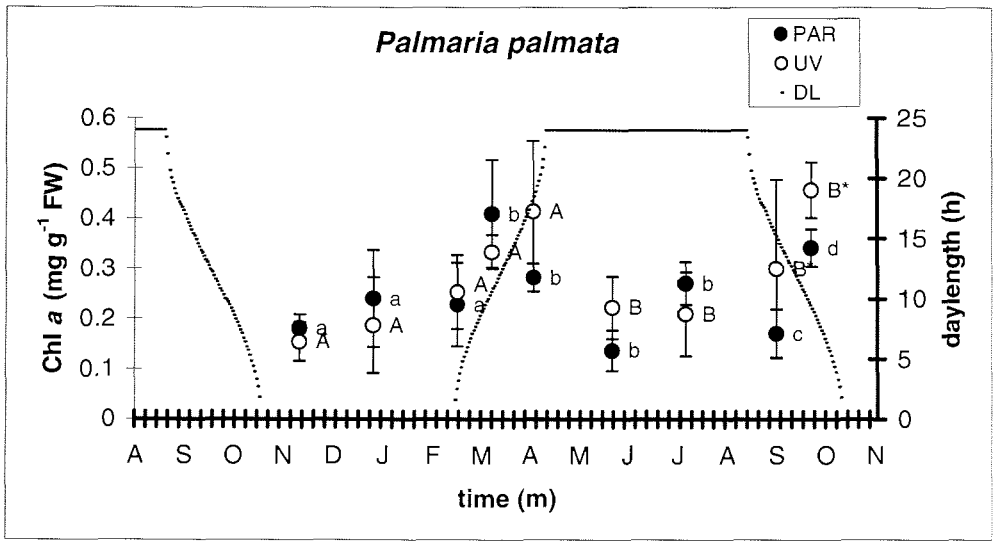

Figure 2a: Chlorophyll *a* (Chl a) concentrations (mg g FW^{-1}) in the course of the season in *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}) and line the day length. UV exposure is indicated by a grey banner. Mean values ±SD of 5 parallels. Statistics see Fig.1

Seasonal variation in pigment and protein content

In darkness, chl *a* content amounted 0.18 to 0.24 mg g FW^{-1} in the control and 0.15 to 0.19 mg g FW^{-1} in UV treated thalli (Fig. 2a). With increasing daylength in spring chl *a* content increased significantly ($p<0.016$) to 0.41 mg g FW^{-1} in the PAR treatment until March and the same amount in UV treated thalli. Later in April, the concentration was already slightly reduced to 0.28 mg g^{-1} FW under PAR (Fig. 2a). In summer, under permanent light, chlorophyll concentration decreased to half the spring concentrations in UV treated thalli and varied between 0.14 and 0.27 mg g FW^{-1} in the PAR treatment (Fig. 2a). In October, chl *a* values rose again to 0.46 mg g FW^{-1} under UV conditions and to 0.34 mg g FW^{-1} ($p<0.035$) in the control (Fig. 2a). In winter and spring, PAR values were slightly higher than in thalli exposed to UV radiation, whereas from April to October UV treated thalli generally exhibited slightly increased values in relation to the control.

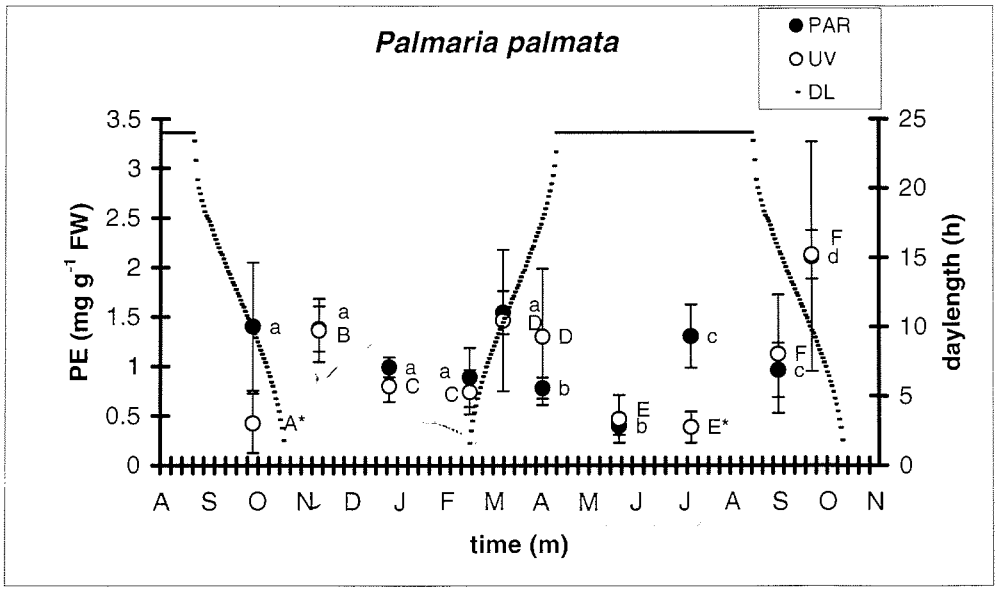

Figure 2b: Phycoerythrin (PE) concentrations (mg g FW^{-1}) in the course of the season in *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}) and line the day length. UV exposure is indicated by a grey banner. Mean values ±SD of 5 parallels. Statistics see Fig.1

The content of the phycobiliproteins phycoerythrin (PE) and phycocyanin (PC) followed a similar pattern as for chl *a*. Highest PE and PC values were measured in spring and autumn, whereas lower values were observed in winter and summer. Highest PE concentrations in *P. palmata* were measured in March with 1.5 mg g FW^{-1} and in October with 2.16 mg g FW^{-1} for both treatments, whereas values in winter and in the permanent light period ranged between 0.36 and 1.30 mg g FW^{-1} (Fig. 2b). Highest PC concentration appeared also in March with 0.12 mg g FW^{-1} for the PAR treatment and 0.13 mg g FW^{-1} for the UV treated thalli as well as in October with 0.17 and 0.18 mg g FW^{-1}, respectively (Fig. 2c). Differences between the two treatments were small and no UV effect could be observed.

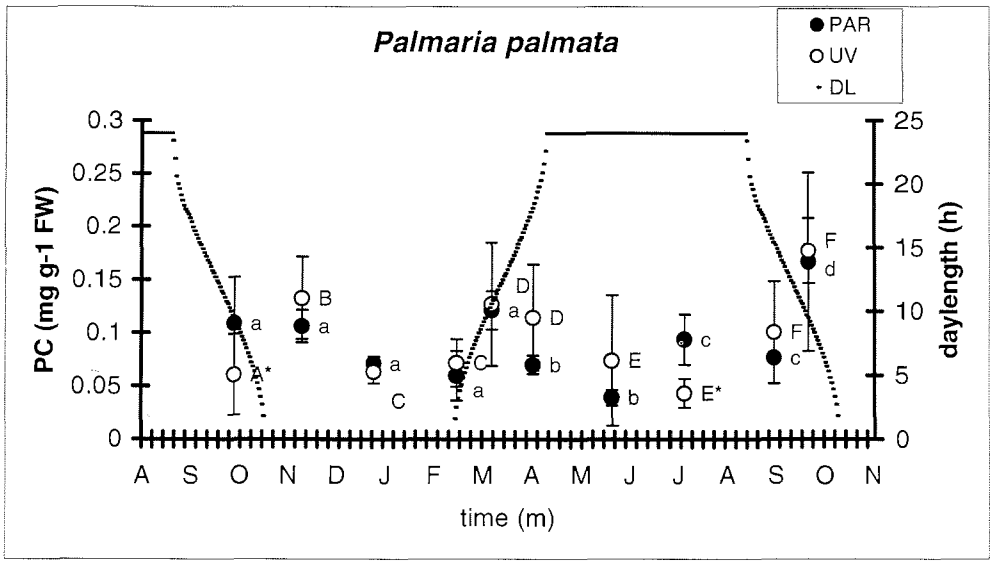

Figure 2b: Phycoerythrin (PE) concentrations (mg g FW^{-1}) in the course of the season in *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}) and line the day length. UV exposure is indicated by a grey banner. Mean values ±SD of 5 parallels. Statistics see Fig.1

Protein content in *P. palmata* varied between 8.04 and 31.28 mg g FW^{-1} in the control and between 6.62 and 25.41 mg g FW^{-1} in UV treated thalli (Fig. 3). Maximum values were measured in spring and autumn when daylength changed rapidly. Protein content in the UV treatment was mostly higher than in the control. Significant differences between the treatments could be observed in October ($p<0.0311$) and February

(p<0.0005, Fig. 3). A significant decrease in protein content to minimum values was found from October to November under both conditions (p<0.0001 for PAR and p<0.0326 for UV treated thalli). From November to December significant decrease was observed under PAER conditions (p<0.0241) as well as from April to May in both treatments (p<0.0016 for PAR and p<0.0004 for UV, Fig. 3). A significant increase in protein content was than observed from February to March (p<0.0002) under PAR and from July to September in thalli exposed to UV (p<0.0148, Fig. 3).

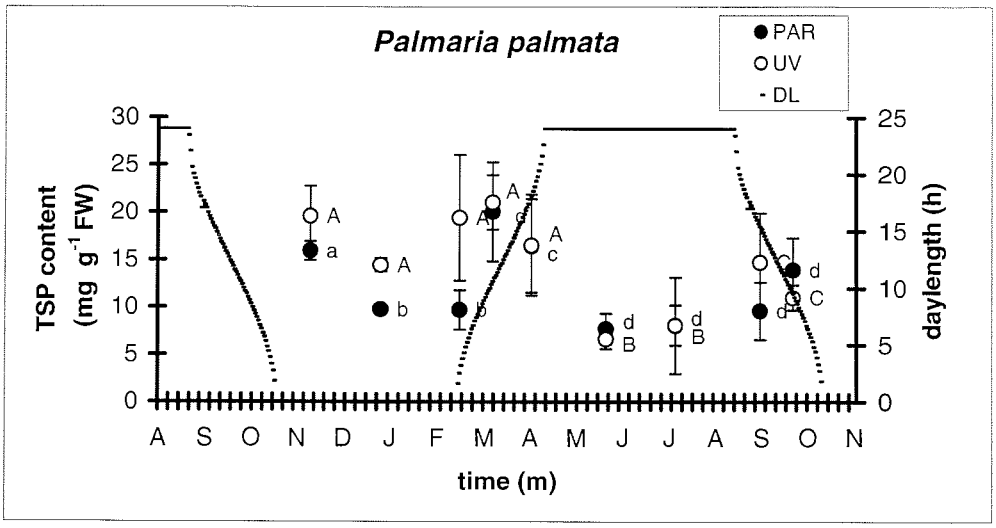

Figure 3: Content of total soluble protein (TSP) (mg g FW^{-1}) in the course of the season in *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}) and line the day length. UV exposure is indicated by a grey banner. Mean values ±SD of 5 parallels. Statistics see Fig.1

Seasonal variation in antioxidative properties

Ascorbic acid content in *P. palmata* varied between 0.2 and 0.54 mg g^{-1} FW in the PAR treatment and between 0.18 to 0.88 mg g^{-1} FW in UV exposed thalli. In general UV treated thalli showed higher values than the PAR treated thalli, differing significantly from each other in February (p<0.003), April (p<0.001) and September (p<0.0001, Fig. 4).

The antioxidative enzymes ascorbate peroxidase and catalase mostly exhibited high activities during the dark period in thalli that had been exposed to UV in the previous season. Significant differences between the PAR and UV treatment were only found in a

few cases because of high standard deviation, especially in samples exposed to UVR. In *P. palmata*, APX activity exhibited values between 3.2 and 5.7 U g^{-1} FW in the control, whereas 12.6 to 17.3 U g^{-1} FW were measured in darkness (Fig. 5a). In UV treated thalli, activities of 2.6 to 6.9 were observed from spring to autumn, whereas winter values exhibited even higher activities between 17.6 and 19.8 U g^{-1} FW (Fig. 5a). For CAT the same pattern was observed, but less pronounced than in APX, and with a much higher standard deviation. Activities ranged between 5.1 and 16.1 U g^{-1} FW for the control and 7.6 and 22 U g^{-1} FW in thalli exposed to UV radiation (Fig. 5b). SOD seemed to be negatively affected by daylengths ≥12 h in both treatments. SOD activity was significantly lower in September and October in UVR exposed thalli, whereas only minor differences were recorded during the rest of the year (Fig. 5c).

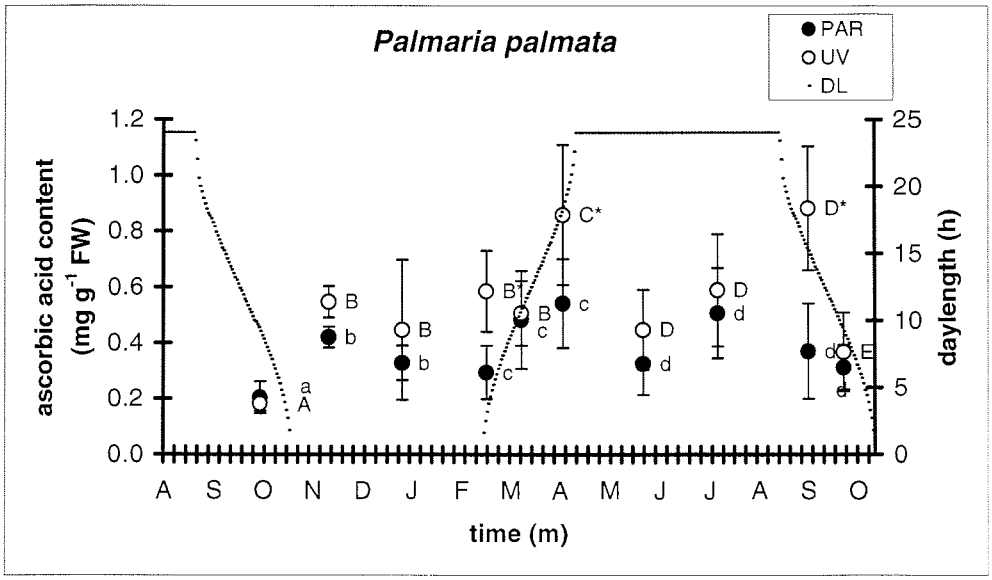

Figure 4: Ascorbic acid content mg g^{-1} FW in the course of the year in *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels. Statistics see Fig.1

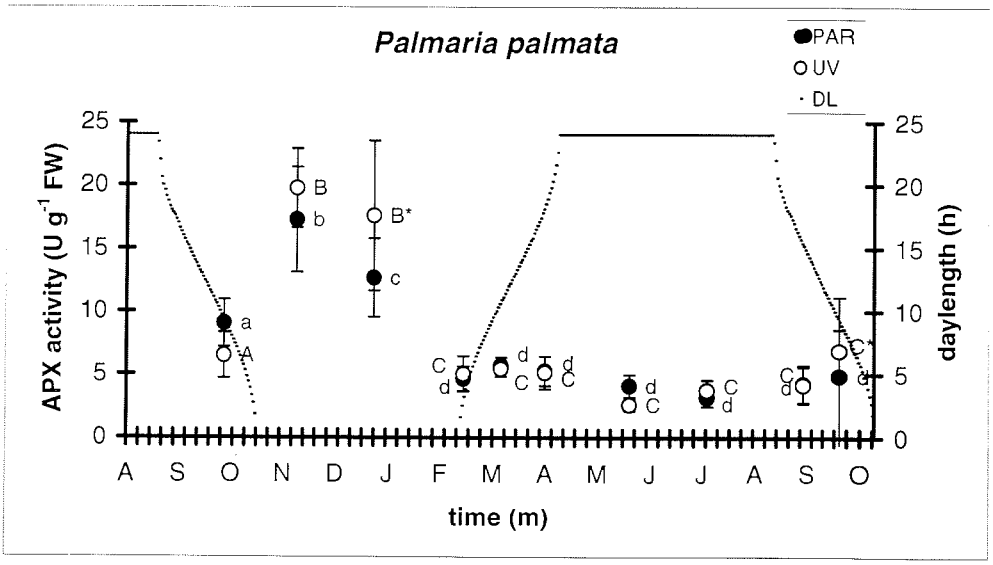

Figure 5: Enzyme activities of a) ascorbate peroxidase (APX) b) catalase (CAT) and c) superoxide dismutase (SOD) as U g FW^{-1} in the course of the year in *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels. Statistics see Fig.1

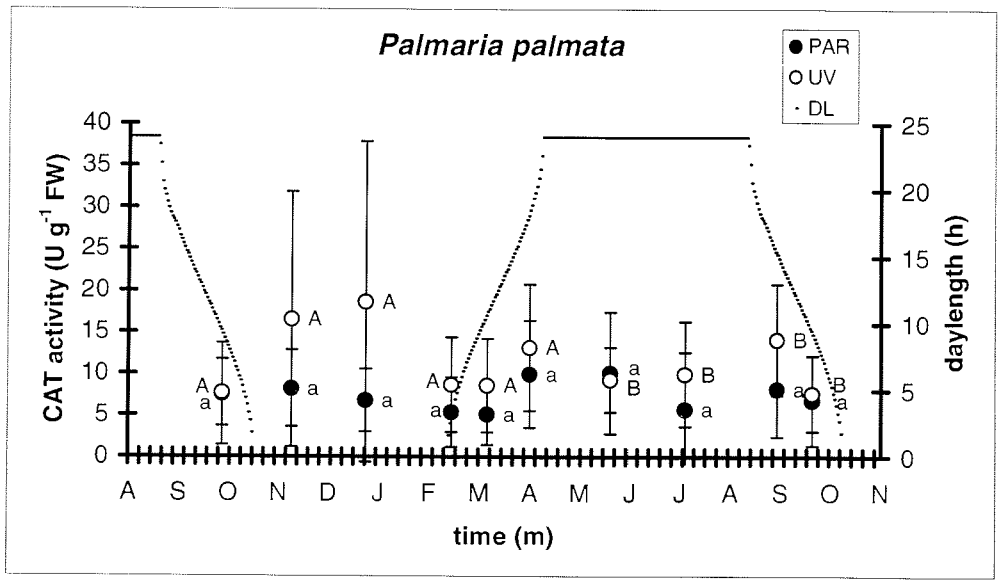

Figure 5b

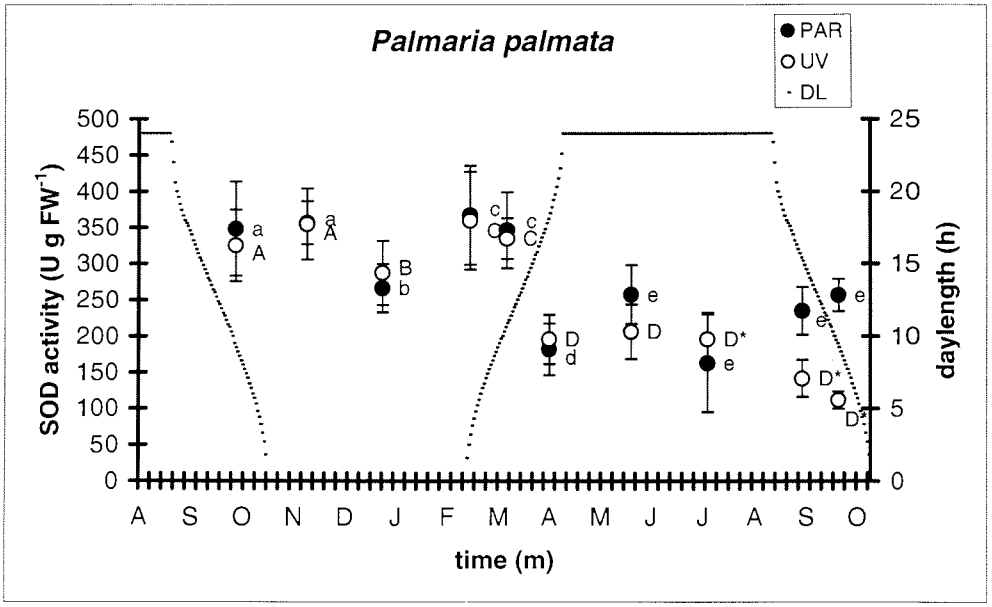

Figure 5c

Seasonal variation in MAA content

Five different MAAs were detected in *Palmaria palmata*, namely shinorine, porphyra-334 (P-334), palythine, asterina-330 and palythinol. Based on percentage of total MAA concentration the most abundant MAAs were shinorine, P-334 and palythine. Total MAA concentration in thalli exposed to UV radiation was generally higher than in the control even in the time period of no UV exposure. There was a clear seasonal pattern in MAA content. In the control, a basic level of MAA concentration of 230 µg g^{-1} DW was maintained from October to February, than rising significantly in March to 560 µg g DW^{-1} and remaining at a level of 320 to 500 µg g^{-1} DW throughout the whole summer period. In thalli exposed to UVR the basic level of 570 µg MAA g^{-1} DW measured in winter was in most cases significantly higher than the PAR control (Fig. 6). In spring after UV onset MAA concentrations strongly increased ($p< 0.0001$) leading to maximum values of 1485µg g^{-1} DW (Fig. 8). In the period of permanent PAR, MAA concentration initially decreased significantly ($p<0.0002$) in April and than remained between 370 and 984 µg g^{-1} DW in UV treated thalli until the end of the experiment, whereas values obtained under PAR amounted 320 to 494 µg g^{-1} DW (Fig. 6).

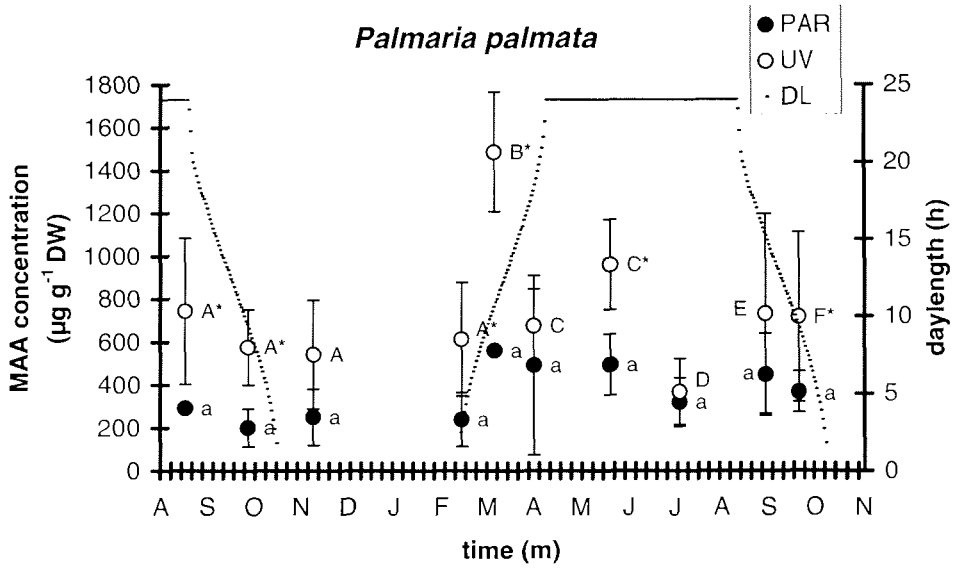

Figure 6: MAA content in the seasonal course of the year in *Palmaria palmata*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels. Statistics see Fig.1

Differences in the composition of MAAs could be observed with respect to the season and the experimental treatments. As an example we choose data from November as being representative for winter and data from end of May representing summer values. In winter nearly 50% of the total MAA content was provided by P-334 with a concentration of 125 µg g^{-1} DW whereas in UV treated thalli it was nearly 60% of the total content with 349 µg g^{-1} DW (Tab. 1). In summer similar percentages with 55% and 52 % were reached in both treatments but the concentration was much higher in the control (275 µg g^{-1} DW) as in UV-exposed thalli (513 µg g^{-1} DW). PAR levels of palythine were 81 µg g^{-1} DW in winter and 161 µg g^{-1} DW in summer. Under UV treatment they doubled to 120 µg g^{-1} DW and 393 µg g^{-1} DW in the two seasons, respectively (Tab. 1). Percentages of palythine were lower in winter with 21% in the PAR treatment and 33% in UV treated thalli, whereas in summer 33% and 40% were recorded for the two treatments, respectively (Tab. 1). Shinorine amounted 28 µg g^{-1} DW under PAR light and 46 µg g^{-1} DW under UV in winter whereas in summer 44 µg g^{-1} DW and 38 µg g^{-1}

DW were measured in the two treatments, respectively (Tab. 1). Asterina-330 and palythinol amounted less than 5% of the total MAA content.

Tab. 1: MAA contents (µg g^{-1} DW) in comparison of winter (Nov) and summer (May) as well as between PAR and PAR+UV treatment in *Palmaria palmata*. Given are mean values ± SD (n=5). n.d.= not detectable. UV samples in winter were exposed to UVR in the previous light period but under the same conditions in winter as the PAR treatment

	Winter		Summer	
MAA (µg g^{-1} DW) % of total	PAR	UV (previous season)	PAR	UV
Shinorine	27.52 ± 46.64 11.0	45.73 ± 26.42 7.8	44.27 ± 10.15 9.0	37.74 ± 9.36 3.8
Porphyra-334	125.02 ± 73.32 49.9	349.42 ± 151.12 59.8	274.58 ± 94.26 55.6	512.52 ± 51.68 52.1
Palythine	81.56 ± 36.51 32.5	119.98 ± 59.60 20.5	161.28 ± 43.30 32.6	392.57 + 128.11 39.9
Asterina-330	9.82 ± 4.71 3.9	16.62 ± 10.99 2.8	12.36 ± 3.30 2.5	24.15 ± 5.99 2.5
Palythinol	6.70 ± 10.06 2.7	9.98 ± 5.39 1.7	1.73 ± 2.81 0.3	9.34 ± 11.09 1.0
Usujirene	n.d.	42.35 ± 28.96 7.3	1.37 ± 0.61 0.3	6.85 ± 4.22 0.7
Total	250.64 ± 130.49	584.14 ± 274.60	494.22 ± 141.09	983.71 ± 184.83

DISCUSSION

Seasonal development and UV effects on growth and photosynthetic pigments

The present study demonstrates that also the seasonal development of Arctic macroalgae can be simulated in the laboratory as previously shown for macroalgae from the Antarctic (Wiencke 1990;Weykam et al. 1997; Gomez & Wiencke C. 1996) and other regions (Lüning 1993). Major events in the phenology of macroalgae such as formation of spores and induction of growth were obtained by simulating the annual cycle of daylength at Ny Ålesund, Spitsbergen, Norway. Of course, there are enormous differences between Arctic and laboratory conditions. Daylengths in the subtidal will differ from the meteorological daylengths used in this study. Moreover the influence of ice cover and turbid meltwater on the radiation regime was not simulated. The irradiances *P. palmata* was exposed to in the laboratory were much lower compared to field values. While in the Arctic summer, irradiance at the water surface of the Kongsfjord may reach 1300 µmol m^{-2} s^{-1} PAR, 19 W m^{-2} UVA and 1.1 W m^{-2} UVB (Bischof et al. 1998), we used only 25 µmol m^{-2} s^{-1} PAR, 2.67 W m^{-2} UVA and 0.23 W m^{-2} UVB. Maximum transmittance for PAR and UVB in the water column as expressed by the 1% depth ranges from 6.2 to 24.2 m and 3.4 to 9 m, respectively (Bischof et al. 1998). But typically these values are often much lower due to turbidity in the Kongsfjord (Hanelt et al. 2001). As *P. palmata* is usually found in a depth range of 2 to 10 m (Karsten et al. 2003) it may experience similar irradiances in its habitat, as applied in this study.

Among macroalgae there are two seasonal growth types, described by Kain (1989) and (Lüning & tom Dieck 1989), season responder or type I, and season anticipator corresponding to type II. Season responders grow under favourable environmental conditions showing growth peaks generally around summer solstice. Season anticipators in contrast grow in a strategic annual rhythm suitable to the species (Kain 1989), growth is not a response to suitable environmental conditions but a response to a trigger. *Palmaria palmata* may be regarded as season anticipator because the formation of new blades occurs in winter and the growth maximum is reached in spring, long before the summer solstice. In mid summer growth is reduced. In the studied and other

species daylength triggers seasonal growth and formation of gametes or spores (Dring 1984; Wiencke 1990). The influence of temperature and nutrients, which do also influence phenology of macroalgae, was not considered in the experiments described here. The growth data indicate that daylength is the trigger for the seasonal development of *P. palmata* and that nutrient concentrations and temperature have a minor influence. Growth rates are in the same range as field data on *P. palmata* from the Kongsfjord (Aguilera et al. 1999b), on young blades of this species from the British Isles (Irvine 1983) and in laboratory cultures (Sagert & Schubert 2000). Specific growth rates in winter may be underestimated by the fresh weight method applied due to high surface to volume ratios of blade initials. In the field, young plants were also observed early in the year as well as growth of older plants was reported to occur in February/March (Irvine 1983). Spore formation was observed from September to winter which is in full agreement to the field observations made by Kain (1982) and Irvine (1983) describing occurrence of tetraspores between November and spring in material from the British Isles.

Chl *a*, PE and PC concentrations measured in this study are comparable to data from a field study at same Arctic location (Aguilera et al. 2002a) and to data on temperate *P. palmata* (Sagert & Schubert 2000). The decrease in pigment concentration in summer may be explained as a normal photoacclimation process, i.e. low concentration under long day and high concentrations under short day conditions. The oscillation of phycobiliproteins within the seasons coincides with the decrease and increase in total soluble protein content. The extension of the daylength seems to be a trigger to start growth and pigment synthesis in *Palmaria palmata* as it is also documented for *Palmaria decipiens* (Lüder et al. 2001). The pigments increase number and/or size of light-harvesting antennae and reaction centres in order to trap as much light energy as possible, and thereby optimising photosynthetic efficiency and accelerating growth. Chl *a* content in *P. palmata* already increases before the start of the light period, indicating a photoperiodic control. This is also reflected by the early decrease in phycobiliproteins at the end of winter before light exposure in spring.

Growth rates were lower but not significantly different in UV treated thalli, which is consistent with growth measurements of Aguilera et al. (1999b) in the same species and other species from the Arctic Kongsfjord showing reduced growth rates under the full sunlight spectrum (PAR+UVA+UVB) in comparison to filtered solar radiation (PAR).
High doses of UVR result in a rapid decrease of thallus absorption. Moreover, significant differences in the absorption peaks of the main pigments were found in *Palmaria palmata* after exposure to elevated UVB radiation (Cordi et al. 1999). Uncoupling of the transfer of energy between PE and Chl *a* by UV is revealed first by a gradual increase of PE fluorescence, followed by a subsequent decrease in parallel with photobleaching of the pigments. A decrease of PE and PC combined with photobleaching could also be observed in the present study when exposure to UVR was accompanied by permanent PAR. There was also a decrease in PE and PC concentration in winter before exposure indicating a photoperiodic controlled down regulation of the antenna pigments. This phenomenon is also described in *P. palmata* and other Arctic macroalgae by Aguilera et al. (2002a). After a minor increase in March and April, concentrations of PE and PC as well as Chl *a* decreased strongly in summer, indicating acclimation to changing environmental conditions, which is also reflected by data from Aguilera et al. (2002a) for mid July. These authors showed that pigment concentrations are down-regulated in the period of high irradiation and high water transparency but then rise again, when turbid meltwater from melting glaciers and snow decrease transmittance of radiation (Hanelt et al. 2001). All these data indicate that PE and PC are sensitive to high radiation stress.

Ascorbic acid and antioxidative enzymes

The activity of antioxidative enzymes exhibited a marked seasonal variation. Curiously, elevated values were observed in winter, although there is no radiation stress and consequently no ROS pressure. This phenomenon may be explained by a photoperiodic control. In winter, the alga maintains a high enzymatic protection capacity against ROS, especially in those samples which were exposed to UVR during the previous season. These specimens may have achieved a certain pre-disposition, anticipating upcoming radiation stress. Such kind of phenomenon is also known for invertebrates like land snails, activating their antioxidant defences during dormance in dryness (estivation) to

be prepared for oxidative stress during arousal (Hermes-Lima. M. et al. 1998). Increased activities of antioxidant enzymes have also been observed under other kind of stress situations in which the actual production of oxyradicals should decrease e.g. anoxia exposure in snakes or freezing in frogs (Hermes-Lima. M. et al. 1998). With increasing daylength, activities of APX and CAT decreased drastically. SOD activity decreased as soon as UV exposure was added to the treatment, indicating some degree of inhibition or even degradation of the enzyme which coincides with a decrease in TSP content. This drastic decrease in enzyme activities during the summer period may be due to an increased radiative stress, as observed for SOD in *Chlorella vulgaris* after long-term exposure to UVB (Malanga & Puntarulo 1995). The reason for this negative effect may be an inhibition of gene expression for this enzyme as observed by Strid (1993) in *Pisum sativum*. A further explanation for the reduction in SOD activity may be the senescence of the thalli. This has been documented for SOD activity in tobacco leaves, when based on fresh weight but not on protein content (Dhindsa &Plumb-Dhindsa 1981). This theory is supported by the slight increase in SOD in September /October in the second year under PAR conditions coinciding with the start of sporulation in this treatment as younger tissues would therefore exhibit higher SOD activities in comparison to older tissues. Also the higher winter values could be explained partly by this theory with respect to the outgrowth of new blades. In tobacco leaves also reduction in protein content as well as CAT activity are correlated to senescence (Dhindsa &Plumb-Dhindsa 1981), which is in agreement with our data.

The contrast of our data to field observations by Aguilera et al. (2002a), who proved a clear seasonal increase in SOD activity in *P. palmata* and also in the green alga *Monostroma* sp., can be explained by the different radiation conditions. Compared to natural solar radiation, the proportion of PAR is underrepresented in our laboratory study, thus photosynthesis is probably not saturated and superoxide is hardly formed by the Mehler-reaction, which represents a major source for ROS. In another laboratory study on *P. palmata*, significant decreases in APX and SOD activities could be shown after 1 and 4 days of exposure to UVR (Aguilera et al. 2002b). In the same study discrepancies between field and laboratory studies concerning SOD and GR activities in the green alga *Acrosiphonia penicilliformis* could be shown (Aguilera et al. 2002b): Whereas artificial UVR had no effects on enzyme activity, a significant reduction was

observed when natural UVR was cut off by selective filter foils in the field (Aguilera et al. 2002b). This supports the theory that the induction of antioxidative enzymes, in addition to a certain basic level of antioxidative activity, is depending on ROS production in response to radiation conditions in the respective experimental set-ups as proposed by Mackerness et al. (1999). This shows that the processes regulating the amounts and activities of antioxidative enzymes are species specific and still unclear. A proof of the regulation mechanisms could be given in experimental designs including polyacrylamid gelelectrophoresis (PAGE) to separate the proteins followed by the identification of the enzyme by Western Blot and quantification via densitometry.

MAAs

In general, thalli exposed to UVR showed higher MAA levels as the control, as it is well described for red macroalgae under natural and artificial UVR (Hoyer et al. 2002b; Karsten et al. 1999a,b; Karsten et al. 2003). The composition of individual MAAs and quantitatively most abundant MAAs in *Palmaria palmata* agree with data from Hoyer et al. (2002b) and Karsten (1999b). MAA composition changed with the season and differed in the treatments of our study. P-334 concentrations were twice as high in the summer period in comparison to the winter and remained increased in UV-treated thalli during the winter period, whereas the percentage of P-334 of the total MAA content remained relatively unchanged. This means that in *P. palmata* P-334 as well as palythine were induced by UVR and PAR in summer as it was already documented for this species in the field by Karsten et al. (1999b) and for Antarctic red macroalgae (Hoyer et al. 2002a). The elevated MAA concentrations in the UV treated thalli in winter can be explained by a lack of degradation or a very slow degradation process which may take several weeks, at least in the red alga *Chondrus crispus* (Kraebs, personal communication). In both seasons palythine was also higher in UV treated thalli in comparison to PAR. Shinorine level remained rather unchanged in the course of the seasons. Asterina-330 increased only under UV exposure in summer indicating wavelength specific induction whereas in Hoyer et al. (2002a) also PAR led to an increase in MAA concentration in the related Antarctic red macroalgae *Palmaria decipiens*. Palythinol was not documented in Hoyer et al. (2002b) but was detectable in low concentrations in our study. Usujirene exhibited highest concentrations in winter in

UV treated thalli but was not detectable under PAR conditions. In summer, comparably low usujirene concentrations were measured, indicating qualitative changes in the MAA inventory. Average values of total MAA content in *P. palmata* is comparably lower as values found in the field, there ranging between 0.5 and 2.8 mg g^{-1} DW depending on radiation treatment, sampling depth and time within the season (Karsten et al. 1999b; Aguilera et al. 2002a). Maximum MAA concentrations of *P. palmata* with 1.5 mg g^{-1} DW in our study were in the same range as initial samples collected in the field which were already loaded-up and therefore no further induction in MAA content was possible (Hoyer et al. 2002b). MAA content in *P. palmata* also rose in the field throughout the summer reaching a plateau in August (Aguilera et al. 2002a). The higher MAA concentrations found in the field point to the importance of UV-absorbing compounds in the natural environment. The increase of MAA concentration in autumn may be a certain kind of lag phase. This phenomenon was also described by Hoyer et al. (2002b) in induction experiments on various red algae, where an additional increase in MAA content was observed although radiation treatment was already terminated. The MAA concentrations in winter remained relatively high indicating a lack of decomposition and therefore not serving as a source of energy in times of no photosynthetic activity as suggested for the phycobiliproteins in Lüder et al. (2001).

In consequence *P. palmata* exhibits an additional increase in total MAA concentration after incubation under PAR and PAR+UVR with the highest concentrations under the full spectrum providing higher protection under higher radiation conditions in spring and summer. Therefore MAAs contribute to a large extent to the maintenance of photosynthesis and growth during times of radiation stress, with their photobiological function as UV sunscreens.

CONCLUSIONS

We conclude that *Palmaria palmata* is well adapted to the radiation conditions of its natural habitat, and that it is well protected by various biochemical substances and mechanisms working in times of environmental stress such as UVR and permanent PAR during Artic summer. The alga starts to increase phycobiliprotein levels in autumn and forms new phycobilisomes, as main light-harvesting antennae. In late winter, *P. palmata* increases chl *a* levels to build up new reaction centres to optimise photosynthetic

apparatus for the coming light season. Consequently photosynthesis and pigment contents are maximal in spring when conditions are most favourable for optimum growth as reflected in nature by the most transparent water body after sea-ice break up, where sunlight can penetrate deep into the water column and before water becomes turbid due to sediment input by melting of glaciers and phytoplankton blooms. In contrast, during summer *P. palmata* reduces the photosynthetic apparatus. This is in agreement with the strong inhibition of maximum quantum yield of photosynthesis and respective maximum photosynthetic rate (ETR_{max}) in *P. palmata* due to high irradiances of PAR and UVR as stated by Hanelt et al. (1997) and Bischof et al. (2002). In consequence of reduced photosynthesis, the metabolism is slowed down concerning the antioxidative enzyme systems. Reduction in the activities of these enzymes may also be due to direct inhibition by permanent PAR or UVR. Ascorbic acid is always kept on a high level, which protects *P. palmata* against ROS, and in combination with the increased concentrations of MAAs also against UVR. *Palmaria palmata* shows a sensitivity of photosynthesis to UVR but its potential to recover is high (Bischof et al. 2002). Although *P. palmata* is known to be photoinhibited, under UVR growth was not significantly affected under the UV treatment in our study. As the differences in the integrative parameter growth between UV treated thalli and the PAR control were only minor, the protection mechanisms against direct and indirect oxidative stress are working most effectively.

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Publication VI

Seasonal variation in ecophysiological patterns in two red macroalgae from Arctic Kongsfjord (Spitsbergen, Norway): A long-term culture study with emphasis on UV protective mechanisms; II: *Devaleraea ramentacea* (L.) Guiry

DUMMERMUTH AL[1], HOYER K[2], KARSTEN U[3], WIENCKE C [1]

[1] Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12
27570 Bremerhaven
email: adummermuth@awi-bremerhaven.de

[2] Present adress:
NMR Laboratory for Physiological Chemistry
Brigham and Women's Hospital, Harvard Medical School
221 Longwood Ave., BLI 247
Boston, MA 02115 USA

[3] University of Rostock,
Institute of Aquatic Ecology,
Albert-Einstein-Str. 3
18059 Rostock, Germany

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Seasonal variation in ecophysiological patterns in two red macroalgae from the Arctic Kongsfjord (Spitsbergen, Norway): A long-term culture study with emphasis on UV protective mechanisms; II: *Devaleraea ramentacea* (L.) Guiry

- Seasonal ecophysiology of *Devaleraea ramentacea* -

Angelika Dummermuth[1*], Kirsten Hoyer [2], Ulf Karsten[3] Christian Wiencke[1]

[1]Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, D-27570 Bremerhaven, Germany

[2] present address:
NMR Laboratory for Physiological Chemistry, Brigham and Women's Hospital, Harvard Medical School, 221 Longwood Ave., BLI 247, Boston, MA 02115 USA

[3] University of Rostock, Institute of Aquatic Ecology, Albert-Einstein-Str. 3, D-18051 Rostock, Germany

* corresponding author:
Angelika Dummermuth
Alfred Wegener Institute for Polar and Marine Research
Am Handelshafen 12, D-27570 Bremerhaven, Germany
Phone: ++49-471-4831-1540
Fax: ++49-471-4831-1425
Email: adummermuth@awi-bremerhaven.de

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ABSTRACT

The influence of seasonally fluctuating Arctic daylengths and ultraviolet radiation (UVR) on growth, pigment concentrations, the antioxidative enzyme activities of ascorbate peroxidase (APX), catalase (CAT) and superoxide dismutase (SOD) as well as the contents of the antioxidant ascorbic acid and of mycosporine-like amino acids (MAAs) in *Devaleraea ramentacea* was investigated in a long-term culture study. Growth rate was optimal in April (spring), formation of tetraspores and new blades occurred from October onwards, classifying *D. ramentacea* as season anticipator. Chlorophyll a and phycobiliprotein concentrations were maximal in spring and autumn, whereas in winter and summer they were lower, indicating an effective acclimation of the photosynthetic apparatus prevailing to radiation conditions. Throughout the whole year, an unchanged ascorbic acid content was maintained under non-UV exposed samples. Even higher values were measured in thalli being exposed to UVR in the previous season, accompanied by high APX activities. This indicates an active scavenging of hydrogen peroxide by the ascorbate cycle. The antioxidative enzyme activities were highest in winter and presumably underlie a photoperiodic control and serve as protection against gigh light stress in spring. MAAs provided additional protection against UVR coinciding with the spring growth maximum. Overall *D. ramentacea* is well protected by present biochemical defence mechanisms against direct an indirect UVR stress as indicated by the fact that UV effects on growth were not evident.

INTRODUCTION

The Arctic is characterised by strong seasonal changes in environmental conditions. This study focuses on the influence of fluctuating daylengths and UVR on the integrative parameter growth, as well as on pigment concentrations and the direct and indirect UV protective systems. The latter include the antioxidative enzymes APX, CAT and SOD, ascorbic acid and UV-absorbing mycosporine-like amino acids (MAAs). The red macroalga *Devaleraea ramentacea*, which is one of the few taxa endemic to the Arctic, was chosen as the object of investigation. A similar study has been performed on the co-existing *Palmaria palmata* (Dummermuth et al. 2003a). These two species have a slightly different distribution depth at its original site of isolation (Kongsfjord, Spitsbergen): *D. ramentacea* is growing from 1 to 8 m depth and *P. palmata* from 2 to 10 m depth {Karsten, Dummermuth, et al. 2003 #2890}.

Recent studies on the seasonal variation in ecophysiological patterns in macroalgae from the Kongsfjord describe the sensitivity of photosynthesis to UVR (Bischof et al. 2002) as well as biochemical defence systems against high light stress (Aguilera et al. 2002). However, both studies deal with field samples covering only the time period from June to August. Seasonal variations in photosynthetic performance were hardly observed in *Devaleraea ramentacea* while *P. palmata* exhibited a high sensitivity to seasonal changes in radiation and also to UV-exposure. Both species are known to contain and synthesise large amounts of UV absorbing MAAs in response to natural and artificial photosynthetic active radiation (PAR) and UVR (Aguilera et al. 2002a; Karsten et al. 1999; Karsten & Wiencke 1999; Hoyer et al. 2002b). The ability to form MAAs correlated with a high capacity of *D. ramentacea* to cope with UV stress, thus preventing UV-induced inhibition of photosynthetic electron transport (Karsten et al. 1999). Both red algae are also known to contain effective scavenging systems for reactive oxygen species (Aguilera et al. 2002b). Among various red macroalgae from the Kongsfjord, *D. ramentacaea* and *P. palmata* showed highest enzyme activities of superoxide dismutase (SOD), glutathione reductase (GR), ascorbate peroxidase (APX) and catalase (CAT) related to their high vertical distribution on the shore (Aguilera et al. 2002).

In both species, the response of protective systems like antioxidative enzymes and MAAs to decreasing daylength and prolonged exposure to darkness is completely unknown. The reaction patterns of perennial algae, like *D. ramentacea*, when being

repeatedly exposed to UVR also imposes the question if these organisms do possess a kind of "memory" involved in the process of acclimation. For this reason we set up a long-term culture study with simulated Arctic daylength conditions including a summer period with artificial UV exposure. Growth measurements were performed, since these integrate positive and negative long-term effects of the experimental treatment. The different biochemical parameters measured should provide insights in the protection mechanisms of *D. ramentacea* in comparison to *P. palmata* which may explain their different sensitivity to UVR and their slightly different distribution depth {Karsten, Dummermuth, et al. 2003 #2890}. The same experimental approach was performed with *Palmaria palmata* (Dummermuth et al. 2003a).

MATERIAL AND METHODS

Tetrasporophytes of *Devaleraea ramentacea* were originally isolated from the Arctic Kongsfjord (Spitsbergen, Norway 78°55,5'N; 11°56,0'E). Algal thalli were then cultivated in the laboratory under seasonally fluctuating daylengths (Wiencke 1990a). Daylength was adjusted according to its seasonal variation at the collection site, in a second treatment radiation conditions were additionally supplemented with artificial UVR. UVR was applied for 10 h per day as soon as daylength exceeded 10 h. Irradiances of UVR were 2.67 W m^{-2} UVA and 0.23 W m^{-2} UVB. Osram daylight fluorescent lamps (L58/W12) adjusted to 25 µmol photons m^{-2} s^{-1}, in combination with Q-Panel UVA-340 fluorescent tubes (Q-panel Company, Cleveland, USA), emitting a spectrum similar to solar radiation in the UVR range between 280 and 340 nm, were used. Spectra emitted by these artificial radiation sources were measured with a Spectro 320 D spectroradiometer (Instrument Systems, Germany). All other conditions were as described in Dummermuth et al. (2003a).

Growth rates were determined by recording fresh weight of algae (n=5) after dry blotting with paper tissue as described by (Wiencke & tom Dieck 1989, see also Dummermuth et al. 2003a). Activities of antioxidative enzymes were determined by grinding samples (0.25-0.35 g FW or 0.010-0.012 g DW) in liquid nitrogen and extracting them with 1-1.5 mL 50 mM potassium phosphate buffer (pH 7.0) containing Complete™ protease inhibitor cocktail (Boehringer, Mannheim, 2 tablets in 100 mL buffer). Extracts were then centrifuged for 15 min at 15,000 r.p.m. and 4 °C.

For determination of photosynthetic pigments, samples were treated in the same way as in Dummermuth et al. (2003a). Determination of phycoerythrin (PE) and phycocyanin (PC) followed the method of Beer & Eshel (1985). Chlorophyll *a* (Chl *a*) was measured spectrophotometrically after (Inskeep &Bloom 1985). Total soluble protein (TSP) content of crude extracts was determined using a commercial Protein Assay (BioRad), based on the method described by Bradford (1976). The measurement of ascorbic acid content followed Foyer et al. (1983) as described by Dummermuth et al. (2003b). Enzyme activities were analysed in the supernatant according to Aguilera et al. (2002b) and modified for use of a microtiter plate spectrophotometer (Spectramax, Molecular Devices, Sunnyvale CA94089, USA) as described by Dummermuth et al. (2003b).
For extraction and analysis of MAAs, a 25% aqueous methanol (v/v) extraction was prepared from 10-20 mg dry weight (DW) of the algal samples. After evaporating to dryness under vacuum (Speed Vac Concentrator SVC 100H), dried extracts were re-dissolved in 100% methanol. Samples were analysed with a Waters high-performance liquid chromatography (HPLC) system according to Hoyer et al. (2002a). All total MAA concentrations are given as means of 5 replicates (±SD) expressed as concentration on a dry weight basis (mg g^{-1} DW).

Mean values and standard deviations were calculated from four to five independent replicates per treatment. Statistical significance was tested with a model 2 two-way ANOVA (Statistica) followed by a Fishers protected least significant difference test (LSD-test), (Sokal &Rohlf 1995). Significance level was at $p<0.05$.

RESULTS

Seasonal variation in growth

In *Devaleraea ramentacea* specific growth rates up to 10 % d^{-1} were observed. In the initial phase of the experiment in September growth rates exhibited around 4 % d^{-1} in both treatments (Fig. 1). In the following winter under darkness growth rates declined significantly. Later, at the end of the winter, growth rates were zero for both treatments (Fig. 1). From September onwards, release of tetraspores could be observed, which grew to 11 mm long germlings within 10 weeks. Blade initials were formed from the basal thallus part from October onwards under both treatments. With increasing daylength in spring, growth rates quickly rose significantly from zero to 3.6

% d^{-1} ($p<0.0001$) under PAR conditions and 3.3 % d^{-1} ($p<0.0001$) in UV exposed thalli and to maximal values in May (Fig. 1). When day length reached 24 h, growth rates in both treatments decreased significantly to 1.4 % d^{-1} in the PAR treatment ($p<0.0001$) and 2.6 % d^{-1} under UV conditions ($p<0.0001$). Under PAR conditions growth rates rose again to 3 % d^{-1} in July and then remained at the same level until September. In October, after UV radiation was turned off and daylength decreased below 10 h, growth rates in both treatments decreased as well, under PAR significantly to 1.8 % d^{-1} ($p<0.459$) and in the UV treated thalli to 0.9 % d^{-1} (Fig. 1).

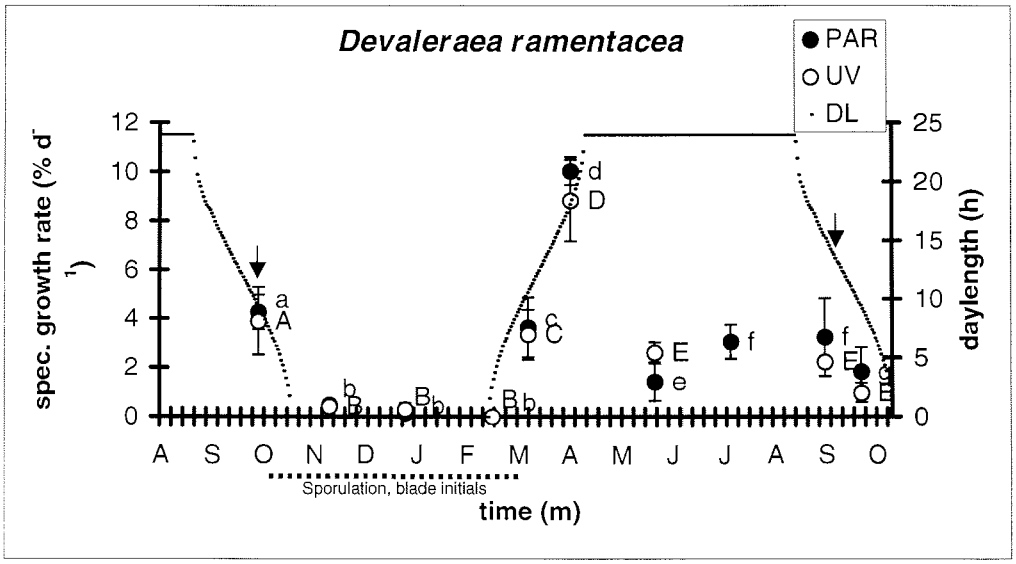

Figure 1: Specific growth rate (% d^{-1}) of *Devaleraea ramentacea* in the course of the season. Filled circles indicate PAR treatment, open circles UV treatment (10 h d^{-1}), line the day length and grey banner the time of UV exposure. Black arrow indicates start of sporulation. Mean values ±SD of 4 parallels. Statistical significance with $p<0.05$ between the treatments is indicated by *, as well as between the sampling in seasonal succession by alphabetic letters in capitals for UV treated thalli and in small letters for the PAR treated thalli.

Seasonal variation of pigments and protein content

Generally, low chlorophyll contents were measured ranging between 0.08 and 0.13 mg g^{-1} FW in November/December and 0.10 and 0.13 mg g^{-1} FW in May to July (Fig. 2a). Only in early spring as well as in late autumn higher values could be observed. In March chlorophyll content reached 0.34 mg g^{-1} FW ($p<0.012$) in the control and 0.29 mg g^{-1} FW in UV treated thalli (Fig. 2a). In October 0.25 and 0.23 mg chl *a* g^{-1}

FW were measured (Fig. 2a), but no significant differences between treatments could be observed. Chl *a* content in UV treated thalli was in general lower as in the control but differences were not statistically significant.

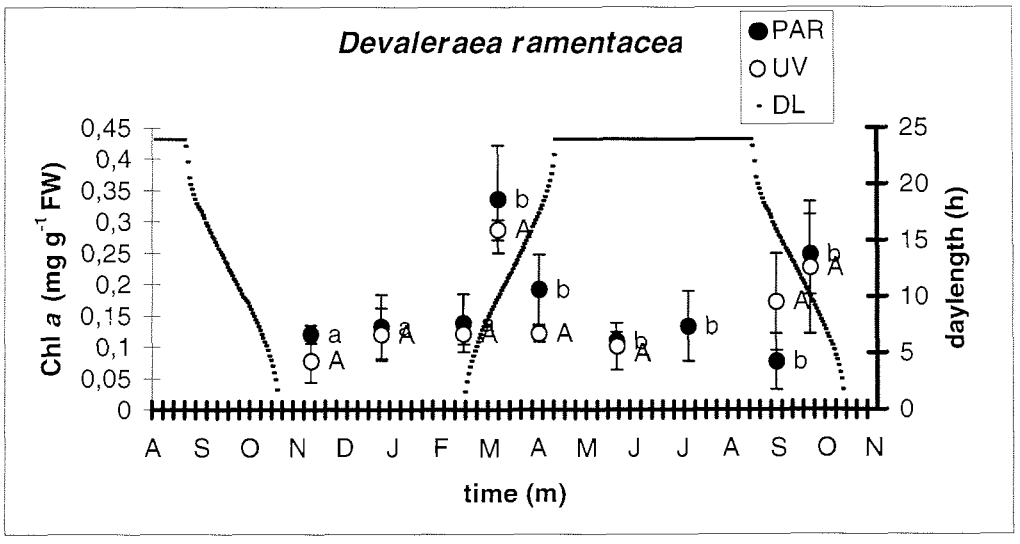

Figure 2a: Chlorophyll *a* (Chl *a*) concentration as mg g^{-1} FW in *Devaleraea ramentacea* in the course of the year. Filled circles indicate PAR treatment, open circles UV treatment (10 h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels.

Phycobiliprotein concentrations were also maximal in spring and autumn with values of 2.10 mg g^{-1} FW in February and 1.62 mg g^{-1} FW in September for PE in the PAR treatment as well as 1.1 mg g^{-1} FW and 0.90 mg g^{-1} FW for UV treated thalli (Fig. 2b). PC concentrations amounted 0.12 mg g^{-1} FW in the PAR treatment and 0.07 mg g^{-1} FW in UV treated thalli in February, as well as 0.11 mg g^{-1} FW and 0.07 mg g^{-1} FW in September for the two treatments, respectively (Fig. 2c). Low pigment concentrations in summer were accompanied with bleaching in both treatments, UV treated thalli showing slightly stronger bleaching.

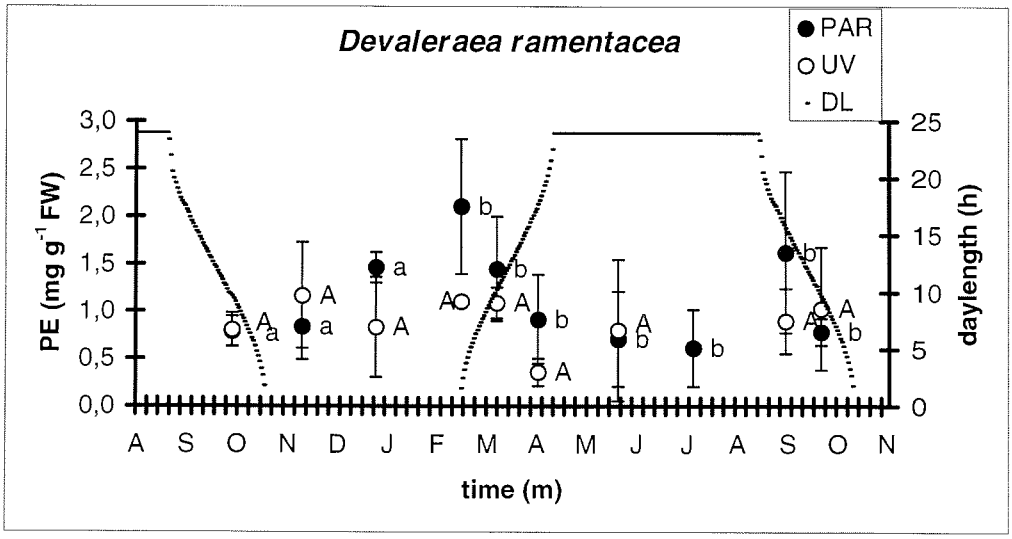

Figure 2b: Phycoerythrin (PE) concentration as mg g^{-1} FW in *Devaleraea ramentacea* in the course of the year. Filled circles indicate PAR treatment, open circles UV treatment (10 h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels.

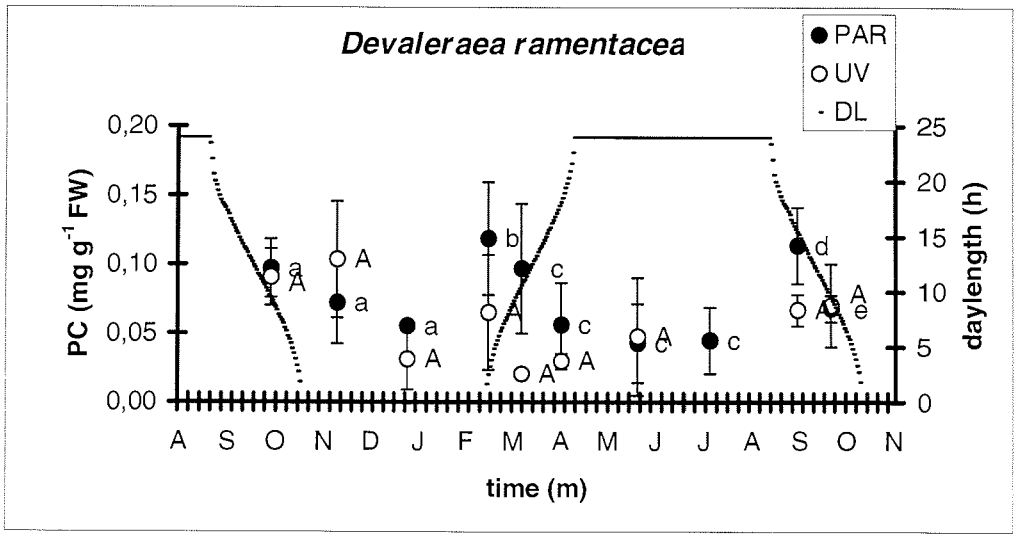

Figure 2c: Phycocyanin (PC) concentration as mg g^{-1} FW in *Devaleraea ramentacea.* in the course of the year Filled circles indicate PAR treatment, open circles UV treatment (10 h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels.

Protein content ranged between 5 and 22 mg g^{-1} FW showing a similar seasonal pattern as the phycobiliprotein concentrations. High protein contents were measured in spring with 18-22 mg g^{-1} FW and 17 mg g^{-1} FW in autumn in the control and 12-19 mg g^{-1} FW in thalli exposed to UV radiation. A significant decrease in protein content was observed from March to April ($p<0.0282$ for PAR and $p<0.0094$ for thalli exposed to UV, Tab. 1). Significant increase in protein content was measured from December to January in the control ($p<0.0441$, Fig. 3).

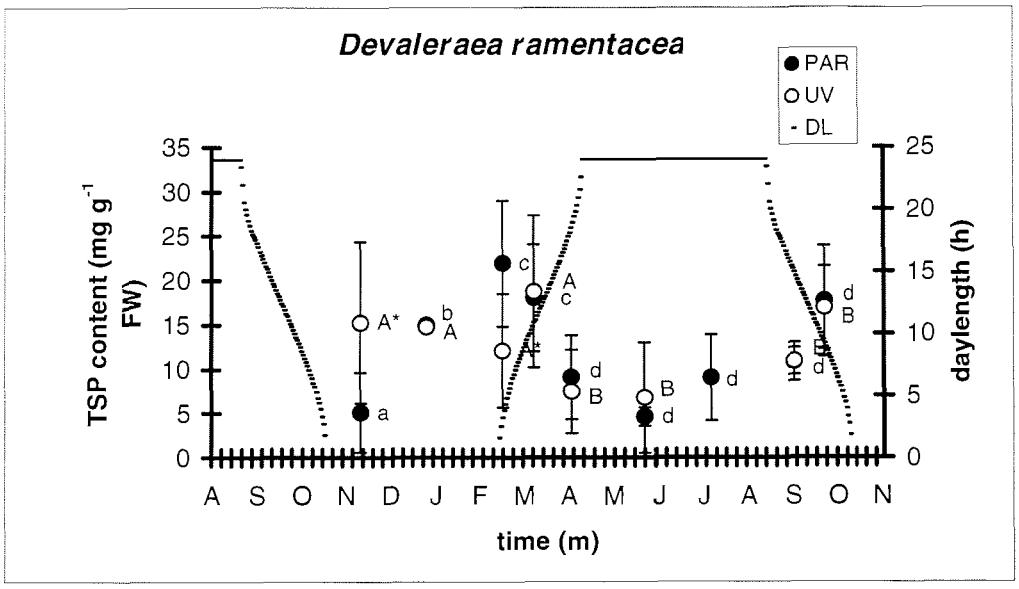

Figure 3: Content of total soluble protein (TSP) (mg g^{-1} FW) in the course of the season in *Devaleraea ramentacea*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}) and line the day length. UV exposure is indicated by a grey banner. Mean values ±SD of 5 parallels. Statistics see Fig.1

Seasonal variation in ascorbic acid and antioxidative enzymes

Ascorbic acid content was elevated in winter/darkness in samples pre-treated with UV in the previous season, differing significantly to the PAR treatment as control from October to February ($p< 0.009$, Fig. 4). High ascorbic acid contents from UV treated thalli in winter ranged from 0.56 to 0.66 mg g^{-1} FW whereas thalli under PAR conditions exhibited lower values with 0.28 to 0.3 mg g^{-1} FW (Fig. 4). In spring ascorbic acid content was reduced to 0.29 mg g^{-1} FW for the UV treated thalli and 0.26 mg g^{-1} FW for the control (Fig. 4). In late summer, when daylength already

decreased, ascorbic acid content rose again in both treatments resulting in values of 0.36 and 0.45 mg g^{-1} FW for the control in June and August, and 0.47 mg g^{-1} FW for UV treated thalli in August (Fig. 4). In September, after UV was turned off, ascorbic acid content decreased significantly to 0.31 mg g^{-1} FW ($p<0.016$) under PAR conditions and to 0.32 mg g^{-1} FW ($p<0.001$) in the UV treatment (Fig. 4).

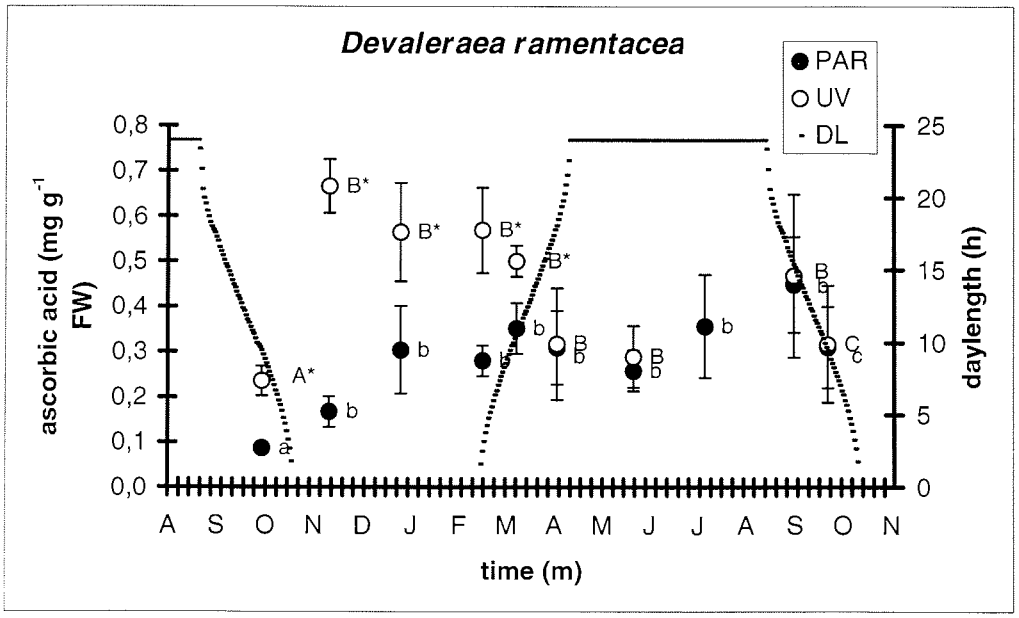

Figure 4: Ascorbic acid content (mg g FW^{-1}) in the course of the season in *Devaleraea ramentacea*. Filled circles indicate PAR treatment, open circles UV treatment (10h d^{-1}) and line the day length. UV exposure is indicated by a grey banner. Mean values ±SD of 5 parallels. Statistics see Fig.1

APX and CAT activity showed a similar pattern with highest values in winter and lowest values in summer. Maximum APX activity was observed in December with 4.2 U g^{-1} FW in the control and 3.4 U g^{-1} FW in UV pre-treated thalli (Fig. 5a). CAT activities in thalli exposed to UV were higher than the control. Especially samples pre-treated with UVR in autumn exhibited high CAT activities in the following winter (Fig. 5b). SOD activity in *D. ramentacea* showed high winter values and significantly decreased activities in summer. When daylength rose rapidly from February onwards accompanied by UVR one month later, SOD activity increased significantly to 375 U g^{-1} FW in the control and to 466 U g^{-1} FW in UV treated thalli. After polar day conditions were reached SOD activity decreased significantly to 226 U g^{-1} FW in the

control ($p<0.0028$) and to 188 U g^{-1} FW in thalli exposed additionally to UV radiation ($p<0.0001$, Fig. 5c). In autumn under declining daylength there was a slight increase in SOD activity in both treatments.

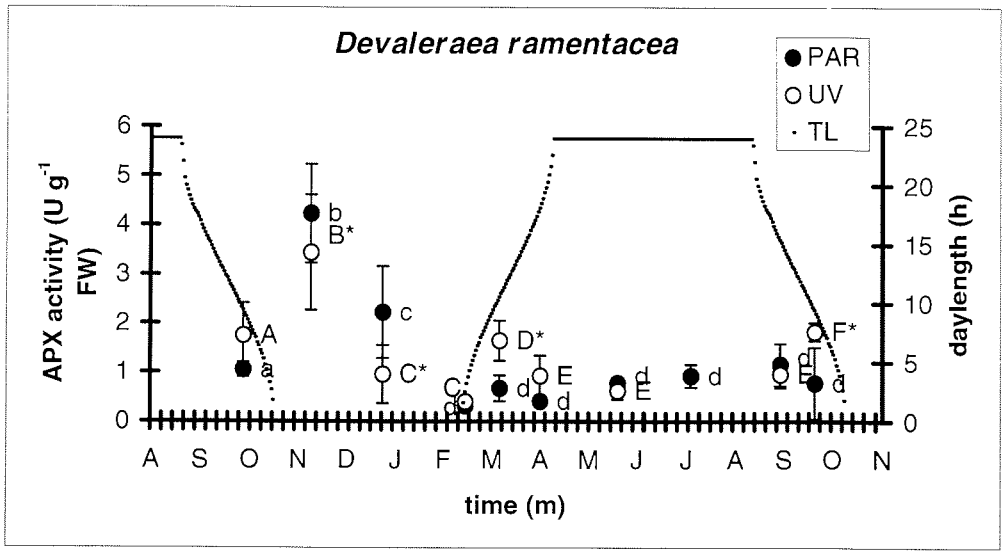

Figure 5: Enzyme activities of a) ascorbate peroxidase (APX) b) catalase (CAT) and c) superoxide dismutase (SOD) as U g FW^{-1} in the course of the year in *Devaleraea ramentacea*. Filled circles indicate PAR treatment, open circles UV treatment (10 h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels.

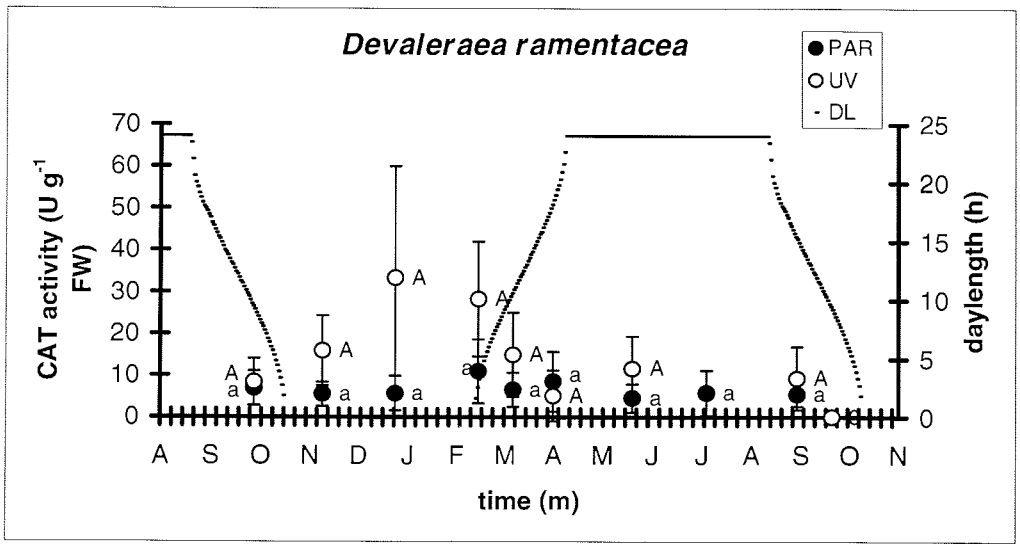

Figure 5b

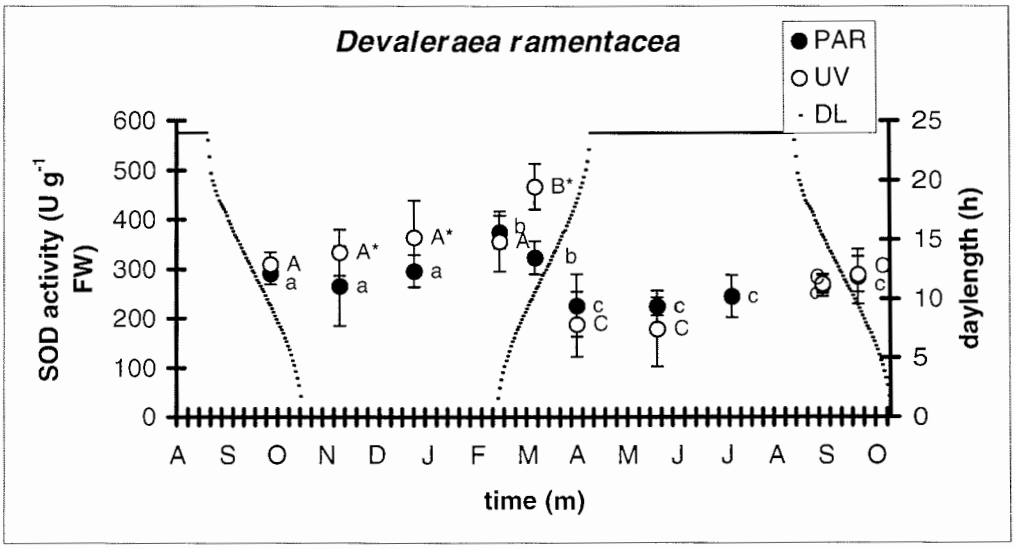

Figure 5c

Seasonal variation of MAAs

In *Devaleraea ramentacea* six different MAAs were identified, namely: Shinorine, porphyra-334, palythine, asterina-330, palythinol and usujirene. The basic level of total MAA concentration in *D. ramentacea* amounted 100 µg g^{-1} DW in winter, in spring concentrations up to 400µg g^{-1} DW were accumulated under PAR conditions (Fig. 6). Until the end of the experiment MAA concentration varied between 91 and 185 µg g^{-1} DW (Fig. 6). Thalli exposed to UV radiation exhibited significantly elevated MAA concentrations in comparison to the PAR control. Basic level in winter amounted 500 µg g^{-1} DW, whereas in spring the MAA concentration rose significantly tomaximal concentrations of 1000 µg g^{-1} DW (Fig. 6). In May, MAA concentration decreased significantly ($p<0.009$) to 637 µ g DW^{-1} and further to 288 µg g DW^{-1} (Fig. 6). In autumn, MAA levels amounted 711µg g DW^{-1} in the first season and 419 µg g^{-1} DW at the end of the experiment (Fig. 6).

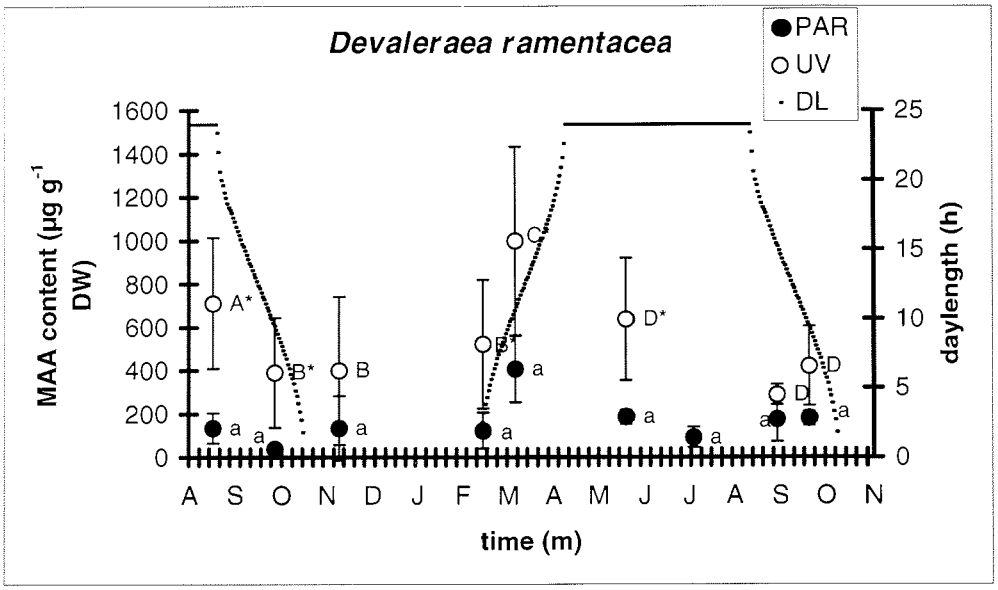

Figure 6: MAA content in the seasonal course of the year in *Devaleraea ramentacea*. Filled circles indicate PAR treatment, open circles UV treatment (10 h d^{-1}), line the day length and grey banner the time of UV exposure. Data are given as mean values ± SD of 5 parallels.

The distribution of the individual MAAs with respect to the different treatments and in the seasonal course of the year is shown for November and end of May as examples. Major MAAs were porphyra-334 (P-334) with 39-59% of the total MAA content and 31-45% for palythine, depending on the treatment and the season (Tab. 1). P-334 showed a 2.3-2.5 fold higher concentration in the UV treatment, even in winter, when algal thalli were kept in darkness. Comparing the seasons, in summer P-334 amounted 254 and 100 µg g^{-1} DW in the two treatments, whereas in winter 186 and 79 µg g^{-1} DW were measured, respectively (Tab. 1). For palythine similar results were obtained, showing a four fold increase in the UV treatments (Tab. 1). Shinorine, asterina-330 (A-330) and palythinol were always present in much lower concentrations <27 µg g^{-1} DW (Tab. 1). Shinorine could not be detected in the PAR treatment in winter, but under UVR 8.3 µg g^{-1} DW were observed in comparison to 13.8 µg g^{-1} DW and 22.8 µg g^{-1} DW in both treatments in summer (Tab. 1). Palythinol, in contrast, could not be detected in the PAR treatment in summer and showed also reduced concentration in the UV treatment in comparison to the winter values (Tab. 1). Usujirene was not detectable in winter but reached 21% of the total

MAA concentration under UV conditions in summer with 136 µg g^{-1} DW in comparison to 15 µg g^{-1} DW under PAR conditions.

Tab. 1: MAA content (µg g^{-1} DW) in comparison of winter and summer as well as PAR and PAR+ UV (10 h UV d^{-1}) treatment in *Devaleraea ramentacea*. Given are mean values + SD (n=5). UV samples in winter were exposed to UVR in the previous light period but under the same conditions in winter as the PAR treatment

	Winter		Summer	
	PAR	UV (previous season) MAA (µg g^{-1} DW) % of total	PAR	UV
Shinorine	-	8.25 ± 4.66 2.1	13.77 ± 2.93 7.4	22.80 ± 3.86 3.6
Porphyra-334	79.13 ± 95.45 59.0	186.36 ± 149.59 46.57	99.88 ± 17.61 53.7	253.80 ± 79.82 39.8
Palythine	49.17 ± 47.12 36.6	178.70 ± 164.88 44.7	57.94 ± 10.94 31.2	261.99 ± 154.25 41.1
Asterina-330	3.38 ± 3.12 2.5	10.82 + 6.91 2.7	4.64 ± 0.97 2.5	16.95 ± 8.6 2.7
Palythinol	4.03 ± 3.11 3.0	26.90 ± 28.41 6.7	-	16.91 ± 9.32 2.7
Usujirene	-	-	15.46 + 7.52 8.3	136.45 ± 123.48 21.4
Total	134.23 ± 148.05	400.21 ± 343.40	185.85 ± 32.91	637.26 ± 282.03

DISCUSSION

Seasonal development and UV effects on growth and pigments

The seasonal development of *Devaleraea ramentacea* could be simulated successfully under laboratory conditions as it was also shown in *Palmaria palmata* (Dummermuth et al. 2003a) from the Arctic and for macroalgae from the Antarctic (Weykam et al. 1997; Wiencke 1990a; b;Gomez &Wiencke C. 1996; Gomez et al. 1995; Dummermuth & Wiencke 2003) and other phytogeographic regions (Lüning 1991; 1993). In *Devaleraea ramentacea* growth was initiated in March and specimens developed new blades from October onwards, while formation of tetraspores was observed from September onwards. Formation of spores and new blades was observed until February/March, mostly in the PAR treatment and also but less under UVR. This growth pattern seemed to be based on photoperiodic control, probably due to an underlying circannual rhythm, which may be triggered by daylength as suggested for *Palmaria decipiens* (Lüder et al. 2001; Wiencke 1996) and recently for *P. palmata* (Dummermuth et al. 2003a). Photoperiodic short-day responses are also responsible for the production of tetraspores, monospores, gametes, new blades and upright thalli in many algal taxa, especially in red and brown macroalgae (Kain & Norton 1990; Lüning 1990).

Growth rates measured for *D. ramentacea* ranged 0.9-3.3 % d^{-1}, and were similar to data obtained for the same species by Bischoff & Wiencke (1993) who used a 18:6 h light:dark cycle. However comparing growth at the same daylength gave only in autumn similar results. In spring, our data were much higher with 8.8 % d^{-1}in the PAR treatment and even 10 % d^{-1}in UV exposed thalli. Growth rates also coincide with data obtained by Novaczek et al. (1990). Maximum growth rate was measured in April before permanent light and summer solstice are reached. This indicates a seasonal growth pattern as season anticipator (Kain 1989). This is further supported by the fact that pigment concentrations were maximal at the same time in order to trap as much light energy as possible, to optimise photosynthetic capacity and accelerate growth. Chl *a* concentrations of *D. ramentacea* are much lower as data reported by Aguilera et al. (2002a) in a seasonal field study at the Kongsfjord, but are in the same range as concentrations measured in *P. palmata* in the same study (Aguilera et al. 2002a). Phycobiliproteins, in contrast, showed similar concentrations

and trends compared to the latter study. The seasonal pattern of phycobiliproteins was more pronounced as in *P. palmata*.

D. ramentacea is endemic to the Arctic environment, whereas *P. palmata* mainly inhabits temperate regions. The Arctic is the northern distribution limit of the latter species and therefore the temperature optima for growth may be different in both species explaining the lower growth rate in *P. palmata* at 0 °C. For *D. ramentacea* from Disko Island growth optimum was ascertained for 5 °C by (Bischoff &Wiencke 1993), whereas the European isolates (Iceland and North Norway) rather showed growth optima at 10 °C (Novaczek et al. 1990).

The effects of UV radiation on the measured parameters were different. Specific growth rate was more or less the same in both treatments, showing no statistical difference and in consequence no UV effects. This points to well functioning protective mechanisms against radiative stress which will be discussed in more detail.

Ascorbic acid and antioxidative enzymes

Clear seasonal and UV effects could be shown for ascorbic acid content . Concentrations increased in winter and decreased in spring and summer. In contrast, ascorbic acid content was lower in the PAR control. This phenomenon may be explained by a higher turn over of ascorbic acid in winter especially in those samples being exposed to UVR in the previous autumn, which might be due to photoperiodic control, as already shown for other organisms (Hermes-Lima. M. et al. 1998). The high level of ascorbic acid in winter is regarded as a "buffer" against high oxidative stress in spring and summer. Ascorbic acid content is comparable to data provided by Aguilera et al. (2002b) on other macroalgal species from the Arctic Kongsfjord, as well as to concentrations measured in temperate *Fucus* species (Collen & Davison 1999) and in *Palmaria palmata* (Dummermuth et al. 2003a).

The seasonal variation in the antioxidative enzymes was specific for each enzyme measured.

APX activity showed elevated values in darkness (November/December) as also observed in *P. palmata* under the same experimental conditions (Dummermuth et al. 2003a), but decreased drastically when exposed to short daylengths under PAR conditions. In autumn, slightly higher APX activities were measured, thus supporting the theory of photoperiodic control, resulting in highest enzyme activities in winter.

APX activities measured in this study are in the same range as field data of *D. ramentacea* and *P. palmata* (Aguilera et al. 2002b). High levels of APX activity were accompanied by high contents of ascorbic acid similar as in *Acetabularia mediterranea* (Castillo et al. 1986). CAT activity was also elevated in winter in samples being exposed to UVR in the previous autumn. These samples exhibited similar high activities as usually found in field samples of *D. ramentacea* from the same location in summer (Aguilera et al. 2002b). Basic CAT activities in this study are slightly lower as CAT activities found in field (Aguilera et al. 2002b) but in the same range as for *P. palmata* (Dummermuth et al. 2003a). CAT activities in winter were obviously higher in *D. ramentacaea* as measured in *P. palmata*, which may reflect its higher distribution on the shore and the higher UVR the former species has to cope with.

For SOD activity we propose an additional mechanism acting together with the photoperiodic control: decreasing activity might be a consequence of radiative stress as previously observed for *Chlorella vulgaris* (Malanga &Puntarulo 1995). This is most obvious in *D. ramentacea*, in which SOD activity decreased significantly right after the onset of UVR. Values even dropped below the control value, which was not observed in *P. palmata*. SOD activity then step wisely recovered in the course of the summer until the initial activity of 300 U g^{-1} FW was reached in autumn. These results are in contrast to SOD activities measured in field samples from the Kongsfjord, in which no significant decrease or increase in SOD activity in consequence of UVR could be shown (Aguilera et al. 2002a). A possible explanation may be that the different radiation conditions applied in the laboratory are lower compared to the field. In consequence photosynthesis may not be saturated, consequently all other metabolic processes are inhibited, including the synthesis of antioxidative enzymes.

MAAs

The MAA concentrations in *D. ramentacea* showed a clear seasonal response pattern, with elevated MAA concentrations in spring, especially in thalli exposed to UVR. In general, the MAA concentrations in the UV treatment were significantly ($p<0.05$) higher as the PAR treatment as it is well known from other studies on red macroalgae under natural and artificial UVR (Hoyer et al. 2002b; Karsten et al. 1999; Karsten & Wiencke 1999; Karsten et al. 1998; 2003). MAA concentrations measured in our study are comparable to field samples from the understorey or greater sampling depths in the Kongsfjord (Hoyer et al. 2002b; Karsten et al. 1999). Karsten

et al. (1999) also discuss seasonal aspects based on higher MAA concentrations in August in comparison to samples from June, which is in accordance with our data. MAA composition varied between the seasons and the treatments in our study. The most abundant MAA was in both seasons P-334, followed by palythine. Since the percentage contribution to total MAAs changed for palythine between winter and summer, a quantitative rearrangement of individual MAAs can be assumed. This is also indicated in *P. palmata* (Dummermuth et al. 2003a). as a common feature both MAAs exhibited 2 to 3 fold higher concentrations in the UV treatment, even in winter, indicating a major induction of MAAs by UVR as it was shown in (Hoyer et al. 2002a; b; Karsten et al. 2003). Shinorine, A-330 and palythinol contributed less than 10 % of total MAAs, and exhibited higher values in UV treated thalli compared to PAR. Palythinol concentrations were higher in winter and could not be detected under PAR in summer, thus also indicting a rearrangement of MAAs in the course of the seasons. This is also the case for usujirene, which is only detectable in samples taken in summer and especially in UV exposed thalli. This indicates induction by this waveband. These results clearly classify *D. ramentacea* as a species exhibiting an additional increase in total MAA concentration after incubation under PAR and PAR+UVR, with the highest concentrations under the full spectrum. The adjustment of MAA composition seems to contribute to protection during the summer period, characterised by high irradiances of PAR and UVR.

CONCLUSIONS

In conclusion, our long-term laboratory experiment demonstrates, that *D. ramentacea* is well adapted to the seasonal changing environmental conditions in the Arctic Kongsfjord. The high tolerance to UVR stated by Bischof et al. (2002) is due to its various biochemical protection mechanisms working in seasons of increased UVR and PAR (Aguilera et al. 2002). Enzyme activities, ascorbic acid and MAA concentrations are stimulated by UVR or triggered by daylength. The synthesis of MAAs and ascorbic acid in combination with high enzyme activities imply an increased resistance to higher radiation conditions as they occur in spring and summer in the field. *Devaleraea ramentacea* exhibited a lower sensitivity against UVR and lower protection mechanisms in terms of ascorbic acid and MAAs indicating good adaptation to its habitat in low water depths in comparison to *P. palmata*. This

may be due to the fact that *D. ramentacea* is endemic to the Arctic and temperature requirements for growth are optimal in this environment whereas Spitsbergen is the northern most distribution limit of *P. palmata*.

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