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The freshwater red alga *Batrachospermum turfosum* (Florideophyceae) can acclimate to a wide range of light and temperature conditions

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Abstract

Batrachospermum turfosum Bory is one of the generalists among the few red algae that have adapted to freshwater habitats, occurring in a variety of primarily shaded, nutrient-poor microhabitats with lotic (running) or lentic (standing) waters. Seasonal variations in water level and canopy cover can expose this sessile alga to widely fluctuating temperatures, solar irradiation and nutrient availability. Here we report on the ecophysiology of *B. turfosum* collected from an ultra-oligotrophic bog pool in the Austrian Alps. Photosynthesis as a function of photon fluence density (PFD) and temperature was studied by measuring oxygen evolution in combination with chlorophyll fluorescence. In addition, the effects of ultraviolet radiation (UVR) on photosynthetic pigments were analysed using HPLC and spectrophotometric methods, and cellular ultrastructure was studied using transmission electron microscopy. We found that *B. turfosum* is adapted to low light, with a light compensation point (I_c) and a light saturation point (I_k) of 8.4 and 29.7 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, respectively, but also tolerates higher PFDs of $\sim 1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, and is capable of net photosynthesis at temperatures between 5°C and 35°C. Exposure to either UV-A or UV-AB for 102 h led to a strong transient drop in effective quantum yield (F/F_M'), followed by an acclimation to about 70% of initial F/F_M' values. Ultrastructural changes included the accumulation of plastoglobules and dilated membranes after UVR treatment. Although all photosynthetic pigments strongly decreased upon UVR exposure and no UV-photoprotectants (e.g. mycosporine-like amino acids) could be detected, the alga was capable of recovering F/F_M' and phycobiliproteins after UVR treatment. In summary, *B. turfosum* tolerates a wide range of irradiation and temperature regimes, and these traits may be the basis for its successful adaptation to challenging environments.

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Author contributions

S. Aigner: algal collection, designing and conducting experiments and manuscript writing; A. Holzinger: performing transmission electron microscopy, discussing and editing manuscript; U. Karsten and I. Kranner: suggestions and advises on experimental and statistical tasks and editing manuscript.

Keywords

dynamic photoinhibition; photoacclimation; photosynthesis; phycobiliproteins; respiration; Rhodophyta; ultrastructure; ultraviolet radiation

Introduction

The majority of red algae (Rhodophyta) are marine organisms, and only about 3% (~180 species) occur in freshwater habitats (Guiry, 2012). Most of these freshwater red algae are found in light- and nutrient-poor running waters and accordingly, their ecophysiological adaptations differ from those of most marine red algae. However, freshwater Rhodophyta are important constituents in river ecosystems, where they can be abundant and widely distributed (Necchi, 2016). Three freshwater red algae, *Compsopogon caeruleus*, *Kumanoa mahlacensis*, and *Batrachospermum turfosum* (Sheath & Vis, 2015), are typical inhabitants of lentic freshwater bodies, such as lakes and ponds. There are also other species with a widespread distribution e.g., *Bostrychia moritziana* and *Chroodactylon ornatum* (Sheath & Vis, 2015), but these are marine invaders inhabiting estuaries and brackish waters, i.e., are not strictly limited to freshwater.

The broad latitudinal and altitudinal distribution of *B. turfosum* is unusual for freshwater red algae, which are usually restricted to elevations below 900 m above sea level (Israelson, 1942; Sheath & Vis, 2015). In North America *B. turfosum* occurs from Alaska to central Mexico (Sheath *et al.*, 1994; Müller *et al.*, 1997) and in Europe is abundant in Finland and Sweden, but rare in central Europe (Eloranta & Kwadrans, 2007). *Batrachospermum turfosum* occurs in mountainous to subalpine microhabitats characterized by dark-coloured, oligotrophic (i.e., nutrient-poor) humic waters (Parker *et al.*, 1973; Kumano, 2002), often attached to solid substrata like stones or wooden branches. In such conditions algae can form colourless hair cells hosting phosphatase enzymes that mediate the uptake of phosphate from organic P-compounds (Gibson & Whitton, 1987), inhibited in the presence of Fe(III). These microhabitats can exhibit significant seasonal fluctuations in temperature and water levels, and light penetration through canopy gaps (Canham *et al.*, 1990). In shallow-water ponds inhabited by *B. turfosum* temperatures can fluctuate from 34°C in the summer (Gregor *et al.*, 2003) to around freezing point in the winter (Müller *et al.*, 1997), and elevated photosynthetic active radiation (PAR) and ultraviolet radiation (UVR) are additional stress factors at high altitudes (Blumthaler *et al.*, 1997).

Numerous studies on photosynthesis of freshwater red algae showed that they are typically adapted to shaded environments, with a low light compensation point (I_c) and light saturation point (I_k) in combination with high photosynthetic efficiency (α) in the light-limiting range (Karsten *et al.*, 1993; Leukart & Hanelt, 1995), and a capability of down-regulating photosynthesis by dynamic photoinhibition (Necchi, 2005; Drerup *et al.*, 2015). Parker *et al.* (1973) described bleaching and disintegration of the thalli after transplantation, and suggested that *B. turfosum* (formerly *B. vagum*) is sensitive to bright light, although related species (e.g., *B. gelatinosum*) can tolerate a wide range of irradiance (Rider & Wagner, 1972). More recent studies on photoacclimation in freshwater red algae showed that

they can adjust components of their photosynthetic apparatus and can decrease chlorophyll *a* contents after high light exposure (Bautista & Necchi, 2007). Ultraviolet radiation can cause damage to nucleic acids, proteins and pigments (Karsten, 2008 and references therein) and can directly damage photosystem II (PSII) by photoinactivation of the oxygen-evolving complex or the D1 protein (Tyystjarvi, 2008; Vass, 2012). In contrast, photosystem I seems to be less affected by UVR absorption, but can suffer oxidative damage through reactive oxygen species (ROS) when photoprotective mechanisms fail (Vass, 2012).

The Rhodophyta are highly diverse in their photoprotective mechanisms, and their capabilities for effective high energy quenching (q_E) of overexcited photosystems are somewhat enigmatic (Bruce & Vasil'ev, 2004), because typical key q_E components are missing (PsbS proteins) or uncommon (xanthophyll cycle). Carotenoid profiles of Rhodophyta have been classified into three groups, with predominantly zeaxanthin, lutein or xanthophyll cycle-related pigments (Schubert *et al.*, 2006). *Batrachospermum* sp. belongs to the second group (Schagerl & Donabaum, 2009). Photoprotective responses to enhanced UVR have been extensively investigated in marine red macroalgae (Bischof *et al.*, 2006), identifying an array of UV-absorbing compounds such as mycosporine-like amino acids (MAAs; Karsten & West, 2000; Hoyer *et al.*, 2001), phenolic compounds (Blunt *et al.*, 2015) and carotenoids (Schubert *et al.*, 2006), but there is little data for freshwater red algae. The phycobilisomes (PBS) in the antenna of red algae contain phycobiliproteins (PBPs), which efficiently absorb PAR and transfer excitons to chlorophyll *a* in PSII. Kaczmarczyk & Sheath (1991) showed that antenna pigments in *Sheathia boryana* (formerly *B. boryanum*) were altered in response to light quality, although without changes in photosynthetic rates. Ultraviolet radiation triggers the dissociation of PBS from the thylakoid membrane (Lao & Glazer, 1996) and further disassembly and breakage of the anchor linker (ApcE; Six *et al.*, 2007), leading to a partially uncoupled energy transfer (Sah *et al.*, 1998).

In summary, hardly any data exist on the photoprotective mechanisms against UVR damage in freshwater red algae, and even less is known about the ecophysiology of the generalist *B. turfosum*. Here, we provide baseline data for light and temperature requirements of photosynthesis and respiration, assessed by oxygen evolution together with measurements of the optimum quantum yield (F_V/F_M), and for photoprotection against UVR by studying the kinetics of quantum efficiency, pigment composition and changes in ultrastructure in response to either UV-A or UV-AB exposure.

Materials and methods

Algal material and environmental parameters

Thalli of *Batrachospermum turfosum* Bory attached to wooden branches were collected from a humic bog pool (Fig. S1) in Austria and identified after Knappe & Huth (2014). The algal material was transported in light-protected boxes to the lab, cleaned of peat particles with MilliQ water (Barnstead, Thermo Fisher Scientific, Waltham, USA), and acclimated at 10–15 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at 6 to 10°C for 4 to 8 days before experimental treatments. Parameters of solar radiation were recorded in the shade and in the light on a sunny, cloudless day on July 18, 2014 (13:00, CET + 1) with a PMA 2100 radiometer (Solar Light, Glenside, USA) equipped with PMA 2132, PMA 2110 and PMA 2106 sensors for PAR, UV-

A and UV-B, respectively. Bog water was sampled for nutrient analysis (Spectroquant test kits, Merck, Darmstadt, Germany), electrical conductivity and pH (WTW, Cond 330i, Weilheim, Germany). Phenol contents were calculated as gallic acid equivalents (GAE) and measured according to Everette *et al.* (2010).

Light and temperature requirements for photosynthesis and respiration

Respiratory oxygen consumption in the dark and photosynthetic oxygen production rates under increasing photon fluence density (PFD; PI curve) were measured with a Fibox 3 oxygen optode (Presens, Regensburg, Germany) at 20°C using a 3 ml thermostatic acrylic chamber (DW1, Hansatech, Norfolk, UK) after Remias *et al.* (2010). Algal filaments were suspended in 2.5% Bold's Basal Medium (BBM), enriched with 0.2 ml NaHCO₃ solution (75 mM; Merck, Darmstadt, Germany) to produce a 3 ml suspension with a final inorganic carbon concentration of about 2 mM. Photon fluence densities (PFDs) from 0 to 1050 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR emitted from a halogen light source were calibrated inside the chamber using a radiometer (QRT1 sensor, Hansatech, Norfolk, UK) and were controlled with combinations of neutral density filters (10%, 25%, 50%, 80%; Nr. 980214, Hansatech, Norfolk, UK).

Photosynthetic oxygen evolution rates at temperatures from 5 to 50°C (in increments of 5°C) were measured with the above-mentioned equipment, by irradiating the algal material with 100 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR using a temperature-controlled water bath (K20/DC 10, Thermo Haake, Karlsruhe, Germany). Oxygen evolution rates were measured for 10 min each in the light, and in the dark after a 20 min pre-acclimation phase for each temperature. F_V/F_M was recorded using a pulse-amplitude modulated (PAM) fluorimeter (PAM 2500, Heinz Walz, Effeltrich, Germany) and a KS-2500 cuvette (Heinz Walz). F_V/F_M measurements were conducted after Schubert *et al.* (2011) with minor modifications, in the same temperature range as oxygen evolution rates using red LED as actinic light ($E_{m_{\max}} = 630 \text{ nm}$) and far red LED ($E_{m_{\max}} = 750 \text{ nm}$) to ensure full oxidation of the electron-transport chain (state 1). Thalli were dark-acclimated for 30 min, followed by a 5-s far-red pulse and another dark incubation for 5 min, and then F_V/F_M was measured with saturating pulses (SP) of $\sim 1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. Oxygen evolution for each PFD increment and time interval was normalized to the concentration of total chlorophyll *a* and dry weight (DW). For this, algal material was filtered after each measurement onto GF/C glass fibre filters (Whatman, Dassel, Germany), immediately frozen in liquid nitrogen, freeze-dried (Lyovac GT2, Leybold, Hanau, Germany), weighed and analysed by high performance liquid chromatography (HPLC; see chromatographic analysis).

Light, confocal laser scanning and transmission electron microscopy

Light and confocal laser scanning microscopy (CLSM) were conducted with a Zeiss Axiovert 200 M microscope (Carl Zeiss AG, Jena, Germany), either in bright field mode or by differential interference contrast (DIC). Images were captured by a Zeiss AxioCam MRc5 camera operated by Zeiss Axiovision (release V 4.7.1.0) software. *Batrachospermum turfosum* cells were excited by an argon laser beam (488 nm) and emission was collected with a long pass filter (LP 560 nm; false coloured red). Z-stack projections were generated, with optimized optical slice distances, covering approximately half of the width of an

individual whorl cell. For transmission electron microscopy (TEM) thalli were chemically fixed as previously described by Holzinger *et al.* (2011) with minor modifications. Briefly, samples were fixed in 1.25% glutaraldehyde in 25 mM sodium cacodylate buffer (pH 6.8) for 1 h, post-fixed with 1% OsO₄ at 4°C for 24 h, rinsed and dehydrated in increasing ethanol concentrations and propylene oxide, and embedded using Spurr's low viscosity embedding kit (Science Services, Munich, Germany). Ultrathin sections were prepared with a Reichert Ultra-microtome (Leica, Wetzlar, Germany), counterstained with 2% uranyl acetate and Reynold's lead citrate, and investigated at a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss AG, Oberkochen, Germany) at 80 kV. Images were taken with a Proscan 2 k SSCCD camera (Proscan Electronic Systems, Lagerlechfeld, Germany) and processed with Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, CA, USA).

Irradiation and quantum efficiency of PSII

For monitoring effective quantum yield F/F_M' before and after UV-exposure, thalli were wetted in 2.5% BBM (pH 5.5) and placed on GF/C glass fibre filters (13 mm; Pall corporation, Dreieich, Germany), followed by mild vacuum filtration (> 99 mPa) for 2 s and rehydration with one drop of BBM. F/F_M' was measured using a red LED as actinic light source ($E_{m_{max}} = 630$ nm; SP ~ 1000 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) at 20 to 25 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 5 to 10 min at ambient temperature ($\sim 22^\circ\text{C}$). Subsequently, the thalli were transferred to sterile multi-well plates (Greiner Bio-One, Frickenhausen, Germany) filled with 4 ml of 2.5% BBM (pH 5.5) and irradiated at 16°C in a temperature-controlled growth cabinet (CLF-Percival, Wertingen, Germany). Algae were exposed for 3, 6, 30, 54, 78 and 102 h at a 18/6 light/dark cycle using a combination of white fluorescent tubes (Alto 17 W, Philips, Amsterdam, Netherlands), UV-A fluorescent tubes (Actinic BL TL-D 18 W/10, Philips, Amsterdam, Netherlands) and UV-B incandescent bulbs (ReptiGlo 25 W/10, Hagen GmbH, Holm, Germany) to provide PAR (400–700 nm), UV-A (320–400 nm) and UV-B (280–320 nm) irradiation, respectively. To obtain the desired irradiation regimes, multi-well plates were covered with transparent cut-off filter foils for 320 nm (type 6088329, Corporate Express, Stuttgart, Germany) and 380 nm (type 2041, Bruksafol, Hammelburg, Germany); the latter foil is transparent for a low amount of UV-A ($< 5\%$) between 380 and 400 nm and the coverlid of the multi-well plates filtered irradiation lower than 290 nm.

Thalli were exposed either to PAR (P), or PAR with UV-A (PA) or PAR with UV-A and UV-B (PAB). PAR was 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for all irradiations, the UV-A was 4.10 W m^{-2} for PA and 5.03 W m^{-2} for PAB, and the UV-B was 0.026 W m^{-2} for PA and 0.132 W m^{-2} for PAB. To ensure uniform irradiation of all samples, multi-well plates were placed on rotating discs (four rotations per min). After UVR exposure for 102 h thalli were allowed to recover for 24 h at 16°C and 20 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR (L36W/77, Osram Fluora, Munich, Germany) without UVR in a diurnal cycle (18L/6D). Thalli were transferred to GF/C glass fibre filters (13 mm; Pall Corp., Dreieich, Germany), F/F_M' was determined as described above, and thalli were frozen in liquid nitrogen and freeze-dried.

Pigment analysis

Freeze-dried thalli were ground with glass beads using a laboratory mill (Tissuelyser II, Qiagen, Venlo, The Netherlands) at 30 Hz for 10 min, and extracted as described by Aigner *et al.* (2013) with minor modifications. The powder was suspended in 4 ml methyl-*tert*-butylether (MTBE, SigmaAldrich, St. Louis, USA) containing 0.1% butylated hydroxytoluene (BHT, SigmaAldrich, St. Louis, USA) to prevent oxidation of pigments. The extract was sonicated for 15 min at 0°C, and then 2 ml of 20% methanol (v/v; Roth, Karlsruhe, Germany) was added, the mixture vortexed and frozen overnight at –20°C for quantitative extraction. This extract was then centrifuged (1,000 g, 5 min) at 4°C to support phase separation of the lipophilic supernatant (MTBE-phase) and the hydrophilic lower (methanol) phase. The upper and the lower phases were separated, evaporated to dryness in a SpeedVac (SPD111V, Thermo Fisher Scientific, Waltham, USA) with a refrigerated vapour-trap (RVT5105, Thermo Fisher Scientific, Waltham, USA), and then re-suspended in, respectively, 500 µl *N,N*-dimethylformamide (DMF, Scharlau, Sentmenat, Spain) and 500 µl methanol (HPLC grade, Roth, Karlsruhe, Germany). The extracts were centrifuged (15,000 g, 45 min, 4°C) prior to injection into the HPLC. Primary pigments in the lipophilic phase and secondary pigments in the hydrophilic phase were separated using an Agilent 1100 Series system (Waldbronn, Germany) equipped with a vacuum degasser, a binary pump, a cooled autosampler (4°C), a diode array detector (DAD) and a fluorescence detector (FLD).

Photosynthetic primary pigments (chlorophylls, carotenoids and phycobiliproteins)

Primary pigments were analysed according to Remias *et al.* (2005) with minor modifications. Primary pigments were separated on a LiChroCART (C18, 100 x 4.6 mm, 5 µm, 120 Å) column (Agilent, Waldbronn, Germany) at 30°C at a flow rate of 1 ml min^{–1} using solvent A (acetonitril:methanol = 74:6) and solvent B (methanol:hexane = 5:1). The system was started at 0% solvent B for 4 min, followed by a gradient to 100% solvent B from 4 to 9 min, which was maintained for 9 min, followed by a 5 min post-run with 100% solvent A. Whole absorbance spectra were recorded each s and primary pigments were identified by retention time and absorption spectra at 440 nm with a DAD, and quantified using a calibration curve of external standards. Chlorophyll *a* was obtained from SigmaAldrich, St. Louis, USA; antheraxanthin and violaxanthin from DHI C14, Centralen, Denmark; zeaxanthin and lutein from Carl Roth, Karlsruhe, Germany; β-carotene from Calbiochem, Darmstadt, Germany. Neoxanthin and chlorophyll *b* from spinach extracts were gathered with a fraction collector (1200 Series, Agilent, Waldbronn, Germany) and concentrations calculated using the specific absorption coefficients.

The phycobiliproteins R-phycoerythrin (PE), R-phyococyanin (PC) and R allophycocyanin (APC) were extracted according to Cornish *et al.* (2013) with minor modifications. Briefly, ground material was suspended at 4°C in 500 µl extraction solution consisting of 74.5 ml Sørensen's phosphate buffer (50 mM, pH 7), 0.5 ml Triton X-100, 10.5 µl β-mercaptoethanol and 10 protease inhibitor tablets (cOmplete, Roche Diagnostics GmbH, Mannheim, Germany). The extracts were vortexed, sonicated for 15 min at 0°C and subjected to two freeze-thaw cycles from –25°C to 4°C. The samples were centrifuged (17,000 g, 20 min) at 4°C and supernatants collected for spectrophotometric analysis. Concentrations were

calculated using the equations of Beer & Eshel (1985) for PC and PE and the equation of Kursar *et al.* (1983) for APC.

Secondary photoprotective compounds (phenolic compounds and MAAs)

To test for the presence of putative photoprotective compounds, secondary hydrophilic phenolic compounds were analysed on a Synergi hydro column (RP18, 150 x 2.0 mm, 4 μ m, 100 Å; Phenomenex, Aschaffenburg, Germany) with a RP-18 guard cartridge (20 x 4 mm) at 25°C at a flow rate of 0.3 ml min⁻¹ and an injection volume of 25 μ l using solvent A (water with 0.5% formic acid) and solvent B (methanol with 0.5% formic acid) starting at 0% solvent B, with a gradient to 100% solvent B within 40 min, followed by a 8 min post run with 100% solvent A. Whole absorbance spectra were recorded each s and DAD detection wavelengths were set to 280 nm and 350 nm, respectively, after Aigner *et al.* (2013).

The presence of MAAs was tested using a Supelcosil LC-NH₂ column (RP 18, 150 x 4.6 mm, 3 μ m; Supelco, St. Louis, USA), protected with a RP-18 guard cartridge (20 x 4.6 mm) at 30°C at a flow rate of 1 ml min⁻¹ using solvent A (0.1% ammonium formate, pH 3.14) and solvent B (methanol), starting with 75% solvent B, followed by a gradient to 30% solvent B within 5 min, then to 0% solvent B from 7 to 8 min, to 30% solvent B at 10 min, and then 75% solvent B at 15 min, followed by a 5 min post run with 100% solvent A. Whole absorbance spectra were recorded each s at wavelengths between 310 nm and 330 nm. MAA standards isolated from marine red algae (asterina-330, mycosporine-glycine, palythine, porphyra-334, and shinorine) were made available from former studies (Karsten & West, 2000).

Statistical analysis

Data for oxygen evolution ($n = 4$), F_V/F_M ($n = 4$), nutrient contents in the bog water ($n = 5$), primary and accessory pigments ($n = 5$), and F/F_M' -measurements ($n = 10$) were tested for significance using a one-way analysis of variance (ANOVA), with a p -value 0.05, $p = 0.01$, and $p = 0.001$, or two-way ANOVA ($p = 0.01$), as appropriate. Data for oxygen evolution and F_V/F_M were further tested by Fisher's LSD *post-hoc* test, and all other data with the Bonferroni *post-hoc* test. All analyses were carried out using SPSS 18.0 (IBM Corp., Somers, NY, USA), Origin Pro 9.1 (Originlab, Northampton, USA) or the software package R (version 3.3.1).

Results

Algal material and environmental parameters

The collection site of *B. turfosum* is characterized by diurnally fluctuating PFDs, which differed about 14-fold, 5-fold, and 3.5-fold for PAR, UV-A, and UV-B, respectively (Table S1) when comparing shaded conditions under the tree canopy with direct insolation. Irradiation, measured on a sunny, cloudless summer day, reached values of $1700 \pm 35 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ PAR, $39 \pm 1.3 \text{ W m}^{-2}$ UV-A and $2.1 \pm 0.04 \text{ W m}^{-2}$ UV-B, typical for this altitude and season, and the habitat received between 700–900 KWh m⁻² a⁻¹ radiant energy over the course of the year (Fig. S2). Solar irradiation at one hour of full exposure at noon in the sun varied by less than 5%, for PAR, UV-A and UV-B whereas in the shade PAR varied

by almost 50%, UV-A by up to 20% and UV-B by about 12%. However, only a small proportion of radiation was able to penetrate through the brown-coloured water (Fig. S3; $K_{d\text{ UV-B}} \sim 0.01$). On the various sampling dates the water had very low conductivity (22.3–24.5 $\mu\text{S cm}^{-1}$) and was acidic-soft (pH values were between 5.7 to 4.6 and water hardness was between 1 and 1.5 dH). The nutrient concentrations were very low for ammonia (0.02–0.04 mg l^{-1}), nitrate (0.05–0.08 mg l^{-1}) and below detection limit for phosphate ($< 0.01 \text{ mg l}^{-1}$), whereas the iron content was very high on all three sampling dates (1.2–2.7 mg l^{-1}), due to the iron-rich sandstone in the first geological formation at the collection site. In the bog pool water phenolic compounds were characterized as polyphenols and the concentrations varied between spring and summer (1.1–1.9 mg l^{-1}). Both iron and phenolic compounds are responsible for the brown colouration of the bog water, which was most pronounced in autumn. Calculations of the proportion of humic acids showed that these compounds are responsible for 20–55% of the brown colouration (Table S2), whereas iron most probably accounted for the remaining absorbance.

Light and confocal laser scanning microscopy (CLSM)

Chloroplasts in whorl cells were ribbon-shaped in a parietal arrangement and formed a spiral, which was corroborated by CLSM images (Z-stacks). The chlorophyll autofluorescence allowed us to illustrate the parietal arrangement in median optical CLSM sections (Fig. S1).

Photosynthetic performance under varying light and temperature regimes

Light requirements for photosynthesis—Increasing PFDs stimulated photosynthetic oxygen production at 20°C in *B. turfosum* up to 275 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, followed by a gentle decline at higher PFDs (Fig. 1). Based on a fit according to Walsby (1997), values for I_c and I_k were 8.4 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ and 29.7 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, respectively, and in the light-limited range α was 0.4978, clearly indicating very low light requirements for photosynthesis (inset of Fig. 1). In the light-saturated range, the maximal net photosynthesis (P_{max}) was $11.9 \pm 0.29 \mu\text{mol O}_2 \text{ h}^{-1} \text{ mg}^{-1}$ chlorophyll *a* and the degree of photoinhibition (β) was determined to be $-0.002 \mu\text{mol O}_2 \text{ h}^{-1} \text{ mg}^{-1}$ chlorophyll *a*, although the alga showed relatively high photosynthetic activity up to 1050 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (for ease of comparison with other studies DW-based calculations are shown in Fig. S4A).

Photosynthetic activity under increasing temperatures—The effect of increasing temperature (from 5°C to 50°C) on photosynthetic oxygen production (at 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$) and respiration (in the dark) together with measurements of F_v/F_m in *B. turfosum* revealed strong differences in sensitivity of the two physiological processes (Fig. 2A). Net oxygen production was observed between 5°C and 35°C, whereas at 25°C the highest oxygen production of $40.7 \pm 11.94 \mu\text{mol O}_2 \text{ h}^{-1} \text{ mg}^{-1}$ chlorophyll *a* was found, which was significantly different from all other temperatures except 30°C ($p < 0.05$). Oxygen production was not detectable above 35°C, and negative net production of oxygen was observed at temperatures above 40°C. Dark respiration (oxygen consumption) was low up to 15°C ($-2.1 \pm 0.9 \mu\text{mol O}_2 \text{ h}^{-1} \text{ mg}^{-1}$ chlorophyll *a*), increased about 2-fold at 20°C, about 3-fold at 25°C, about 10-fold above 30°C (Fig. 2B), where it was greatest ($-20.2 \pm 3.1 \mu\text{mol O}_2 \text{ h}^{-1} \text{ mg}^{-1}$ chlorophyll *a*). Chlorophyll *a* concentration did not change significantly during

the temperature treatment (Table S3) and DW-based calculations for oxygen evolution are shown in Fig. S4B. The highest F_V/F_M values were observed at 10°C (0.584 ± 0.028), which significantly declined between 10°C and 20°C ($p < 0.05$). F_V/F_M further decreased, dropped to approximately 30% at 40°C (0.202 ± 0.105) and reached 0 at 45°C. The highest ratio between photosynthesis and respiration was 4 to 4.5 between 15°C and 25°C. Ratios of 2.5 to 3, and 1.5 to 2, respectively, were found between 5°C and 10°C, and between 30°C and 35°C (Fig. 2B).

Response to ultraviolet radiation

Kinetics of effective quantum yield—The decrease in F/F_M' , expressed as a percentage of initial F/F_M' of untreated thalli (P: 0.48 ± 0.002 ; PA: 0.48 ± 0.005 ; PAB: 0.48 ± 0.006) was compared with F/F_M' values of thalli irradiated with P, PA und PAB. All three irradiation treatments differed significantly from each other ($p < 0.01$; Fig. 3). Within each treatment, algae exhibited a decrease in F/F_M' between 0 h and 6 h, and those treated with PAB showed a significantly sharper transient drop in F/F_M' compared to PA at 3 h ($p < 0.01$). Under PA and PAB F/F_M' values significantly increased between 6 h and 54 h of treatment ($p < 0.01$), then plateaued at around 70% of their initial values (PA: $73.2 \pm 10.3\%$, PAB: $75.3 \pm 5.5\%$) until the end of UVR exposure at 102 h when P-irradiated algae reached $95.8 \pm 3.8\%$ of initial values (Fig. 3).

None of the treatments damaged or chronically affected the alga. After a recovery period of 24 h (without UVR) F/F_M' of thalli from all treatments increased almost to the initial values (P: $101.8 \pm 4.7\%$; PAB: $94.8 \pm 4.7\%$; PA: $86.8 \pm 8.0\%$).

Photosynthetic primary pigments—Untreated thalli contained chlorophyll *a* ($8.33 \pm 1.86 \text{ nmol mg}^{-1} \text{ DW}$), lutein ($3.04 \pm 0.91 \text{ nmol mg}^{-1} \text{ DW}$), zeaxanthin ($0.27 \pm 0.07 \text{ nmol mg}^{-1} \text{ DW}$; Fig. 4A) and trace amounts of α - and β -carotene (Table S3). After 102 h chlorophyll *a* levels dropped by about two-thirds, which was significant for both, PA and PAB ($p < 0.001$), compared to P ($p < 0.01$). Upon irradiation, lutein contents were halved compared to 0 h ($p < 0.05$), whereas zeaxanthin contents remained unchanged. During the 24 h recovery period (without UVR) chlorophyll *a* levels partially recovered after the P and PAB treatments, but not after PA (P: $p < 0.05$, PA: $p < 0.01$). Lutein levels fully recovered after all treatments, and under PAB the zeaxanthin content slightly increased compared to 0 h ($p < 0.05$; Fig. 4A).

Phycobiliproteins were represented by APC and PC, whereas PE was not detectable with the spectrophotometric method used (Fig. 4B). All irradiation treatments led to a decrease in PC ($p < 0.05$), which was most pronounced for PAB that declined by two thirds ($p < 0.001$). In PAB-exposed algae APC was halved ($p < 0.001$). During the 24 h recovery phase, PC and APC remained unchanged in P-treated thalli and decreased further in PA-treated ones, but significantly increased after PAB treatment (Fig. 4B). The presence of putative photoprotective secondary metabolites such as phenolic compounds and/or MAAs was tested for, but none were detected.

Transmission electron microscopy—Whorl cells of *B. turfosum* exposed for 102 h under P conditions had intact chloroplasts with few plastoglobules and abundant cytosolic

floridean starch (Fig. 5). In contrast, cells exposed for 102 h to PA (Fig. 6) exhibited dense accumulations of plastoglobules, and the thylakoid membranes occasionally showed dilations. However, numerous starch grains were apparent in the cell centre. Exposure to PAB (102 h) also resulted in accumulation of plastoglobules and cytoplasmic lipid bodies (Fig. 7). After recovery (24 h), cells did not change in ultrastructural appearance (Fig. 8) when compared to P. After recovery from PA (Fig. 9) thylakoid membranes were arranged in a more parallel manner, but plastoglobules and starch granules could still be observed (Fig. 9). A similar effect of the recovery was observed for PAB-treated cells (Fig. 10), with a reduced number of plastoglobules and parallel thylakoid membranes. Additionally, these cells contained electron-dense bodies with a diameter of up to 1 μm .

Discussion

This study shows that *Batrachospermum turfosum* is adapted to a broad range of temperature and irradiance conditions, including UVR, which are typically found in habitats at elevated altitudes and northern latitudes characterized by ultra-oligotrophic waters that may contain unfavourable organic and inorganic compounds.

Light and temperature requirements for photosynthesis

The photosynthetic performance of *B. turfosum* is characterised by very low I_k and I_c values together with a high photosynthetic efficiency even at low PFDs (Fig. 1), in agreement with reports on other freshwater red algae (Karsten *et al.*, 1993; Leukart & Hanelt, 1995; Necchi, 2005). However, *B. turfosum* also tolerates PFDs of $\sim 1000 \mu\text{mol photons m}^{-2} \text{s}^{-1}$. PFDs between 1000 and 2400 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ were also reported to occur in the habitats of *Compsopogon caeruleus* and *Batrachospermum delicatulum* (Necchi & Zucchi, 2001). Tolerance to this wide range of light conditions is supported by the plasticity of the photosynthetic apparatus (Necchi, 2005), which in freshwater red algae can vary between populations (Necchi & Vis, 2005), between field collected and cultured specimens (Necchi, 2005; Drerup *et al.*, 2015) and between algae acclimated to low light and high light conditions (Bautista & Necchi, 2007).

The temperature dependence of photosynthesis was assessed by maximum chlorophyll *a* fluorescence and oxygen evolution from PSII (Gilbert *et al.*, 2000), showing oxygen production in a broad temperature range between 5°C and 35°C (Fig. 2a), in accordance with the temperatures reported for *B. turfosum* habitats (Müller *et al.*, 1997; Gregor *et al.*, 2003). Highest photosynthetic efficiency between photosynthesis and respiration (P:R ratio) was found between 15 and 25°C (Fig. 2b), supporting previous reports on other *Batrachospermum* species and for *C. caeruleus* (Necchi & Zucchi, 2001; Necchi, 2004). At temperatures above 20°C oxygen consumption started to increase, and was maximal at 30°C, indicative of unfavourable conditions for net photosynthesis. However, *B. turfosum* can also withstand high temperatures of up to 35°C for short periods, as would be expected for an alga occurring in standing waterbodies in which temperatures were reported to fluctuate up to 34°C (Gregor *et al.*, 2003). The two methodological approaches for the assessment of photosynthesis, i.e. through chlorophyll *a* fluorescence and oxygen measurements provided different results (Fig. 2a), as reported earlier (Hanelt *et al.*, 1994; Gilbert *et al.*, 2000).

F_V/F_M steadily declined with increasing temperatures above 15°C, to approximately 60% of its initial signal at 35°C, where the P:R ratio was still positive, to about 30% at 40°C, where no oxygen production was detectable. Therefore, the increase in oxygen consumption at 40°C is probably induced by higher mitochondrial activity masking net photosynthesis. Above 40°C, photosynthetic activity collapsed, as reported for higher plants (Pospisil *et al.*, 1998), presumably due to increasing thermal denaturation of proteins such as the D1 of PSII. In summary, in *B. turfosum* optimal conditions for photosynthesis are low light, around 100 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$, and temperatures between 15 and 25°C, although photosynthetic activity was also recorded at much higher PFDs, and at lower and higher temperatures, supporting euryoecious adaptation.

Dynamic photoinhibition in response to ultraviolet radiation

The effective quantum yield of thalli exposed to PA and PAB differed significantly from P, and from each other throughout the entire exposure period (Fig. 3). In UVR-treated thalli, but not in the P-treated thalli, F/F_M' showed a significant, rapid, transient decrease, suggesting that PSII was impaired, followed by an increase, indicative of photoacclimation, and then F/F_M' plateaued. Similar kinetics were also described for higher plants (Tyystjarvi, 2008) and cyanobacteria (Allakhverdiev & Murata, 2004), in which the initial decline in F/F_M' was suggested to be due to damage to PSII components, such as the oxygen-evolving complex, and to insufficient repair mechanisms (Vass, 2012). Furthermore, damage to PSII by UV-A alone is generally lower compared to UV-AB, although both have similar effects on PSII (Vass, 2012). In *B. turfosum* the initial drop in F/F_M' was greater in PAB-irradiated thalli than in PA-irradiated ones, but in both treatments thalli were able to acclimate, as shown by their ability to reach about 70% of their initial F/F_M' values. In marine red algae UVR contributed to dynamic photoinhibition, resulting in a 30% drop in photosynthetic performance in *Lithophyllum incrustans* and *Sphaerococcus coronopifolium* (Figueroa & Gomez, 2001), and in young gametophytes of *Gelidium floridanum* (Schmidt *et al.*, 2012). When *B. turfosum* thalli were allowed to recover in the absence of UVR, they almost re-established their initial F/F_M' values (Fig. 3). Interestingly, PAB-irradiated thalli recovered better than PA-irradiated ones, as also observed in the red alga *Gracilaria lemaneiformis* (Xu & Gao, 2010). UV-B-induced repair mechanisms for D1 and D2 proteins were also found in cyanobacteria (Vass *et al.*, 2013). Taken together with the mostly intact ultrastructure after the recovery phase (Figs 5–10), it appears that UVR did not incur chronic photoinhibition in the conditions tested, providing further evidence for the presence of a flexible, plastic photosynthetic apparatus.

The pigment composition of *B. turfosum* is dominated by chlorophyll *a* and lutein and it also contains small amounts of zeaxanthin (Fig. 4A), typical of Batrachospermales (Schagerl & Donabaum, 2009). Exposure to P, PA, and PAB induced a strong decline in chlorophyll *a* and lutein concentrations, also in the P-treated controls in which no significant changes in F/F_M' were found. This indicates that the alga suffered some stress, or acclimated by down-regulating photosynthesis, in response to the treatment, even at the dim light of 20 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ used. The phycobiliproteins, APC and PC, were also strongly affected by UVR, whereby PAB-irradiation had the greatest effect, especially on APC, of which the concentration was halved. In contrast, P- and PA-irradiation had only a small effect on APC,

whereas PC was also significantly lowered. Figueroa & Gomez (2001) suggested that state transitions in combination with cyclic electron transport by photosystem I greatly contribute to non-photochemical quenching in red algae. Direct involvement of PBS in energy dissipation was shown in *Porphyridium cruentum* (Kana *et al.*, 2014), but the contribution of PBS to q_E in *B. turfosum* remains to be elucidated. In the recovery phase, PBS composition remained unchanged in P-irradiated thalli, whereas APC significantly increased in the PAB-irradiated thalli. Upon recovery of *B. turfosum* PBPs re-synthesis was apparently enhanced in thalli treated with PAB compared to PA. High photosynthetic plasticity and fast re-synthesis of PBPs were also described for polar marine red algae after long periods of darkness (Lüder *et al.*, 2002). In the present study, re-synthesis of PBPs was observed although the experiments were conducted under nutrient-poor conditions. In contrast, another generalist red alga, *C. caeruleus*, an indicator of nutrient-enriched environments, suffered from nitrogen starvation, which caused PBS degradation (Bautista & Necchi, 2014). Therefore, the capacity to re-synthesize PBPs could reflect the adaptation of *B. turfosum* to ultra-oligotrophic environments.

Algae from the eulittoral are known to synthesize numerous UV-photoprotective compounds, mainly MAAs (Karsten, 2008), in response to elevated UVR, whereas algae from deeper waters lack this capability (Hoyer *et al.*, 2001). The presence of such UV-sunscreens can alleviate ultrastructural damage, including to chloroplasts, as shown for *Palmaria decipiens* and *Bangia atropurpurea* (Poppe *et al.*, 2003; Holzinger *et al.*, 2004). In *B. turfosum* no putative UV-sunscreens, like MAAs or phenolic compounds, were found. However, the alga may benefit from UV-absorbing organic and inorganic compounds, such as polyphenolic compounds and iron, which are abundantly present in bog water. On the other hand, these compounds can lead to ROS formation (Roach *et al.*, 2015).

Batrachospermum spp. are characterised by a thick extracellular mucilage, which is why they are sometimes named “frog spawn”. This mucilage could act as a physical barrier for ROS and polyphenols. Moreover, the dense whorls of *B. turfosum* will provide some self-shading, which may also contribute to photoprotection. Nonetheless, *B. turfosum* has a notable tolerance of UVR compared to shade-adapted marine red algae from the lower sublittoral (I_c values $< 30 \mu\text{mol m}^{-2} \text{s}^{-1}$), which were extremely sensitive to mild UVR treatment (e.g., *Phycodrys austrogeorgica*; Poppe *et al.*, 2003), and showed no or only weak recovery (Bischof *et al.*, 2006). By contrast, the ultrastructure of *B. turfosum* was still intact after the irradiation treatments applied although accumulation of plastoglobules was observed (Figs 5–10). Plastoglobules are lipoprotein particles inside chloroplasts that accumulate upon stress, including high light, senescence and desiccation (Austin II *et al.*, 2006; Bréhélin *et al.*, 2007; Holzinger *et al.*, 2011), and that have also been suggested to function as reserves in red algae (Schmidt *et al.*, 2012).

In conclusion, *B. turfosum* is adapted to low light, but also tolerates high PFDs, at least in the short-term, and is capable of net photosynthesis at a wide range of temperatures. Moreover, *B. turfosum* can acclimate to UVR by dynamic photoinhibition, and although it does not use MAAs for photoprotection, is capable of recovering F/F_M' and PBPs after UVR treatment. These traits of *B. turfosum* may facilitate its broad occurrence in ultra-oligotrophic water bodies, such as bog pools, from northern latitudes to subalpine regions, characterized by widely fluctuating light and temperature conditions.

Supplementary information

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Effects of photon fluence rates on oxygen evolution of *Batrachospermum turfosum*. The inset shows the PI curve in low light. Data were fitted according to the model of Walsby (1997). All measurements were conducted at 20°C (n = 4, means $\pm$ SD).

Fig. 2. Temperature dependence of photosynthesis in *Batrachospermum turfosum*. **A** Oxygen production at $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$, dark-respiration and optimum quantum yield (F_v/F_m). Negative values are shown as stacked bars and significant differences between means are marked with different letters. **B** Ratio between photosynthesis and respiration as a function of temperature. Significant differences between mean values were calculated by one-way ANOVA followed by the Fisher's LSD *post hoc* test ($p < 0.05$; $n = 4$, means $\pm$ SD).

Fig. 3.

Changes in effective quantum yield (F/F_M') in *Batrachospermum turfosum* in response to irradiation. P, PAR; PA, UV-A; PAB, UV-AB. Data points ($n = 10$, means $\pm$ SD) labelled with the same letter do not differ significantly from each other (two-way ANOVA and Bonferroni's *post-hoc* test; $p < 0.01$). Light (18 h) and dark (6 h) phases are indicated by grey and black shading, respectively, for the irradiation treatments, and by white and black shading for the recovery phase.

Fig. 4.

Primary and accessory pigments in *Batrachospermum turfosum* upon irradiation and recovery. P, PAR; PA, UV-A; PAB, UV-AB. Thalli were exposed to irradiation treatments (P, PA or PAB) for 102 h, followed by 24 h of recovery in the absence of UVR ($n = 5$, means $\pm$ SD). Significances between control (0 h) and irradiated samples were tested by one-way ANOVA followed by Bonferroni's *post-hoc* test. Statistical differences between means are indicated by 1, 2 and 3 asterisks for $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively. **A** Primary pigments **B** Phycobiliproteins. PC, phycocyanin; APC, allophycocyanin.

Figs 5–10.

Transmission electron micrographs of *Batrachospermum turfosum* whorl cells upon irradiation and recovery. P, PAR; PA, UV-A; PAB, UV-AB. Thalli were exposed to P, PA and PAB (A, B and C, respectively) for 102 h followed by recovery in the absence of UVR (D, E and F). **Fig. 5.** Parietal chloroplasts with plastoglobules (arrow) and cytoplasmic starch grains; **Fig. 6.** Accumulation of plastoglobules (arrow). **Fig. 7.** Plastoglobules (arrow), cytoplasmic lipid bodies, **Fig. 8.** Chloroplast with parallel arranged thylakoid membranes. **Fig. 9.** Intact chloroplasts. **Fig. 10.** Reduced number of plastoglobules (arrow), electron

dense body in the cell cortex. Abbreviations: CW cell wall, ED electron dense body, L lipid body, N nucleus, S starch, V vacuole. Scale bars: 1 μm .