© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

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

Lindsay A. Green[1, 2*] and Christopher D. Neefus[1]

[1]Department of Biological Sciences, University of New Hampshire, 46 College Road, Durham, NH 03824, U.S.A.

[2]Present address: Department of Biological Sciences, University of Rhode Island, 120 Flagg Road, Kingston, RI 02881, U.S.A.

*Corresponding author lindsaygreen@mail.uri.edu, +16032644638

ACCEPTED MANUSCRIPT

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

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; Fv/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

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

of photosystem II (F_v/F_m) was assessed for each blade 3 hours 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.

Keywords: *Porphyra umbilicalis*; freezing; viability; zonation; preservation; ecophysiology

1.1 Introduction

Studies have linked the zonation of intertidal seaweeds with tolerance to environmental stresses (Wiltens *et al*., 1978; Dring and Brown, 1982; Smith *et al.,* 1986; Kim *et al*., 2008; Sampath-Wiley *et al*., 2008; Karsten 2012). 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 (Smith *et al*., 1986; Abe *et al*., 2001; Zou and Gao, 2002; Kim and Garbary, 2004; Liu, 2009; Kim *et al*., 2013).

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

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) *Porphyra 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). 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).

1.2 Materials and Methods

1.2.1 Genetic Identification of Cultured Material

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

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'-CACTATTCTATGCTCCTTA 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 *Porphyra umbilicalis* neotype on GenBank (**KF478756**) using MegaAlign v. 7.1 (DNA Star Inc.) to confirm species identification.

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

1.2.2 Freezing Experiment Description

Neutral spores of *Porphyra umbilicalis* were isolated from 8 different thalli from populations at Hilton Park, Dover, New Hampshire (NH; n=4; 43º7'11.8" N, 70º49'37.8" W) and Wallis Sands State Beach, Rye, NH (n=4; 43º9'55.2" N, 70º35'28.6" W) and initially cultured at 10ºC 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 15ºC 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 minutes (30% absolute water content, AWC) or 4 hours (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 hours 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.

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

After the allotted drying time, blades were weighed again to obtain a dehydrated weight and immediately frozen at -20ºC or -80ºC 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 x freezing temperature x 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 (15ºC, 30-60 µmol photons m^{-2} s^{-1}, 12:12 L: D) and allowed to recover for 3 hours. 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.

1.2.3 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: $SGR=100* \ln [(L_2/L_1)/(t_2-t_1)]$, 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 hour 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 minutes for dark adaptation and a far red pulse prior to measurement.

1.2.4 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

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

main plots and week as the sub plot (Federer and King, 2007). Photosynthetic efficiency of PSII 3 hours 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.).

1.3 Results

1.3.1 Growth Rate

The effect of AWC on the growth rate of *Porphyra 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 -80ºC 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 -80ºC with 30% AWC (p=0.033). There was no significant difference in the growth rates of blades frozen at -20ºC 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 significantly lower growth rate (7.17 ±0.23% day^{-1}) than all other treatments with the exception of blades frozen for 1 month with 30% AWC. The highest average post-rehydration growth rate was recorded from blades frozen at -80ºC with 5% AWC for 6 months (8.41 ±0.23% day^{-1}), but this growth rate was not significantly higher than blades frozen for 3, 6, or 12 months at either 5% or 30% AWC (Fig. 2).

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

Growth rates of *P. umbilicalis* blades changed significantly during the 4-week post-rehydration period ($F_{3,1460}$=24.66, p<0.001). Blades experienced peak growth two weeks after rehydration (8.92 ± 0.163% day^{-1}), followed by a small decline in weeks 3 (8.04 ±0.16% day^{-1}) and 4 (7.23 ±0.16% day^{-1}). The slowest weekly growth rate was observed one week post-rehydration (7.06 ±0.16% day^{-1}). Post-hoc analysis revealed that growth rates during weeks 1 and 4 were not significantly different, while growth during the second week was significantly higher than all other weeks (week 1: p<0.001, week 2: p=0.011, week 4: p<0.001).

1.3.2 Photosynthetic Efficiency of PSII

1.3.2.1 Short-Term Post-Rehydration Recovery (3 hours): Photosynthetic efficiency of PSII (F_v/F_m) 3 hours after rehydration was significantly affected by AWC ($F_{1,365}$=3.9, p=0.049), freezing temperature ($F_{1,365}$=10.17, p=0.002), and the length of freezing ($F_{3,365}$=2.68, p=0.047); these effects were independent of one another. Blades frozen with 5% AWC had a significantly higher F_v/F_m (0.6 ±0.01) than blades with 30% AWC (0.56 ±0.01) 3 hours after rehydration. Further, blades frozen at -80ºC showed a higher F_v/F_m (0.6 ±0.1) following short-term recovery than blades frozen at -20ºC (0.56 ±0.01). Blades frozen for 6 months had a significantly higher F_v/F_m than blades frozen for 12 months (p=0.03; Table 1).

1.3.2.2 Long-Term Post-Rehydration Recovery (4 weeks): The length of freezing was the only factor that significantly affected the F_v/F_m of *Porphyra umbilicalis* 4 weeks after rehydration ($F_{3,360}$=3.63, p=0.013). While the pattern was identical to that seen after 3 hours of rehydration, blades frozen at all 4 time points had an increase in F_v/F_m between 3 hours post-freezing and 4 weeks post-rehydration (Table 1). Post-hoc analysis showed that blades frozen for 6 months had a significantly higher F_v/F_m 4 weeks post-rehydration than blades frozen for 12 months (p=0.008; Table 1).

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

1.4 Discussion

Our results show that *Porphyra umbilicalis* can survive freezing for up to 12 months without the use of cryoprotectants and recover to grow normally. We found no practical difference in post-rehydration growth rates between the tested AWCs and freezing temperatures (Fig. 1). Interestingly, the post-rehydration growth rate of *P. umbilicalis* was significantly lower after 1 month frozen than after 3, 6, or 12 months frozen (Fig. 2). These blades were frozen along with the 3, 6 and 12 month blades from the same cultures, placed in the same freezers, and thawed at four separate time points (see methods), eliminating the possibility that this response is a result of unique conditions during freezing or thawing of these blades. At present the mechanisms for the deleterious effect of short-term freezing are unknown. Further, growth rates of *P. umbilicalis* blades frozen for up to 12 months were equivalent to growth rates recorded in a previous study on non-frozen blades at 15°C under a 12:12 L:D photoperiod (6.7 ±0.43% day^{-1}; Green 2014).

Desiccation, freezing, and thawing all invariably lead to cellular damage. Boroda *et al*. (2014) reported that the red alga *Porphyridium purpureum* (Bory de Saint-Vincent) K.M. Drew & R. Ross showed no discernible growth for 5-6 days after thawing from cryopreservation at -196°C, but recovered to grow faster than control cultures. Although much less pronounced, a lag in initial growth post-rehydration was also documented in our study and is likely a result of the energetic cost of repairing damage that occurred during desiccation, freezing, and/or thawing.

Biochemical processes are temperature dependent and the rate of processes decreases with decreasing temperature. Although denaturation can occur during storage at -20°C, all biochemical processes are insignificant at temperatures of -80°C and below (Meryman, 1956). Thus, if denaturation was occurring in the blades stored at -20°C but not in those at -80°C it

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

could be reflected in the F_v/F_m immediately following rehydration. While we found that the F_v/F_m was significantly lower in blades stored at -20°C, the difference was actually quite small and it disappeared after 4 weeks of recovery. Duration of freezing might also be expected to impact the degree of denaturation. Overall, we found that freezing duration had very little effect on F_v/F_m although it was slightly lower in blades frozen for 12 months than blades frozen for 1, 3, or 6 months (Table 1). Interestingly, the difference was independent of freezing temperature and it did not disappear after 4 weeks of recovery (Table 1). Perhaps more importantly, our reported F_v/F_m values were similar to those reported in a previous study on non-frozen blades (0.65 ±0.004 for blades grown at 15°C and 12:12 L:D; Green 2014).

Freezing survival has been strongly linked to water content in seaweeds, and thus to desiccation tolerance (Chen *et al*., 2007; Lin *et al*., 2010; Wang *et al*., 2011). Desiccation stress can lead to a disruption in the transfer of photochemical energy in the photosynthetic apparatus (especially between PSI and PSII and in the oxygen-evolving complex), causing the formation of dangerous reactive oxygen species or ROS (Wiltens *et al*., 1978). ROS are highly reactive molecules that can interact with most cellular components and cause considerable damage including protein destabilization (Sampath-Wiley *et al*., 2008). Studies have shown that photosynthesis in desiccation tolerant seaweeds increases during emersion, most likely as a response to air CO_2 availability (Blouin *et al*., 2011). Studies have also shown an upregulation of glutathione reductase, catalase, and carotenoids in *Porphyra umbilicalis* (Sampath-Wiley *et al.*, 2008) and increases in the concentration of ascorbate, β-carotene, glutathione reductase, and catalase in *Mastocarpus stellatus* (Stackhouse) Guiry (Collén and Davison, 1999) in response to emersion. Catalase, glutathione reductase, ascorbate, β-carotene, and to a lesser extent carotenoids, act as antioxidants and ROS scavengers, neutralizing reactive molecules before

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

damage can occur (Collén and Davison, 1999; Sampath-Wiley *et al*., 2008). We propose that the upregulation of photosynthesis, antioxidant synthesis, and ROS scavenger formation, as a response to desiccation stress, while not measured in this study, are partially responsible for the physiological tolerance of *P. umbilicalis* to long-term freezing. These mechanisms further explain the significant difference in F_v/F_m 3 hours post-rehydration that we found between blades with 5% and 30% AWC. Blades dried to 5% AWC were exposed to desiccation for a considerably longer time (4 vs. 0.5 hours), presumably allowing for increased accumulation of protective molecules that play an important role in recovery following rehydration.

Another critical process in survival from freezing stress is the rate of freezing and thawing (Kuwano *et al*., 1993, 1996). Slow freezing tends to favor the formation of extracellular ice crystals, while rapid freezing results in the formation of ice crystals predominately inside the cell (Meryman, 1956); intracellular ice formation is considered universally lethal (Guy, 1990). Thawing rates are also critical to survival and rapid thawing prevents the growth of ice crystals and minimizes cellular damage (Lin *et al*., 2010). In this study, *P. umbilicalis* blades were frozen and thawed nearly instantaneous (L. Green, *pers. obs.*). Therefore, we propose that the survival of *P. umbilicalis* exposed to long-term freezing was dependent on: 1) the reduction in water content prior to freezing to minimize the amount of intracellular ice formation, 2) the induction of protective mechanisms (*i.e.* synthesis of antioxidants and ROS scavengers) prior to freezing to minimize the damage upon rehydration, and 3) the rapid thawing of blades following freezing to prevent growth of intracellular ice crystals.

Our results are the first to demonstrate quantitatively 100% survival of gametophytic blades of foliose Bangiales exposed to long-term freezing stress for up to 12 months without using cryoprotectants. Recently, *Porphyra umbilicalis* has been the focus of research aimed at

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

the development of a nori aquaculture industry in the Northwest Atlantic (Blouin *et al*., 2007). Cryopreservation has been reported to be a useful means of preserving strains of important seaweeds with little effort (Kuwano *et al*., 1993, 1996). An important implication of this research is that frozen storage can be used as a preservation method for seed stock of *P. umbilicalis* for aquaculture. Following our methods, mass quantities of small blades can be produced at one time and then frozen in household freezers (-20°C). This method makes it practical for aquaculture facilities to set-up their own nori nursery and not rely on an outside source of seed stock, which has been an obstacle limiting the development of a nori aquaculture industry in the Northwest Atlantic (Green 2014).

1.6 Acknowledgements

We would like to acknowledge Drs. Leland Jahnke, Arthur Mathieson, Charles Yarish, and David Berlinsky for their valuable feedback on this manuscript. Partial funding was provided by the New Hampshire Agricultural Experiment Station. This is Scientific Contribution Number XXXX. This work was supported by the USDA National Institute of Food and Agriculture Hatch Project 223365. This research was also funded by a grant from New Hampshire Sea Grant (R/CFR-14, C.D. Neefus)

1.7 References

Abe, S., Kurashima, A., Yokohama, Y., Taneka, J., 2001. The cellular ability of desiccation tolerance in Japanese intertidal seaweeds. Bot. Mar. 44, 125-131.

Blouin, N.A., Brodie, J.A., Grossman, A.C., Xu, P., Brawley, S.H., 2011. *Porphyra*: a marine crop shaped by stress. Trends Plant Sci. 16(1), 29-37.

Blouin, N., Xiugeng, F., Peng, J., Yarish, C., Brawley, S.H., 2007. Seeding nets with neutral spores of the red alga *Porphyra umbilicalis* (L.) Kützing for use in integrated multi

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

271 trophic aquaculture (IMTA). Aquaculture 270, 77-91.

272 Boroda, A.V., Aizdaicher, N.A., Odintsova, N.A., 2014. The influence of ultra-low temperatures
273 on marine microalgae cells. J. Appl. Phycol. 26, 387-397.

274 Bray, T.L., Neefus, C.D., Mathieson, A.C., 2006. Morphological and molecular variability of
275 *Porphyra purpurea* (Roth) C. Agardh (Rhodophyta, Bangiales) from the Northwest
276 Atlantic. Nova Hedwigia 82, 1-22.

277 Brodie, J., Irvine, L.M., 2003. Seaweeds of the British Isles, Volume 1 Rhodophyta, Part 3B
278 Bangiophycidae. The National History Museum, London.

279 Chen, C.S., Weng, L., Wang, L., Ji, D.H., Xie, C.T., Xu, Y., 2007. Influence of desiccation and
280 cold preservation on the survival of *Porphyra haitanensis* and unwanted alga. Acta
281 Oceanol. Sin. 29(2), 131-136.

282 Clokie, J.J.P., Boney, A.D., 1980. *Conchocelis* distribution in the Firth of Clyde: estimates of the
283 lower limits of the photic zone. J. Exp. Mar. Biol. Ecol. 46, 111-125.

284 Collén, J., Davison, I.R., 1999. Stress tolerance and reactive oxygen metabolism in the intertidal
285 red seaweeds *Mastocarpus stellatus* and *Chondrus crispus*. Plant Cell Environ. 22, 1143-
286 1151.

287 Conover, W.J., Iman, R.L., 1981. Rank transformation as a bridge between parametric and
288 nonparametric statistics. Am. Stat. 35(3), 124-129.

289 Dring, M.J., Brown, F.A., 1982. Photosynthesis of intertidal brown algae during and after
290 periods of emersion: a renewed search for physiological causes of zonation. Mar. Ecol.
291 Prog. Ser. 8, 301-308.

292 Dudgeon, S.R., Davison, I.R., Vadas, R.L., 1989. Effect of freezing on photosynthesis of
293 intertidal macroalgae: relative tolerance of *Chondrus crispus* and *Mastocarpus stellatus*

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

(Rhodophyta). Mar. Biol. 101(1), 107-114.

Federer, W.T., King, F., 2007. Variations on split plot and split block experimental designs. Wiley-Interscience, Hoboken.

Figueroa, F.L., Salles, S., Aguilera, J., Jimenez, C., Mercado, J., Vinegla, B., Flores-Moya, A., Altamirano, M., 1997. Effects of solar radiation of photoinhibition and pigmentation in the red alga *Porphyra leucosticta*. Mar. Ecol. Prog. Ser. 151, 81-90.

Frazer, A.W.J., Brown, M.T., Bannister, P., 1988. The frost resistance of some littoral and sub-littoral algae from southern New Zealand. Bot. Mar. 31(5), 461-464.

Green, L.A., 2014. Physiological studies of cultured New England nori, *Porphyra umbilicalis* Kützing and *Pyropia leucosticta* (Thuret) Neefus & J. Brodie, and implications for their use in integrated multi-trophic aquaculture systems. Ph.D. Dissertation, University of New Hampshire.

Guy, C.L., 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 187-223.

Karsten, U., 2012. Seaweed acclimation to salinity and desiccation stress, in: Wiencke, C., Bischof, K. (Eds.), Seaweed Biology: Novel Insights into Ecophysiology, Ecology, and Utilization. Springer, New York, pp. 87-107.

Kim, J.K., Kraemer, G.P., Yarish, C., 2008. Physiological activity of *Porphyra* in relation to eulittoral zonation. J. Exp. Mar. Biol. Ecol. 365, 75-85.

Kim, J.K., Kraemer, G.P., Yarish, C., 2013. Emersion induces nitrogen release and alteration of nitrogen metabolism in the intertidal genus *Porphyra*. PLoS One 8(7), 1-14.

Kim, K.Y., Garbary, D.J., 2004. Fluorescence responses of photosynthesis to extremes of hyposalinity, freezing and desiccation in the intertidal crust *Hildebrandia rubra*

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

(Hildenbrandiales, Rhodophyta). Phycologia 45(6), 680-686.

Klein, A.S., Mathieson, A.C., Neefus, C.D., Clain, D.F., Taylor, H.A., Teasdale, B.W., West, A.L., Hehre, E.J., Brodie, J., Yarish, C., Wallace, A.L., 2003. Identification of north-western Atlantic *Porphyra* (Bangiaceae, Bangiales) based on sequence variation in nuclear SSU and plastid *rbc*L genes. Phycologia 42(2), 109-122.

Kuwano, K., Aruga, Y., Saga, N., 1993. Cryopreservation of the conchocelis of the marine alga *Porphyra yezoensis* Ueda (Rhodophyta) in liquid nitrogen. Plant Sci. 94, 215-225.

Kuwano, K., Aruga, Y., Saga, N., 1996. Cryopreservation of clonal gametophytic thalli of *Porphyra* (Rhodophyta). Plant Sci. 116, 117-124.

Lin, A.P., Wang, G.C., Shen, S.D., Yang, F., Pan, G.H., 2010. Two specific causes of cell mortality in freeze-thaw cycle of young thalli of *Porphyra yezoensis* (Bangiales, Rhodophyta). J. Phycol. 46, 773-779.

Liu, Y.C., 2009. Mechanism for differential desiccation tolerance in *Porphyra* species. Ph.D. Dissertation, Northeastern University.

Lundheim, R., 1997. Ice nucleation in seaweeds in relation to vertical zonation. J. Phycol. 33, 739-742.

Meryman, H.T., 1956. Mechanics of freezing in living cells and tissue. Science 124 (3221), 515-521.

Mols-Mortensen, A., Neefus, C.D., Nielsen, R., Gunnarsson, K., Egilsdóttir, S., Pedersen, P.M., Brodie, J., 2012. New insights into the biodiversity and generic relationships of foliose Bangiales (Rhodophyta) in Iceland and the Faroe Islands. Eur. J. Phycol. 47(2), 146-159.

Ott, F.D., 1966. A selected listing of xenic cultures. Systematics-Ecology Program No. 72 (Mar. Biol. Lab., Woods Hole, MA), 1-45.

ACCEPTED MANUSCRIPT

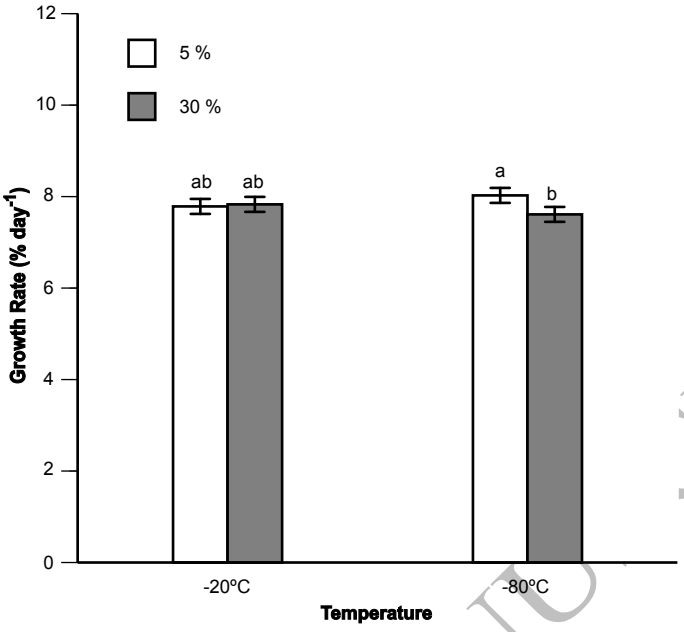
© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

340 Sampath-Wiley, P., Neefus, C.D., Jahnke, L.S., 2008. Seasonal effects of sun exposure and
341 emersion on intertidal seaweed physiology: fluctuations in antioxidant contents,
342 photosynthetic pigments, and photosynthetic efficiency in the red alga *Porphyra*
343 *umbilicalis* Kützing (Rhodophyta, Bangiales). J. Exp. Mar. Biol. Ecol. 361, 83-91.
344 Smith, C.M., Satoh, K., Fork, D.C., 1986. The effects of osmotic tissue dehydration and air
345 drying on the morphology and energy transfer in two species of *Porphyra*. Plant Physiol.
346 80, 843-847.
347 Teasdale, B., West, A., Taylor, H., Klein, A., 2002. A simple restriction length polymorphism
348 (RFLP) assay to discriminate common *Porphyra* (Bangiophyceae, Rhodophyta) taxa
349 from the Northwest Atlantic. J. Appl. Phycol. 14, 293-298.
350 Wang, W.J., Wang F.J., Zhu, J.Y., Sun, X.T., Yao, C.Y., Xu, P., 2011. Freezing tolerance of
351 *Porphyra yezoensis* (Bangiales, Rhodophyta) gametophyte assessed by chlorophyll
352 fluorescence. J. Appl. Phycol. 23, 1017-1022.
353 Wiltens, J., Schreiber, U., Vidaver, W., 1978. Chlorophyll fluorescence induction: an indicator of
354 photosynthetic activity in marine algae undergoing desiccation. Can. J. Bot. 56, 2787-
355 2794.
356 Zou, D., Gao, K., 2002. Effects of desiccation and CO_2 concentrations on emersed
357 photosynthesis in *Porphyra haitanensis* (Bangiales, Rhodophyta), a species farmed in
358 China. Eur. J. Phycol. 37, 587-592.
359

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

360 **Figures:**

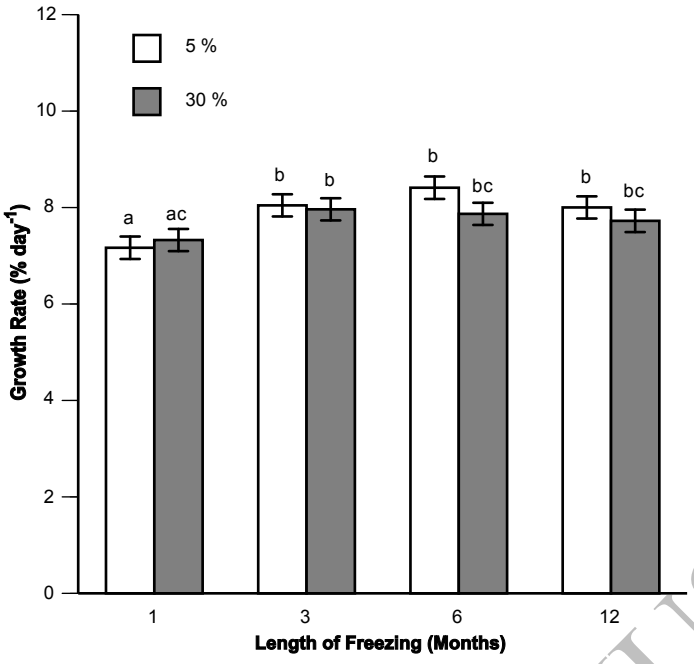


361

362 Fig. 1: Post-rehydration growth rate (% growth day^{-1}) of *Porphyra umbilicalis* blades exposed to
363 long-term freezing at -20ºC and -80ºC with either 5% or 30% absolute water content (AWC) at
364 the time of freezing (mean ± SE). Bars with a letter in common are not significantly different
365 (α=0.05). Although analysis was performed on rank transformed data, original data and standard
366 errors are graphed with letters derived from post-hoc Tukey's analysis of the rank transformed
367 data.

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001



368

369 Fig. 2: Post-rehydration growth rate (% growth day^{-1}) of *Porphyra umbilicalis* blades exposed to
370 long-term freezing for 1, 3, 6, and 12 months with either 5% or 30% absolute water content
371 (AWC) at the time of freezing (mean ± SE). Bars with a letter in common are not significantly
372 different (α=0.05). Although analysis was performed on rank transformed data, original data and
373 standard errors are graphed with letters derived from post-hoc Tukey's analysis of the rank
374 transformed data.

375

ACCEPTED MANUSCRIPT

© 2014. This manuscript version is made available under the CC-BY-NC-ND 4.0 license
http://creativecommons.org/licenses/by-nc-nd/4.0/ doi:10.1016/j.jembe.2014.10.001

376 Table 1: Photosynthetic efficiency of PSII (F_v/F_m) 3 hours and 4 weeks post-rehydration in
377 blades frozen for 1, 3, 6, or 12 months. Boxes with a letter in common (within each column) are
378 not significantly different (α=0.05). Although analysis was performed on rank transformed data,
379 original data and standard errors are shown with letters derived from post-hoc Tukey's analysis
380 of the rank transformed data.

Length of Freezing (Months)	**F_v/F_m** **3 hours post-rehydration**	**F_v/F_m** **4 weeks post-rehydration**
1	0.59 ± 0.01^{ab}	0.63 ± 0.01^{ab}
3	0.58 ± 0.01^{ab}	0.63 ± 0.01^{ab}
6	0.58 ± 0.01^{b}	0.63 ± 0.01^{b}
12	0.56 ± 0.01^{a}	0.61 ± 0.01^{a}

381

ACCEPTED MANUSCRIPT