

Acclimation of the intertidal red alga *Bangiopsis subsimplex* (Stylonematophyceae) to salinity changes

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Abstract

The effect of salinity on growth, photosynthetic performance and osmotic acclimation was investigated in the eulittoral red algal species *Bangiopsis subsimplex* (Stylonematophyceae). The strain grew in a broad salinity range between 1 and 70 psu showing optimum growth between 10 and 50 psu. The saturation point I_k of the photosynthesis irradiance curves ranged between 153 and 83 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at all salinities and indicates an adaptation of *B. subsimplex* to moderate radiation conditions. Adjustments on the photosynthetic level (non-photochemical quenching) were sufficient to prevent damage to the photosynthetic apparatus as F_v/F_m values were constantly high (>0.7) even when grown at the most hypo- and hypersaline conditions. As main low molecular weight carbohydrates, *B. subsimplex* contains the heteroside digeneaside and the polyol sorbitol. Digeneaside concentration was low and almost unchanged after hypersaline treatment ($<20 \mu\text{mol g}^{-1} \text{DW}$), i.e. it did not play a role in osmotic acclimation. By contrast, sorbitol levels increased linearly from 150 to 380 $\mu\text{mol g}^{-1} \text{DW}$ with increasing salinities between 5 and 60 psu, indicating its important function as an osmolyte and compatible solute under hypersaline conditions. The data presented are consistent with the natural habitat of *B. subsimplex*, i.e. the upper eulittoral zone.

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1. Introduction

The red algal species *Bangiopsis subsimplex* (Montagne) Schmitz is a warm-temperate to tropical taxon newly assigned to Stylonematophyceae, which is one of the ancestral clades within the red algal lineages (Yoon et al., 2006). It typically grows in the upper littoral zone on marine rocky shores in Puerto Rico (Børgesen,

1915), Guyana (Taylor, 1960), India, Pakistan and Sri Lanka (Umamaheswararao and Sreeramulu, 1964; Silva et al., 1996; West et al., 2005), Japan (Yoshida et al., 1990) and Fiji (Yoshida et al., 1990; South and Skelton, 2003). In the upper intertidal zone, this species experiences wide fluctuations in external salinity due to ebb and flow, sometimes associated with long periods of desiccation, as well as high insolation of the visible and ultraviolet range.

Although biogeographical data are available for decades, the general biology and ecology of *B. subsimplex*

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is almost unstudied. Some ecological field data of this species have been collected from the rocky shore of Visakhapatnam (India) (Umamaheswararao and Sreeramulu, 1964; Rao and Rangaiah, 1991). However in a recent investigation, West et al. (2005) provided for the first time detailed information on morphological and ultrastructural features, of the reproductive biology, phylogeny and some chemical characters. The occurrence of the sugar alcohol sorbitol and the heteroside digeneaside (α -D-mannopyranosyl-(1–2)-glycerate) as main low molecular weight carbohydrates (LMWCs) is one of their most interesting observations in *B. subsimplex*.

Most orders of the Florideophyceae typically synthesise and accumulate as main photosynthetic and reserve product the heteroside floridoside (α -D-galactopyranosyl-(1–2)-glycerol). In contrast, members of the order Ceramiales generally synthesise and accumulate instead of floridoside the chemically related digeneaside (Kremer, 1978). More recent studies, however, indicate a more complex picture on the diversity and distribution of LMWCs among the Rhodophyta. Members of the Bangiophyceae, the sister class of the Florideophyceae, such as the commercially important genus *Porphyra* contain in addition to floridoside two isomeric forms, D-isofloridoside (α -D-galactopyranosyl-(1–1)-D-glycerol) and L-isofloridoside (α -D-galactopyranosyl-(1–1)-L-glycerol) (Karsten et al., 1993; Meng and Srivastava, 1993). Other biochemical exceptions in the major carbohydrates are recorded in the Ceramiales, with taxa such as the with mangroves associated genera *Bostrychia* and *Caloglossa* which are characterised by the sugar alcohols sorbitol, dulcitol and mannitol, compounds that are otherwise very unusual in the red algae (Karsten et al., 1992a,b). Most interesting, all known LMWCs found in advanced Ceramiales are also evident in the ancestral Rhodellophyceae, a class consisting of morphologically primitive unicellular and filamentous forms (Karsten et al., 1999, 2003). These data suggest that compared to derived Rhodophyta, a higher biochemical diversity exists in the anabolic pathways for the main photosynthetic products of ancestral taxa.

Osmotic acclimation in response to salinity changes is a fundamental mechanism of salinity tolerance that conserves the stability of the intracellular milieu (homeostasis), and hence is essential for maintaining an efficient functional state in the cells (Kirst, 1990). Particularly under changing salinities, the intracellular osmotic adjustment has to follow and compensate the external fluctuations in salt concentration. Under hypersaline conditions, the accumulation of osmotically

active substances by uptake or biosynthesis can be observed, in contrast to excretion or degradation under hyposaline conditions (Kirst, 1990).

The major inorganic osmolytes in most algae include potassium, sodium and chloride (Karsten and Kirst, 1989; Mostaert et al., 1995). Cellular concentrations of these inorganic osmolytes are adjusted easily and quickly with low metabolic energy costs, especially compared to the cost of organic osmolyte biosynthesis or degradation (Kirst, 1990). However, protein and organelle function (e.g. ribosomes, mitochondria), membrane integrity and structural macromolecules in algae are adversely affected by increased ion concentration (Kirst, 1990). The biosynthesis and accumulation of organic osmolytes in the cytoplasm permits the generation of low water potentials without incurring metabolic damage (Yancey, 2005). Therefore, the organic osmolytes are termed “compatible solutes” to indicate that their presence, even at high concentrations, does not interfere with metabolic activity (Brown and Simpson, 1972; Karsten et al., 1996a).

In the red algae, the main photosynthetic product often represents also the main organic osmolyte. Floridoside compensates for hyperosmotic conditions in numerous red algae (Wiencke and Lauchli, 1981; Reed, 1990 and references therein; Karsten et al., 1993). Biochemical exceptions to this general red algal acclimation patterns have been shown. While members of the mangrove red algal genus *Bostrychia* compensate osmotic stress by the biosynthesis and accumulation of sorbitol and dulcitol (Karsten et al., 1992b), members of the genus *Caloglossa* and the unicellular alga *Dixoniella grisea* utilize mannitol as major organic osmolyte (Karsten et al., 1992a; Mostaert et al., 1995; Eggert et al., 2007).

Since the report on sorbitol and digeneaside in *B. subsimplex* was surprising (West et al., 2005), the major aim of the present study was to evaluate their physiological function in osmotic acclimation. In addition, salinity effects on growth and photosynthetic performance were investigated to evaluate the ecological success of this little known red alga in the intertidal zone.

2. Materials and methods

2.1. Algal material and culture conditions

The marine red alga *B. subsimplex* (Montagne) Schmitz was collected from the rocky upper eulittoral zone at Vuda Park, Visakhapatnam, India on 25th November 1999 by Dr. G.M.N. Rao and sent to Dr. F.

Ott, Topeka, Kansas, USA (culture no. MO1560). The latter sent the culture to the 3rd author (JAW culture no. 4283).

The stock culture of *B. subsimplex* was grown in sterile filtrated brackish Baltic Sea water (membrane filter, 0.2 μm , NL16, Schleicher and Schuell, Dassel, Germany) adjusted to a salinity of 33 psu with artificial sea salt (WIMEX, Wiegand Ltd, Krefeld, Germany) and enriched with full-strength Provasoli's nutrients (Starr and Zeikus, 1993). All experimental media were enriched with 2 mM NaHCO_3 to ensure sufficient inorganic carbon supply over the course of treatments. Cultures were maintained in 500 ml Pyrex dishes at 20–22 °C, 10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ of photosynthetic active radiation (400–700 nm) provided by daylight lamps (Osram L18W/19, München, Germany) under a light/dark cycle of 18/6 h. The algae were grown in continuous immersion culture. The medium was changed every 2 weeks.

All osmolalities used in the salinity experiments described below were prepared from Baltic Sea water (12 psu). Hypersaline media were adjusted by adding artificial sea salt (WIMEX, Wiegand Ltd, Krefeld, Germany) and hyposaline media were diluted with distilled water.

2.2. Growth measurements as a function of salinity

Relative growth rates (RGRs) of exponentially growing *B. subsimplex* were determined using an *in vivo* fluorimeter (Hansatech MFMS, Norfolk, UK) according to the technique of Karsten et al. (1996b). *Bangiopsis* cultures were grown in a full marine medium of 33 psu and in various hyposaline media (1, 5, 10, 20 psu) and hypersaline (40, 50, 60, 70 psu) media. All culture conditions and media preparation were as described above. The algae were incubated in 30 ml polyacryl (transparent, nontoxic) petri dishes (5 cm diameter, Kleinfeld, Hannover, Germany) filled with 15 ml of the experimental media. The increase of biomass, i.e. the exponential increase of the chlorophyll fluorescence signal, was followed at 1 day intervals for 14 days.

2.3. Photosynthetic measurements as a function of salinity

After treatment of *B. subsimplex* for 72 h with hypo- (5, 15 psu), hypersaline (45, 60 psu) and full marine (33 psu) media, *in vivo* chlorophyll fluorescence characteristics were measured at 20 °C with a pulse-amplitude modulated fluorometer (PAM 2000, Walz,

Effeltrich, Germany). The system is based on the principle devised by Schreiber et al. (1986). The nomenclature used in this study is according to Kromkamp and Forster (2003).

The maximum photosystem II (PSII) efficiency F_v/F_m , the effective PSII quantum efficiency $\Delta F/F_m'$ and the non-photochemical quenching (NPQ) were determined. F_0 was measured with a red measuring light pulses ($\sim 0.3 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 650 nm), and F_m and F_m' were determined with an 800 ms completely saturating white light pulse ($\sim 9200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$). The actinic light was provided by the red LED (650 nm) of the fluorometer.

After application of a 5 s far red pulse ($\sim 30 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, 735 nm) used to oxidise the electron transport chain, the algae were darkened for 5 min. Then F_v/F_m was determined. Subsequently algae were exposed to 10 different photon flux densities (PFDs) up to 370 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ and $\Delta F/F_m'$ and NPQ were determined each time after 2 min light exposure. The PFD range was wide enough to obtain light-saturated relative electron transport rates in *B. subsimplex*. The relative PSII electron transport rate (rETR) was calculated as follows:

$$\text{rETR} = \Delta F/F_m' \cdot \text{PFD} \quad (1)$$

where $\Delta F/F_m'$ = effective PSII quantum efficiency and PFD = photon flux density.

Photosynthesis-irradiance (PI) curves (as rETR vs. PFD) were determined and fitted to the equation of Webb et al. (1974):

$$\text{rETR} = \text{rETR}_{\text{max}} \cdot \left(1 - e^{\left(\frac{-\alpha \cdot \text{PFD}}{\text{rETR}_{\text{max}}}\right)}\right) \quad (2)$$

where rETR = relative electron transport rate, rETR_{max} = maximum relative electron transport rate, α = initial linear slope alpha and PFD = photon flux density.

The saturation point of the PI curve I_k was calculated by dividing rETR_{max} by the initial slope alpha (Henley, 1993).

2.4. Low molecular weight carbohydrates as a function of salinity

B. subsimplex was treated for 72 h with hypo- (5, 15 psu), hypersaline (45, 60 psu) and full marine (33 psu) media. After 72 h exposure algal samples were harvested, blotted dry using several layers of paper tissue. This material was kept under dry, cool and dark conditions prior extraction. Prior to the chemical analysis, all samples were oven-dried at 50 °C

overnight. This treatment did not affect the low molecular weight carbohydrate concentrations, and such samples could be stored for many months without any degradation when kept under dry, cool and dark conditions.

For the qualitative carbohydrate identification using ^{13}C -nuclear magnetic resonance spectroscopy (^{13}C NMR), approximately 150 mg DW of an algal sample grown at 33 psu was extracted in 5 ml of 70% ethanol (v/v) for 3 h in a water bath at 70 °C. After centrifugation at 6200×g, the supernatant was evaporated to dryness *in vacuo* and re-dissolved in 0.5 ml of D₂O (99.98%) for NMR spectroscopy. The ^{13}C NMR spectra were recorded with a Bruker AVANCE 500 spectrometer (^{13}C : 125.7 MHz; spectral width 30,000; pulse duration 11 μs; number of scans 30,000). Chemical shift values δ (in ppm) are given relative to the signal for internal TMS ($\delta=0$). The calibration of spectra was carried out using the signals of added dioxane (δ (^{13}C)=67.6). A typical ^{13}C NMR spectrum of a *B. subsimplex* extract with the signals of the carbon atoms is presented in Fig. 5(A).

Sorbitol and digeneaside were separated and quantified by high pressure liquid chromatography (HPLC). For these analyses, 15–30 mg DW of the algal samples was extracted in 3 ml of 70% ethanol (v/v) for 3 h in a water bath at 70 °C. After centrifugation for 5 min at 6200×g, 700 μl of the supernatant were evaporated to dryness *in vacuo* (Speed Vac Concentrator SVC 100H). Dried extracts were re-dissolved in 700 μl distilled water, sonicated for 5 min and vortexed for 30 s. After centrifugation at 16,000×g, the supernatants were analysed with an isocratic Agilent HPLC system equipped with a differential refractometer. Carbohydrates

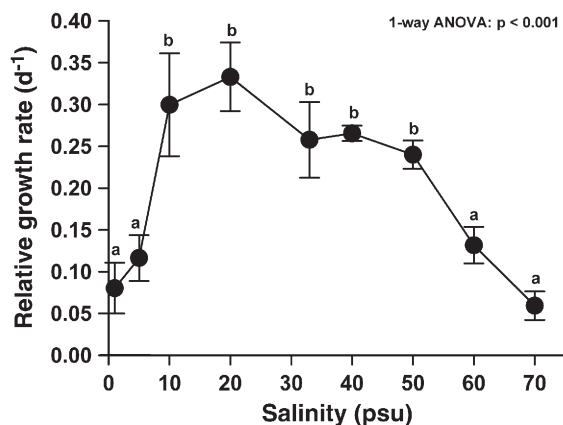


Fig. 1. The effect of salinity on relative growth rates of *Bangiopsis subsimplex*. Data represent means and standard deviations ($n=3$). Significant differences among salinities were tested by one-way ANOVA and the p -value is given. Different letters represent significant differences among salinities as revealed by Tukey's *post hoc* test.

Table 1

Effects of salinity on the maximal quantum yield of PSII (F_v/F_m), the maximal relative electron transport rate (rETR_{max}), the initial slope alpha and the light saturation point of the photosynthesis–irradiance curves (as rETR vs. PFD)

| Salinity (psu) | F_v/F_m (r.u.) | rETR _{max} (r.u.) | Alpha (μmol photons ⁻¹ m ² s) | I_k (μmol photons m ⁻² s ⁻¹) |
|----------------|------------------|----------------------------|---|---|
| 5 | 0.727±0.027 a | 126.8±9.9 c | 0.829±0.012 a | 153±14 c |
| 15 | 0.715±0.007 a | 120.0±9.5 bc | 0.851±0.019 a | 141±13 bc |
| 33 | 0.729±0.004 a | 111.5±13.0 bc | 0.869±0.034 a | 129±16 bc |
| 45 | 0.719±0.005 a | 93.1±6.5 ab | 0.831±0.028 a | 112±4 ab |
| 60 | 0.709±0.009 a | 67.4±13.7 a | 0.818±0.032 a | 83±20 a |
| | $p=0.430$ | $p<0.001$ | $p=0.215$ | $p=0.001$ |

Data represent means and standard deviations of $n=3$. Significant differences among salinities per parameter were tested by one-way ANOVA and the p -values are given. Different letters per parameter represent significant differences among salinities as revealed by Tukey's *post hoc* test.

were separated on a stainless-steel Phenomenex Rezex ROA-Organic Acid column (300×7.8 mm I.D.) protected with a Phenomenex Carbo-H⁺ guard cartridge (4×3 mm I.D.). The mobile phase was 0.5 mM H₂SO₄ that run isocratically at a flow rate of 0.4 ml min⁻¹ and a temperature of 75 °C. LMWCs were identified by comparison of retention times with those of standard compounds, and quantified by peak areas. While sorbitol was verified using a commercial standard (Sigma-Aldrich, Taufkirchen, Germany), digeneaside was isolated from the red alga *Ceramium rubrum* (Hudson) C. Agardh (Karsten et al., 2005). All concentrations are expressed as μmol g⁻¹ DW.

2.5. Data analysis

All experiments were carried out in triplicate and all data represent the mean and standard deviation of 3 replicate measurements. Salinity effects on all parameters (RGR, LMWC, PAM-fluorescence) were analysed using one-way ANOVA. The Tukey-test was used to find *a posteriori* homogeneous sub-groups of means that differed significantly.

The non-photochemical quenching NPQ and the relative electron transport rates rETR showed a parabolic relationship which was fitted to the non-linear model ($y=a+bx+cx^2$) using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA, www.graphpad.com). The coefficient

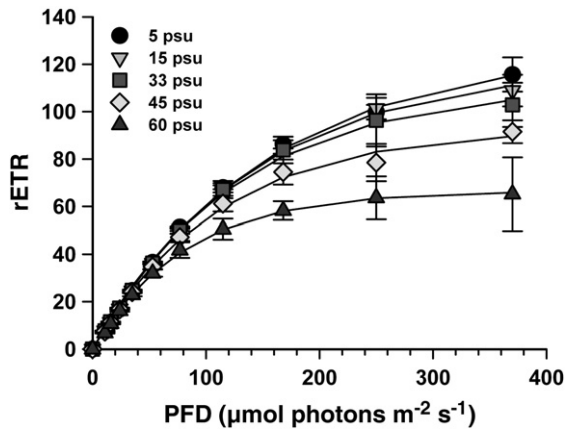


Fig. 2. The effect of salinity on photosynthesis–irradiance curves (as rETR vs. PFD) of *Bangiopsis subsimplex*. Algae were acclimated to 5, 15, 33, 45 and 60 psu for 72 h. Data represent means and standard deviations ($n=3$) and were fitted to the model of Webb et al. (1974).

of determination is given for all fits. The increase of sorbitol content as a function of salinity was additionally described using the linear regression model. The 95% confidence band of the curve was calculated, i.e. the true best-fit curve lies within that band with a probability of 95%. The coefficient of determination (r^2) and the slope of the regression model with the respective standard error are given.

3. Results

Salinity had a highly significant effect on growth of *B. subsimplex* ($p < 0.001$; Fig. 1). Growth rates were highest and statistically similar between 10 and 50 psu, reaching values between 0.24 and 0.33 day^{-1} . Treatment with salinities < 10 psu and > 50 psu led to strong reduction in growth. However, even 24% and 18% of the maximal RGR were achieved at 1 psu and 70 psu, respectively (Fig. 1).

Salinity had no effect on the maximum PSII efficiency F_v/F_m of *B. subsimplex* treated for 72 h with the different salinities ($p = 0.430$; Table 1). However, the PI curves differed clearly (Fig. 2). Even though the initial slope alpha was independent of salinity (mean values: 0.82–0.87 $\mu\text{mol photons}^{-1} \text{m}^2 \text{s}$, $p = 0.215$, Table 1), the maximum relative electron transport rates rETR_{max} decreased considerably with rising salinity ($p < 0.001$, Table 1). rETR_{max} at 60 psu was only half as much compared to 5 psu. Accordingly, also the saturation point of the PI curves I_k decreased with salinity ($p = 0.001$, Table 1). Linear electron transport of *B. subsimplex* growing at 5 psu was saturated at 153 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ compared to 83 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 60 psu.

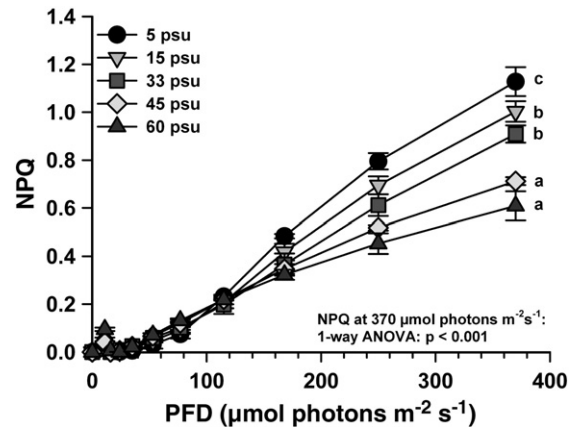


Fig. 3. The effect of salinity on the non-photochemical quenching (NPQ) as obtained from photosynthesis–irradiance curves (as rETR vs. PFD) of *Bangiopsis subsimplex*. Algae were acclimated to 5, 15, 33, 45 and 60 psu for 72 h. Data represent means and standard deviations ($n=3$). Significant differences of NPQ at the highest PFD among salinities were tested by one-way ANOVA and the p -value is given. Different letters represent significant differences among salinities as revealed by Tukey's *post hoc* test.

The non-photochemical quenching of chlorophyll fluorescence (NPQ) increased with PFD during the PI curves in all *B. subsimplex* treated for 72 h with the different salinity media (Fig. 3). Up to 100 $\mu\text{mol photons}$

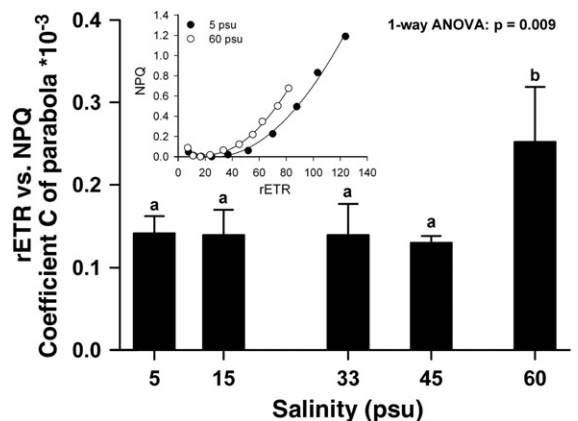


Fig. 4. The effect of salinity on a coefficient of the non-photochemical quenching (NPQ) and the respective relative electron transport rate (rETR) as obtained from photosynthesis–irradiance curves (as rETR vs. PFD) of *Bangiopsis subsimplex*. Algae were acclimated to 5, 15, 33, 45 and 60 psu for 72 h. NPQ and rETR show a parabolic relationship at all salinities (as it is shown for 5 and 60 psu in the inset). NPQ vs. rETR were fitted by a polynomial second order equation ($Y = A + B \cdot X + C \cdot X^2$, with $Y = \text{NPQ}$ and $X = \text{rETR}$). Data represent means and standard deviations ($n=3$) of the parameter C of the parabola, which is a measure of the steepness of the curve. Significant differences among salinities were tested by one-way ANOVA and the p -value is given. Different letters above the bars represent significant differences among salinities as revealed by Tukey's *post hoc* test.

$\text{m}^{-2} \text{s}^{-1}$, NPQ was low (<0.2) in all salinity treatments. Between 100 and 370 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, NPQ increased without saturation at all salinities. However, increase was steeper at low salinities compared to high salinities. Accordingly, the maximal NPQ values measured at the highest PFD (370 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) differed between the salinity treatments ($p < 0.001$). Maximal NPQ values of algae kept at 5 psu was highest with 1.1 and lowest at 45–60 psu with 0.6–0.7, respectively. Intermediate salinities had also intermediate maximal NPQ values.

Next to absolute changes of NPQ, also its relative changes with respect to electron transport rates rETR are informative. The parabolic model fits the data of NPQ vs. rETR with large coefficients of determination ($r^2 > 0.92$ for most data sets, insert of Fig. 4). NPQ increased with rETR at rates larger than an electron transport rate of 20. The parameter C of the model reflects the steepness of the curve, and was similar for all cultures of *B. subsimplex* treated for 72 h with 5, 15, 33 and 45 psu (Fig. 4). However, NPQ increased faster in *B. subsimplex* treated with 60 psu, i.e. the parameter C was almost twice as much.

The ^{13}C NMR spectrum of an extract of *B. subsimplex* clearly showed the dominant presence of the sugar

alcohol sorbitol. This polyol exhibited six characteristic resonances at 60.8, 61.2, 68.0, 69.4, 69.5 and 71.3 ppm (Fig. 5A). The presence of digeneaside could not be documented by NMR because of the relative insensitivity of the used method and because of the small amount of biomass extracted for the analysis. However, it was possible to separate and quantify sorbitol and digeneaside by using the described HPLC method, which has been developed particularly for the heteroside (Fig. 5B). Digeneaside and sorbitol showed retention times of 13.8 min and 17.9 min, respectively, well apart from each other to guarantee full separation of both compounds. Sorbitol was the quantitatively dominant carbohydrate.

After treatment of *B. subsimplex* with salinities from 5 to 60 psu, the digeneaside concentration remained on a very low level $<20 \mu\text{mol g}^{-1}$ DW. But an increase in salinity led to a significant decrease (one-way ANOVA: $p = 0.005$, Fig. 6), i.e. digeneaside contents were lowest at 60 psu, reaching only half of the concentration compared to the other salinity treatments. In contrast, sorbitol concentrations were overall 10-fold higher and salinity treatment led to a significant increase of this polyol in *B. subsimplex* (one-way ANOVA: $p < 0.001$, Figs. 6 and 5B). Sorbitol values increased linearly

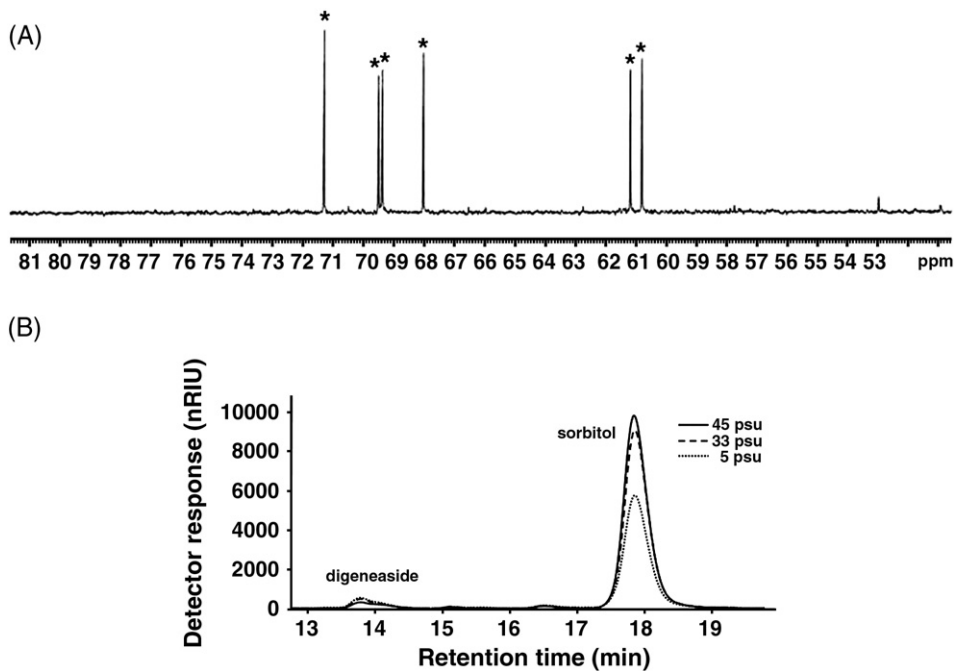


Fig. 5. (A) Representative ^{13}C NMR spectrum of a 70% ethanol extract in D_2O solution of *Bangiopsis subsimplex*, which was grown at 33 psu. Asterisks mark the six resonances of D-sorbitol. (B) HPLC chromatograms obtained from ethanolic extracts of three samples of *Bangiopsis subsimplex* treated for 72 h with 5 psu (dotted line), 33 psu (dashed line) and 45 psu (solid line). Each chromatogram shows two peaks at a retention time of 13.8 min representing digeneaside and of 17.8 min representing sorbitol. A similar biomass of 21–25 mg DW was extracted for all three samples.

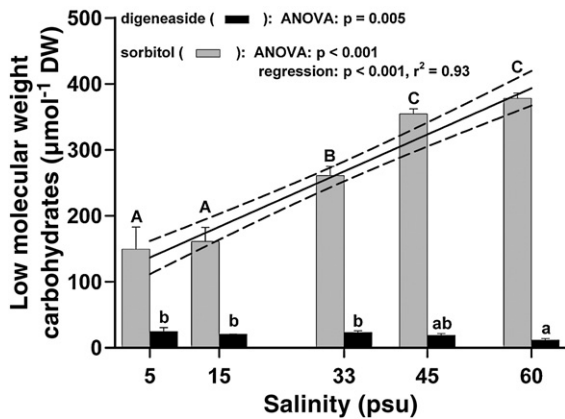


Fig. 6. The effect of salinity on the intracellular concentrations of sorbitol and digeneaside in *Bangiopsis subsimplex* after treatment for 72 h with salinities ranging from 5 to 60 psu. Data represent means and standard deviations ($n=3$). The best fit-line of the linear regression model (sorbitol vs. salinity) and the 95% confidence band are shown. Significant differences among salinities for digeneaside and sorbitol content were tested by one-way ANOVA and the p -values are given. Different letters above the bars (digeneaside: lower case letters; sorbitol: capital letters) represent significant differences among salinities as revealed by Tukey's *post hoc* test.

between 5 and 60 psu from 150 to 380 $\mu\text{mol g}^{-1}$ DW with a mean slope of $47 \pm 4 \mu\text{mol g}^{-1}$ DW per 10 psu (regression model: $p < 0.001$, $r^2 = 0.93$).

4. Discussion

B. subsimplex (Stylonematophyceae) typically inhabits the upper littoral zone on marine rocky shores in south-east Asia. It is an annual species with a growth period from October/November to February/March reaching up to 40% cover on the rocky shore of Visakhapatnam (India), the site where it was collected. Algal turfs of *B. subsimplex* coincide with periods of strongly changing submergence times between 9 and 12 h and salinities of 20–33 psu (Umamaheswararao and Sreeramulu, 1964). These ecological data already point to an ability of this alga to withstand wide fluctuations in the external salinity due to the tidal regime due to strong seasonal changes in tropical regions such as evaporation and precipitation.

The data of Umamaheswararao and Sreeramulu (1964) are well supported by the laboratory results. Under optimum temperature (20–22 °C) and low light conditions ($5\text{--}10 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), *B. subsimplex* grows in a broad salinity range between 1 and 70 psu. Growth rates at the most hypo- and hypersaline conditions were still larger than 0.05 day^{-1} , i.e. about 20% of the maximal rate measured at 20 psu. This is a

typical feature for a euryhaline alga and is a characteristic trait of benthic species inhabiting the upper littoral zone, such as *B. subsimplex* on the shore in Visakhapatnam (India), the red alga *Bostrychia simpliciuscula* in a mangrove swamp (Karsten et al., 1994b) or the green alga *Prasiola crispa* in the supralittoral of the Antarctic peninsula (Jacob et al., 1991).

Exposing algae to hypo- or hypersaline stress can affect many sites in their photosynthetic apparatus resulting in significant effects on the photosynthetic performance (Sudhir and Murthy, 2004). In the red alga *Porphyra perforata*, hyperosmotic salinities inhibited photoactivation of electron flow on the reducing side of PSI and electron flow on the oxidizing side of PSII (Satoh et al., 1983). High salinity increased carotenoid content in the cyanobacterium *Synechocystis* sp. PCC 6803, but decreased the content of phycocyanine (Schubert et al., 1993), both decreasing energy transfer to the PSII reaction centres. Hypoosmotic stress in the green alga *Dunaliella tertiolecta* and hyperosmotic stress in *Chlamydomonas reinhardtii* induced decreased PSII activity associated with state 2 transition leading to stimulated cyclic PSI activity (Gilmour et al., 1984; Endo et al., 1995). However, depending on the stress level, all these effects not necessarily lead to photoinhibition. Instead, all responses can be described as regulatory mechanisms to avoid photodamage by increasing non-radiative energy dissipation and non-assimilatory electron transport. Photoinhibition can be prevented as long as these mechanisms work effectively and the balance between energy conversion and energy consumption is kept. Thus, adverse salinity effects leading to an up-regulation of energy dissipation are not necessarily mirrored in a decreased maximum quantum efficiency of PSII (F_v/F_m).

In *B. subsimplex*, growth rates were high between 10 and 50 psu but significantly decreased at 5 and 60 psu. However, photosynthesis showed no inhibition over the entire salinity range, i.e. F_v/F_m values were consistently high. Also Lu and Zhang (1999) observed only small effects on F_v/F_m in the salt-stressed cyanobacterium *Spirulina platensis* when exposed to low light. A stress-independence of F_v/F_m has also been shown for other abiotic factors, e.g. for sub-optimal temperature effects in the green alga *Valonia utricularis* (Eggert et al., 2003a).

The independence of F_v/F_m from salinity during the 72 h treatment indicates that *B. subsimplex* can at least temporarily cope with hypo- and hypersaline stress by switching on regulatory mechanisms such as non-radiative energy dissipation. Dissipated light energy is lost for the algal metabolism and can explain discrepancies between salinity effects on growth and F_v/F_m in

B. subsimplex. Additionally, growth rates were recorded over 14 days, i.e. salinity stress lasted much longer than in the experiments for the photosynthetic performance (72 h). The maximum quantum efficiency of PSII (F_v/F_m) is widely used as an algal “health” indicator in ecophysiological studies, i.e. the resistance to environmental stress is assessed as the degree of F_v/F_m depression (UV-tolerance: e.g. van de Poll et al., 2003; Roleda et al., 2004, temperature tolerance: e.g. Eggert et al., 2003b). However, the data presented here clearly document that it is possible only to a limited extent to extrapolate effects on F_v/F_m to long-term physiological processes, such as growth and survival. A lack of correlation between the rate of photosynthetic O₂-production and F_v/F_m has already been observed under some conditions in red algae (Hanelt and Nultsch, 1995). Dring et al. (1996) reported an imperfect correlation between F_v/F_m and growth rate in mature sporophytes of three *Laminaria* species, growth rate was clearly reduced at lower UV-radiation dosages than those required for long-term reductions in F_v/F_m .

Maximal photosynthetic rates, measured as $rETR_{max}$, were similar between 5 and 33 psu, but steadily decreased at hyperosmotic conditions of 45 and 60 psu. However, $rETR$ rates are only apparent rates and for a proper calculation of absolute ETR rates the exact amount of quanta absorbed by PS II would have to be known (Bilger et al., 1995). However, this has not been determined in this study. In contrast to $rETR_{max}$ and the initial slope alpha of the PI curves, the irradiance saturation point I_k is independent of the absorption features and therefore a convenient indicator of the photoacclimation status. At PFDs < I_k , rate of photochemistry at PSII is linearly related to incident irradiance. Above I_k , this linearity breaks down as non-photochemical processes are up-regulated (Kroon et al., 1993). PSII related electron transport of *B. subsimplex* was saturated at 153 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ when grown at 5 psu. I_k decreased by almost 50% at the most hypersaline condition. Measured I_k -values were 10 times higher than the irradiance level the algae were acclimated to (10 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). Cultivation conditions were therefore below the range of PFDs to which the alga can fully acclimate. Thus, *B. subsimplex* is adapted to moderate irradiance conditions over the entire salinity range. These are comparable to field measurements of intertidal macroalgae (50–400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, Gómez et al., 2004).

Non-photochemical quenching of chlorophyll fluorescence (NPQ) mirrors processes of non-radiative energy dissipation of excessively absorbed light energy. Several mechanisms may be involved at the level of the

antenna system and the PSII reaction centres, i.e. energy dissipation by carotenoids, state-transition, spillover of excitation energy and photoinhibition-associated quenching (Krause and Weis, 1991; Horton et al., 2005). It was proposed that state transition is predominantly involved in NPQ of red algae leading to redistribution of the absorbed energy between PSII and PSI thereby avoiding imbalances in the rate of turnover of the two photosystems. For *Kappaphycus alvarezii* it has recently been shown that state-transition-like processes are involved in NPQ (Schubert et al., 2004). In contrast, Delphin et al. (1998) concluded from studies with the unicellular red alga *Rhodella violacea* that NPQ commonly associated with state 2 transition is in fact triggered by the formation of a transthylakoid ΔpH and the result of an energy-dependent type mechanism. The photoprotective role against photoinhibition of a large energy-dependent quenching has been shown for *R. violacea* (Ritz et al., 1999). According to Bruce and Vasil'ev (2004), red algae do not possess an active xanthophyll cycle and PsbS protein. However, zeaxanthin, antheraxanthin and violaxanthin and interconversions between these pigments do occur at least in some red algae (*Gracilaria birdiae*: Ursi et al., 2003; *Delesseria lancifolia*: Marquardt and Hanelt, 2004; *Gracilaria* sp.: Andersson et al., 2006). Thus, the molecular mechanisms of NPQ in red algae are still under discussion and it is still uncertain whether a truly functional xanthophyll cycle in this group of organisms exists.

Which molecular mechanism is responsible for NPQ in *B. subsimplex*, i.e. energy-dependent quenching and/or quenching due to state transition, has not been elucidated in this study. Nevertheless, as F_v/F_m was independent of the salinity treatment, no down-regulation of PSII occurred. NPQ mechanism was inactive at PFDs < 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, indicating light-saturation of photosynthetic electron transport. PFDs > 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ cause an up-regulation of energy dissipation, i.e. NPQ increased at all tested salinities with increasing PFD. Measured light-saturation values of photosynthetic electron transport I_k (83–153 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) coincide with the onset of NPQ up-regulation. These results are in accordance with the theory that NPQ based mechanisms are required at saturating irradiances (Falkowski and Raven, 1997). Similar results have been shown for *Heterocapsa pygmaea* (Kroon et al., 1993) and *Gracilaria domingensis* (Andersson et al., 2006). As NPQ did not saturate in the tested PFD range, *B. subsimplex* can cope with 370 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, i.e. this PFD is not photoinhibitory for the alga. Highest

NPQ values measured at $370 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ranged between 0.6 and 1.2 and were in the same magnitude as low light-acclimated *R. violacea* cells shifted to high light conditions (Ritz et al., 2000). However, the slope of NPQ increase was steeper at hyposaline than at hypersaline conditions, i.e. dissipation of excess energy is more strongly activated at 5 psu than at 45 and 60 psu. Thus, the 5 psu samples with the highest rETR_{max} also show the highest level of energy dissipation. This relationship remains controversial, however, photoprotection might be provided by alternative electron flow, which are both the cyclic electron flow within PSII (Miyake and Okamura, 2003) or the water–water cycle (Asada, 2004).

However, next to absolute changes in NPQ, also relative changes of NPQ with respect to rETR are informative. Küster et al. (2005) described the ratio between increasing NPQ and rETR with increasing actinic PFD as a relative measure for short-time acclimation. In *B. subsimplex*, NPQ increased relative to rETR in a parabolic manner at all salinities. The slope of the curve was very similar between 5 and 45 psu, however, was considerably higher at 60 psu. This indicates an up-regulated protection capacity for PSII only at the highest salinity tested.

B. subsimplex contains two low molecular weight carbohydrates (LMWCs), D-sorbitol and digeneaside. Using ^{13}C NMR analysis, it could be verified that D-sorbitol is the main LMWC synthesised by this species which is in agreement to the first report of West et al. (2005). Digeneaside contents of *B. subsimplex* treated with salinities from 5 to 60 psu remained always at low levels ($<20 \mu\text{mol g}^{-1} \text{DW}$). Although Kirst and Bisson (1979) reported an almost 2-fold accumulation of digeneaside in *Centroceras clavulatum* after increasing the salinity from 26 to 51 psu, these authors considered the contribution of this heteroside to the internal osmotic potential of the cells as low and therefore physiologically unimportant. In addition, the digeneaside concentration was completely unaffected after exposure to a range of salinities in *Caloglossa lepreurii* and in *Hypoglossum barbatum* (Karsten et al., 1994a, 2005). Therefore, neither published work nor the present study indicate digeneaside to play more than a minor role in osmotic acclimation of red algae. Nevertheless, a pronounced digeneaside accumulation in response to salt stress has been shown for bacteria (e.g. *Thermococcus* sp.: Lamosa et al., 1998).

In contrast to digeneaside, this is the first experimental proof for a linear increase of the intracellular sorbitol concentration as a function of salinity in an ancestral red alga. Sorbitol concentrations increased

from 150 to $380 \mu\text{mol g}^{-1} \text{DW}$ between 5 and 60 psu. Therefore it is reasonable to assume that this polyol plays an important role in osmotic acclimation in *B. subsimplex*. At hyposaline conditions sorbitol is degraded or transferred into osmotically inactive storage compounds. At hypersaline conditions, it acts as an osmolyte and as a compatible solute, i.e. protecting enzymes under hyperosmotic stress (Karsten et al., 1996a). However, despite sorbitol accumulation, growth rates decreased considerably in *B. subsimplex* at 60 psu. Biosynthesis and accumulation of organic osmolytes are energy requiring processes which cause a drain on metabolites needed for cell growth.

In conclusion, the data presented characterise *B. subsimplex* as a euryhaline organism which is well adapted to a broad salinity range between 1 and 70 psu. This broad salinity tolerance is physiologically supported by the ability to synthesise and accumulate the sugar alcohol sorbitol under hypersaline conditions as organic osmolyte and compatible solute. Adjustments on the photosynthetic level are sufficient to prevent damage to the photosynthetic apparatus as F_v/F_m values were constantly high when grown at hypo- and hypersaline conditions. Adjustments include up-regulation of absolute NPQ at low salinities and up-regulation of relative NPQ high salinities. The results support the ecological success of *B. subsimplex* in the upper eulittoral zone in south-east Asia.

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