ELSEVIER

Journal of Experimental Marine Biology and Ecology
289 (2003) 103–121

Journal of EXPERIMENTAL MARINE BIOLOGY AND ECOLOGY

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Responses of marine macroalgae to hydrogen-peroxide stress

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Received 11 August 2002; received in revised form 19 December 2002; accepted 19 January 2003

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.

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doi:10.1016/S0022-0981(03)00042-X

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 directly 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 Aebi (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

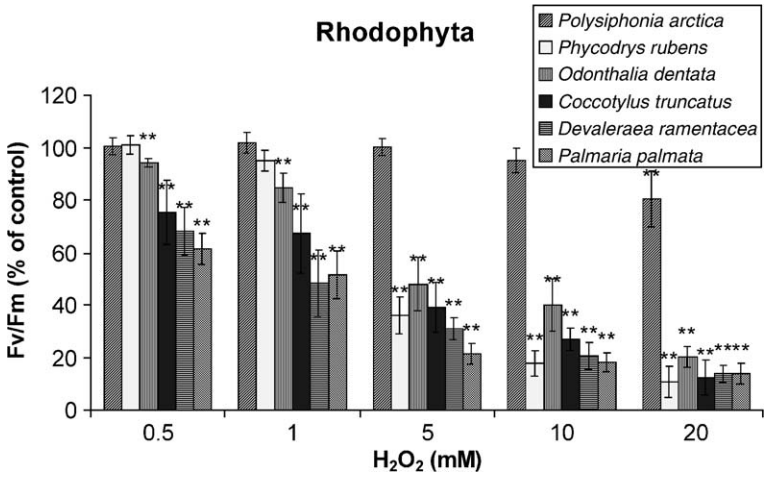


Fig. 1. The effect of increasing H_2O_2 concentrations on the maximum quantum yield of six red macroalgae from Kongsfjorden, Spitsbergen, mean values ± S.D., $n = 10$. F_v/F_m values of the control were between 0.54 and 0.57 for all the red algae. *, **: Significant differences to the control value at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

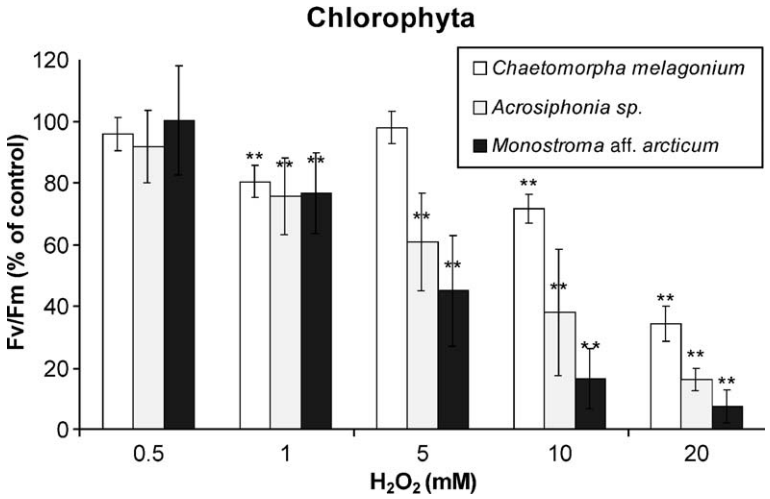


Fig. 2. The effect of increasing H_2O_2 concentrations on the maximum quantum yield of three green macroalgae from Kongsfjorden, Spitsbergen, mean values ± S.D., $n = 10$. F_v/F_m values of the control were 0.61 for *C. melagonium*, 0.71 for *Acrosiphonia* sp. and 0.49 for *M. arcticum*. *,**: Significant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

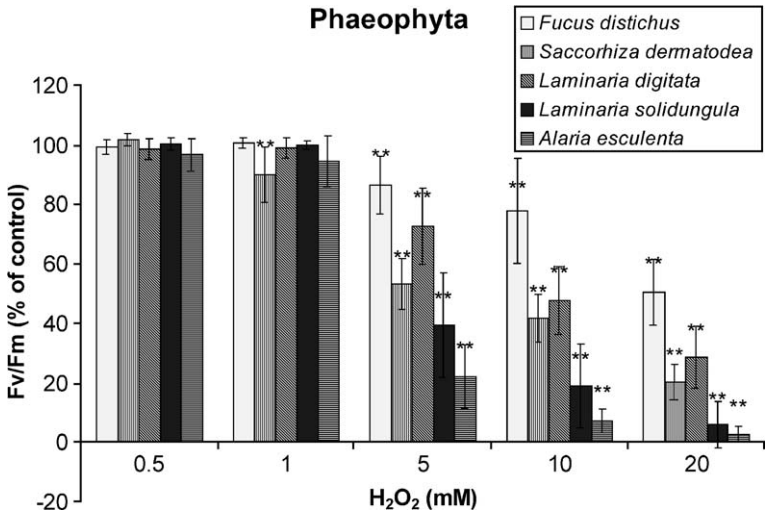


Fig. 3. The effect of increasing H_2O_2 concentrations on the maximum quantum yield of five brown macroalgae from Kongsfjorden, Spitsbergen, mean values ± S.D., $n = 10$. F_v/F_m values of the control were between 0.70 and 0.77 for all brown algae. *,**: Significant differences to the control value at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

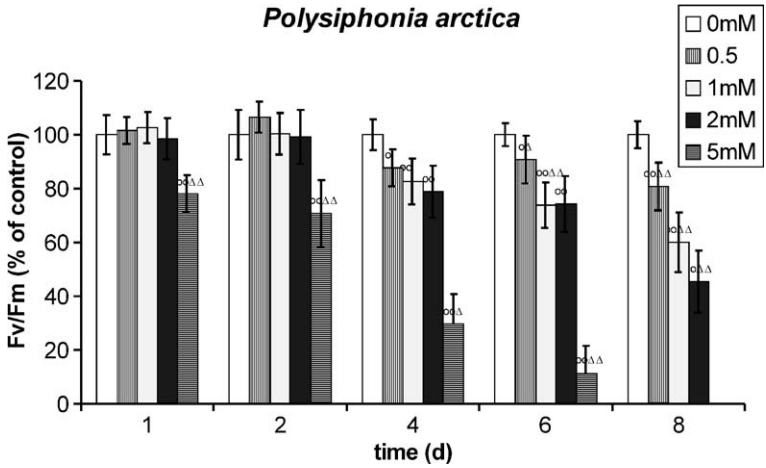


Fig. 4. F_v/F_m mean values ± S.D. (% of control) of *P. arctica* after incubation in a series of ascending hydrogen peroxide concentrations in relation to incubation time, $n=10$. F_v/F_m values of the daily controls were between 0.54 (day 8) and 0.61 (day1). ○,○○: Significant differences to the control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

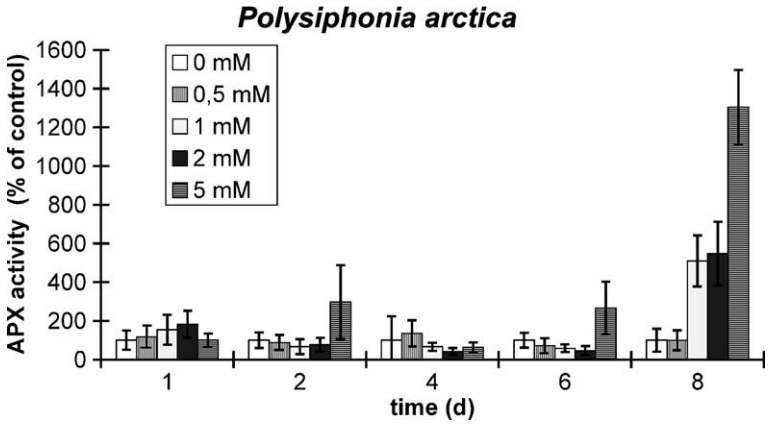


Fig. 5. Kinetic of APX activity under H_2O_2 stress in *P. arctica* (% of control, related to U mg TSP^{-1}), mean values ± S.D., $n = 5$.

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

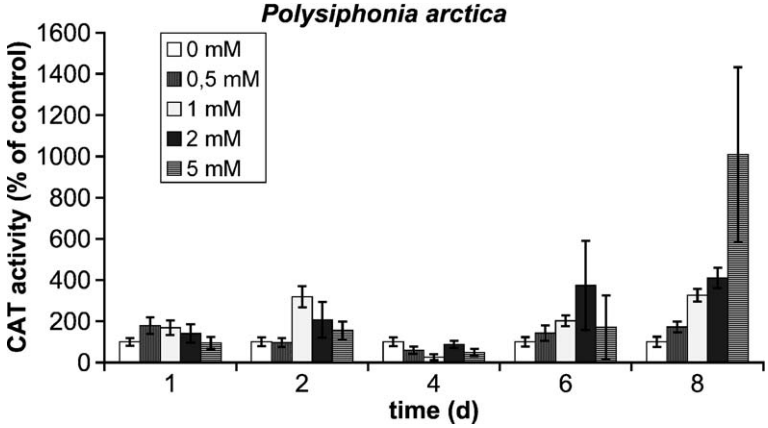


Fig. 6. Kinetic of CAT activity under H_2O_2 stress in *P. arctica* (% of control, related to U mg TSP^{-1}), mean values ± S.D., $n = 5$.

($p < 0.0$ 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

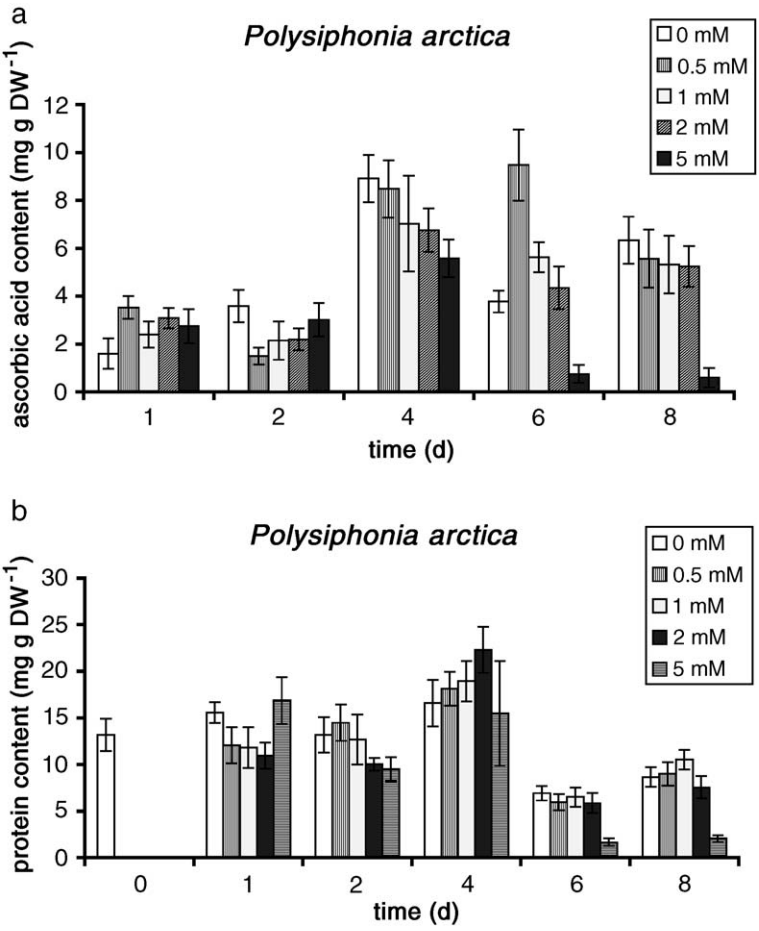


Fig. 7. (a) Ascorbic acid content (mg g DW^{-1}) and (b) protein content (mg g DW^{-1}) in *P. arctica* after incubation in a series of ascending hydrogen peroxide concentrations in relation to incubation time, $n = 5$.

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

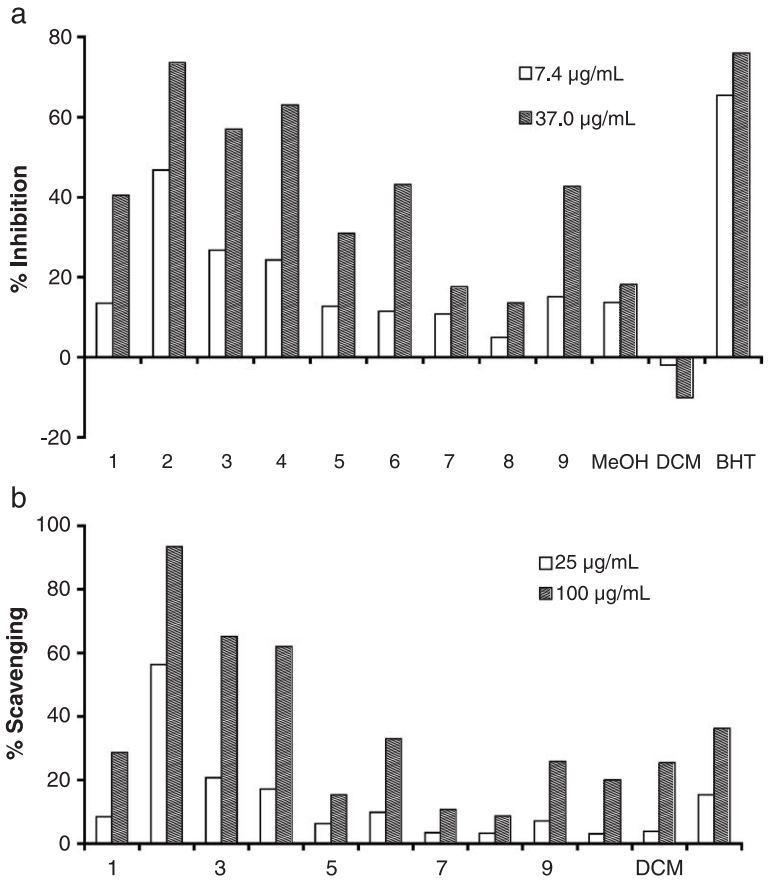


Fig. 8. (a) Inhibition of linolenic acid methyl ester oxidation by *P. arctica* extracts and acetone soluble fractions in the TBARS assay, positive control: butylated hydroxytoluene (BHT). $\%\text{Inhibition} = 100 - (A_{\text{sample}} - A_{\text{sample blank}}) \times 100/(A_{\text{control}} - A_{\text{blank}})$.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

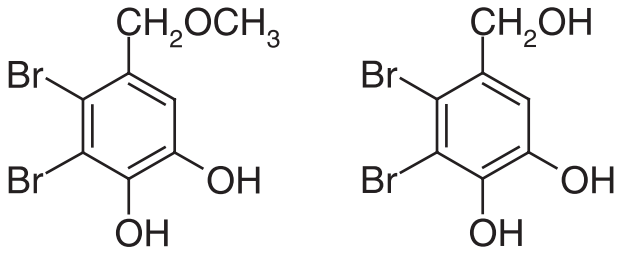
measured with 25–86% of the control. From day 6 onwards, CAT activity increased with increasing H_2O_2 concentration to a 10-fold higher value. Maximum CAT activity was found after 8 days in 5 mM H_2O_2 with 34 U mg TSP^{-1}. CAT activities differed significantly to the control, as well as the treatments 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. Schriek (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.

Acknowledgements

The authors would like to thank the diving team (Heike Lippert, Stefan Kremb, Tanja Michler and Stefanie Bröhl) for support by sampling, as well as the material from

Koldewey station and the Ny Ålesund International research monitoring facility. This project was financially supported by the German Ministry of Education and Research (BMBF; Project: "MONA"; 03FO229A). [**SS**]

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