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The effects of short- and long-term freezing on *Porphyra umbilicalis* Kützing (Bangiales, Rhodophyta) blade viability

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ABSTRACT

Seaweeds inhabiting the upper intertidal zone are subjected to temperature, light, and water stresses and vertical distribution has been linked to environmental tolerance. Previous studies have also attributed successful recovery from freezing stress in intertidal seaweeds to desiccation tolerance. *Porphyra umbilicalis* Kützing is an aseasonal red alga inhabiting the mid to upper intertidal zone in temperate and subarctic regions of the North Atlantic. It is a member of the economically important group of foliose Bangiales, and has been documented to only reproduce asexually via neutral spores in the Northwest Atlantic. The goal of this study was to assess the effects of freezing on the viability of small blades of *P. umbilicalis*. Cultured blades of *P. umbilicalis* (4.8 ± 0.22 mg) were air dried to 5% or 30% absolute water content (AWC) and frozen for 1, 3, 6, or 12 months at −80 °C or −20 °C. Following freezing, blades were rehydrated and the growth rate of each blade was measured weekly for 4 weeks. Photosynthetic efficiency of photosystem II (F_v/F_m) was assessed for each blade 3 h and 4 weeks post-rehydration. Overall, there was 100% blade survival and all blades continued to grow after rehydration. Although the conditions under which the blades were frozen did have statistically significant effects on post-rehydration growth rate and F_v/F_m, in general the differences were quite small. Post-rehydration growth rates ranged from 7.06 to 8.03 ± 0.16% day^{-1}. AWC had an effect on post-rehydration growth rates for blades frozen at −80 °C, but not blades frozen at −20 °C. The length of freezing had a somewhat greater effect on blades with 5% AWC than blades with 30% AWC. Growth rates peaked two weeks post-rehydration followed by a small decline in weeks 3 and 4. F_v/F_m values following freezing were generally similar to those recorded in previous studies on non-frozen blades; however, blades frozen for 6 months performed better than blades frozen for 12 months. Overall, these results indicate that short- and long-term freezing have little physiological effect on blades of *P. umbilicalis*. Therefore, freezing may be a viable method for preservation of *P. umbilicalis* for aquaculture.

1. Introduction

Studies have linked the zonation of intertidal seaweeds with tolerance to environmental stresses (Dring and Brown, 1982; Karsten, 2012; Kim et al., 2008; Sampath-Wiley et al., 2008; Smith et al., 1986; Wiltens et al., 1978). Seaweeds inhabiting the upper intertidal zone are subjected to a wide variety of temperature, light, and water stresses, most notably freezing and desiccation. It has been shown that species growing in the upper intertidal zone can survive extensive desiccation and osmotic stress with little physiological damage (Abe et al., 2001; Kim and Garbary, 2004; Kim et al., 2013; Liu, 2009; Smith et al., 1986; Zou and Gao, 2002).

Seaweeds that survive during the winter months in the intertidal zone of the temperate Northwest Atlantic can encounter freezing stress during low tide. Damage from freezing occurs as a result of intracellular ice formation that can cause damage to cellular membranes and reduce the transfer of energy from the antennae to the reaction center in the photosynthetic apparatus (Dudgeon et al., 1989). Seaweeds inhabiting the upper intertidal zone are more cold tolerant (Frazer et al., 1988) and have lower ice nucleation temperatures (Lundheim, 1997) than seaweeds inhabiting the lower intertidal or shallow subtidal zones.

Porphyra umbilicalis is an aseasonal red seaweed inhabiting the mid to upper intertidal zone in temperate and subarctic regions of the North Atlantic (Brodie and Irvine, 2003). *P. umbilicalis* is a foliose species in the family Bangiaceae and is unique in that it has been documented to only reproduce asexually in the Northwest Atlantic (Blouin et al., 2007).

Abbreviations: α, *p*-value; ANOVA, analysis of variance; AWC, absolute water content; CO_2, carbon dioxide; DNA, deoxyribonucleic acid; dNTP, deoxynucleotide triphosphates; dsDNA, double-stranded deoxyribonucleic acid; F_v/F_m, photosynthetic efficiency of photosystem II; HS, high sensitivity; HSD, honestly significant difference; ln, natural log; Mg^{+2}, magnesium; NH, New Hampshire; NH_4Cl, ammonium chloride; PAM, pulse amplitude modulated; PCR, polymerase chain reaction; PSI, photosystem I; PSII, photosystem II; *rbc*L, large subunit of the ribulose-bisphosphate carboxylase gene; *rbc*S, small subunit of the ribulose-bisphosphate carboxylase gene; ROS, reactive oxygen species; SGR, specific growth rate; VSE, Von Stosch enriched; W_t, dehydrated weight of blade; W_d, dry weight of blade after 24 hours in a drying oven at 80 °C; W_o, fresh weight of the blade.

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Sporelings and full-grown blades of *P. umbilicalis* are commonly found throughout the winter months in the Northwest Atlantic when they are exposed to sub-freezing temperatures twice a day during low tide (L. Green, *pers. obs.*). Previous studies have reported the responses of *Porphyra* to freezing (Chen et al., 2007; Lin et al., 2010; Wang et al., 2011), but none have looked at the viability of sporelings exposed to freezing for up to 12 months or at the tolerance of *P. umbilicalis*. Since *P. umbilicalis* from the Northwest Atlantic lacks the conchocelis phase, which is assumed to persist for multiple years in the subtidal zone (Clokie and Boney, 1980), tolerance of the blade phase to environmental conditions determines the success of the species. The goal of this study was to determine the viability of blades of *P. umbilicalis* after both short-term (1 month) and long-term frozen storage (3–12 months).

1.1. Materials and methods

1.1.1. Genetic identification of cultured material

The genus *Porphyra* contains many species that have very similar morphologies, which makes them difficult to identify without the use of genetic markers. Therefore, DNA barcoding was used to confirm the identity of all cultures used in the study. DNA was extracted using a Puregene™ Isolation Kit per manufacturer's instructions. A 298 base pair segment from the 3′ end of the *rbc*L gene and *rbc*L-*rbc*S spacer was used for identification and was amplified with the forward primer RBCL5RC (5′-GTGGTATTCATGCTGGTCAAA-3′; Klein et al., 2003; Mols-Mortensen et al., 2012) and the reverse primer RBCSPC (5′-CACT ATTCTATGCTCCTTA TTKTTAT-3′; Teasdale et al., 2002).

Polymerase chain reactions (PCR) were performed in 50 μL volumes that contained 10 μL of Taq buffer (Promega GoTaq® Flexi Green), 0.2 mM Mg^{+2}, 1 μL dNTP mixture, 1 μL (20 mM) of each primer, 0.25 μL Taq polymerase (Promega GoTaq® Flexi), and 4 μL of extracted DNA solution following the PCR protocol of Bray et al. (2006). The PCR products were gel-purified by electrophoresis on a SYBR®Safe treated low melting point agarose gel (Ultrapure™, Invitrogen™) and the agarose was digested using agarase (Sigma A6306, 1.5 μL). The concentration of DNA was determined using a dsDNA HS Assay Kit and Qubit® fluorometer (Invitrogen™) per the manufacturer's instructions. Appropriate volumes of DNA and primers were sent to the University of New Hampshire's Hubbard Center for Genome Studies where the samples were sequenced with an Applied Biosciences 3130 DNA Analyzer.

The raw sequence chromatograms were manually trimmed and proofread in Chromas v. 2.2 (Technelysium, Pty. Ltd.) and sequences were aligned and assembled in Seq Man Pro v. 7.2.1 (DNA Star Inc.). Following alignment, sequences were compared to the *rbcL* sequence of the *P. umbilicalis* neotype on GenBank (**KF478756**) using MegaAlign v. 7.1 (DNA Star Inc.) to confirm species identification.

1.1.2. Freezing experiment description

Neutral spores of *P. umbilicalis* were isolated from 8 different thalli from populations at Hilton Park, Dover, New Hampshire (NH; $n = 4$; 43°7′11.8″ N, 70°49′37.8″ W) and Wallis Sands State Beach, Rye, NH ($n = 4$; 43°9′55.2″ N, 70°35′28.6″ W) and initially cultured at 10 °C and 30–60 μmol photons m^{-2} s^{-1} under a 12:12 (Light:Dark) photoperiod with light supplied by 4 cool white fluorescent bulbs (Phillips, T12). After blades reached a visible size, they were transferred to 1 L Erlenmeyer flasks and cultured at 15 °C and 30–60 μmol photons m^{-2} s^{-1} under a 12:12 (L:D) photoperiod with light supplied by 2 cool white fluorescent bulbs (Phillips, T8). Cultures were maintained in Von Stosch Enriched (VSE) seawater (modified from Ott, 1966) with NH_4Cl used as the source of nitrogen. Filter-sterilized (1 μM, Pall® Life Sciences) air was supplied continuously using an aquarium air pump (Aquatic Eco-Systems Inc., Model SL94).

Once the blades reached an appropriate size (average 4.8 ± 0.22 mg) the blotted-dry fresh weight was recorded (Mettler Toledo AG204 ± 0.1 mg) and blades were allowed to air dry at room temperature (approx. 18 °C) in individual open containers for 30 min (30% absolute water content, AWC) or 4 h (5% AWC), respectively. AWC was calculated as: $AWC = (W_t - W_d) / (W_o - W_d) \times 100$, when W_t is the dehydrated weight of the blade, W_d is the dry weight of the blade after 24 h in a drying oven at 80 °C, and W_o is the fresh weight of the blade before dehydration. The conversion factor from fresh weight to dry weight was calculated using separate *P. umbilicalis* blades from each of the cultures and was determined to be 4.14 (fresh weight/dry weight). The calculated AWCs were 4.72% (±1.08) and 30.57% (±1.52) for the 5% and 30% AWC treatments, respectively.

After the allotted drying time, blades were weighed again to obtain a dehydrated weight and immediately frozen at −20 °C or −80 °C for 1, 3, 6, or 12 months in individual 1.7 mL microcentrifuge tubes. Three blades from each of the 8 parent cultures were frozen for each treatment combination ($n = 24$ for each of the 16 AWC × freezing temperature × freezing time treatment combinations). To eliminate the effect of the date of freezing, blades from two cultures were frozen on each of the following dates: 7/18/2012, 7/26/2012, 9/8/2012, and 9/15/2012.

Following the designated freezing time, blades were immediately plunged into 125 mL of aerated sterile VSE seawater at pre-freezing conditions (15 °C, 30–60 μmol photons m^{-2} s^{-1}, 12:12 L:D) and allowed to recover for 3 h. Following the recovery period, initial photosynthetic efficiency of photosystem II or PSII (F_v/F_m) and blotted-dry fresh weights were recorded. Blades were then cultured for 4 weeks with media changes occurring weekly.

1.1.3. Growth rate and photosynthetic efficiency of PSII

The fresh weight of each blade was recorded weekly for 4 weeks. Specific growth rate (SGR), hereafter referred to as growth rate, was calculated using the equation: $SGR = 100 \times \ln[(L_2 / L_1) / (t_2 - t_1)]$, where L_2 and L_1 are the blade weight at times t_2 and t_1, respectively.

Photosynthetic efficiency of PSII (F_v/F_m) was measured twice for each blade, once after a 3 h recovery period and once at the end of the 4 week experiment. Measurements were taken using a white-light PAM (pulse amplitude modulated) fluorometer (Junior-PAM, Heinz Walz GmbH) following a modified protocol from Figueroa et al. (1997) using a minimum of 10 min for dark adaptation and a far red pulse prior to measurement.

1.1.4. Statistical analyses

Growth rate was analyzed as a split-plot analysis of variance (ANOVA) with absolute water content (2 levels), freezing temperature (2 levels), and length of freezing (4 levels) as the main plots and week as the sub plot (Federer and King, 2007). Photosynthetic efficiency of PSII 3 h and 4 weeks post-rehydration were analyzed separately using a standard ANOVA with a fully factorial design (Federer and King, 2007). The response variables (growth and F_v/F_m) did not conform to the assumptions of normality and were rank transformed prior to analysis (Conover and Iman, 1981). All post-hoc comparisons were made using the Tukey's HSD test, which has been shown to be effective on rank transformed data (Conover and Iman, 1981). All analyses were performed in SYSTAT 13.00.05 (Systat, Inc.).

1.2. Results

1.2.1. Growth Rate

The effect of AWC on the growth rate of *P. umbilicalis* was dependent on the temperature at which the blades were frozen ($F_{1,3} = 29.86$, $p = 0.012$; Fig. 1). Highest growth was observed in blades frozen at −80 °C with 5% AWC (8.03 ± 0.16% day^{-1}). Post-hoc analysis showed that the growth rate of these blades was significantly higher than blades frozen at −80 °C with 30% AWC ($p = 0.033$). There was no significant difference in the growth rates of blades frozen at −20 °C with 5% AWC compared with 30% AWC (Fig. 1).

The effect of AWC on post-rehydration growth rate was also dependent on the length of freezing ($F_{3,3} = 12.37$, $p = 0.034$; Fig. 2). Post-hoc analysis revealed that blades frozen for 1 month with 5% AWC had a