Effects of temperature, light level, photoperiod, and ammonium concentration on *Pyropia leucosticta* (Bangiales, Rhodophyta) from the Northwest Atlantic

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Abstract: Seaweed aquaculture in the Northwest Atlantic is a growing industry that is currently based on winter-spring kelp production. Aquaculture of *Pyropia leucosticta,* a species of economically valuable nori, could provide a spring-summer crop and diversify the industry. The objectives of this study were to determine the optimum conditions for the production of the foliose blade phase and the conditions for advancement from the microscopic conchocelis to the foliose blade phase of *P. leucosticta*. Foliose blades were grown under a matrix of temperatures (10º, 15º, and 20ºC), photoperiods (8:16, 12:12, and 16:8 Light:Dark) and light levels (30, 60, 110, and 250 µmol photons m^{-2} s^{-1}) for a period of one month. Free-living conchocelis was grown under a matrix of temperatures (10º, 15º, and 20ºC), photoperiods (8:16, 12:12, and 16:8 L:D) and ammonium concentrations (20 and 500 µM) for 8-12 weeks. Blades grew optimally at 10° to 15°C, ≥110 µmol photons m^{-2} s^{-1} and ≥12 hours of light in the day, with growth rates of over 18% day^{-1} recorded. Phycobilin content of blades significantly decreased with increasing daylength, while protein content significantly decreased with increasing light level. Conchospore release and germination was observed after approximately 40 days under all tested photoperiods, temperatures, and ammonium concentrations and none of these treatments significantly affected

the time until germination. Overall, this study provides important background information required for the establishment of *Pyropia leucosticta* aquaculture in the Northwest Atlantic.

Keywords: *Pyropia leucosticta*, seaweed aquaculture, physiology, life history, mariculture

Introduction

Foliose Bangiales species (nori or laver) have long been the focus of intense investigations both from a biological and molecular perspective (Drew 1949; Krishnamurthy 1969; Conway and Cole 1977; Cole and Conway 1980; Sidirelli-Wolff 1992; Orfanidis 2001; Klein et al. 2003; Broom et al. 2004; Bray et al. 2006; Kim et al. 2007; Brodie et al. 2008; Sutherland et al. 2011; Mols-Mortensen et al. 2012). The genus *Pyropia* is not only the most speciose of the foliose Bangiales genera, but also contains the widest geographical distribution and largest morphological variation among its members (Sutherland et al. 2011). The genus *Pyropia* also contains some of the most economically valuable seaweeds in the world (Yarish et al. 1998; Pereira and Yarish 2010; Sutherland et al. 2011), most notably the commercially produced *P. yezoensis* (Ueda) M.S. Hwang & H.G. Choi and *P. haitanensis* (T.J. Chang & B.F. Zheng) N. Kikuchi & M. Miyata (FAO 2014).

Pyropia leucosticta (Thuret) Neefus & J. Brodie is commonly found in the British Isles, Greenland (Mols-Mortensen et al. 2012), Iceland, from Norway to the Canary Isles, and from Canada to North Carolina in the United States (Brodie and Irvine 2003). The life history of *P. leucosticta* consists of an annual foliose blade phase (haploid) and an alternating microscopic conchocelis phase (diploid), which is presumed to persist for multiple years (Clokie and Boney 1980). The foliose phase is typically present from spring to summer in the Northwest Atlantic and is found growing epiphytically, especially on fucoid seaweeds, in the lower intertidal and shallow subtidal zones (Brodie and Irvine 2003).

Global demand for nori has been increasing over the past several decades as a result of expanding seaweed markets (Israel 2010), especially from the increased popularity of sushi that utilizes nori as the main wrapper. Along with the increase in global demand, interest in domestication of strains of indigenous nori in the Northwest (NW) Atlantic (U.S.A.) for commercial cultivation has risen (Yarish et al. 1998). Currently, commercial production of seaweed here is limited to winter and spring production of kelp (Schmitt 2013), leaving a large gap in production throughout the spring and summer months. Cultivation of *Pyropia leucosticta* has the potential to fill this seasonal production gap, while adding diversity to the seaweed industry in the NW Atlantic.

Commercial production of any species requires a thorough understanding of its life history and the conditions required for production. *Pyropia leucosticta* is a monoecious species, meaning that male and female gametangia are found on the same blade. Male gametes are released and land on female gametangia. Fertilized female gametes divide and form zygotospores which when released develop into the microscopic filamentous conchocelis phase. In nature, the conchocelis grows on old mollusk shells, living barnacles, and calcareous stones (Brodie and Irvine 2003). Triggered by changes in environmental conditions, the filaments develop conchosporangial branches that produce and release conchospores. Conchospores develop into the haploid foliose phase, thus completing the life history (Drew 1949).

In this study, we investigated the effects of temperature (10º, 15º, and 20ºC), light level (30, 60, 110, and 250 µmol photons m^{-2} s^{-1}), and photoperiod (8:16, 12:12, and 16:8 Light:Dark) on the growth of the foliose blade phase of cultured *Pyropia leucosticta* from the Gulf of Maine in the NW Atlantic. Further, we investigated the effects of temperature (10º, 15º, and 20ºC), photoperiod (8:16, 12:12, and 16:8 L:D), and ammonium concentration (20 and 500 µM NH_4^+)

on the growth of the free-living conchocelis phase of *P. leucosticta* and determined the time until conchospore release. The overall goal of these experiments was to provide the necessary background knowledge required for successful cultivation of *P. leucosticta* in the NW Atlantic.

Materials and Methods

Culturing

Blades of *Pyropia leucosticta* were collected from a large population at Wallis Sands State Beach in Rye, New Hampshire, U.S.A. (43º9'55.2" N, 70º35'28.6" W) from June of 2011 to July of 2012. Collections were made at low tide from the low to mid intertidal zone, where *P. leucosticta* grows epiphytically on fucoid seaweeds. Samples were placed on ice and transported to the laboratory. After arrival at the laboratory, blades were processed for spore release and isolation.

Using a dissecting microscope and sterile razor blades, the fertile portions of the blades were excised, cleaned, wrapped in sterile, damp paper towels, and placed in the dark at 4ºC overnight. The following morning, the sections were submerged in sterile seawater at 10ºC and 10-50 μmol photons m^{-2} s^{-1} light to induce spore release. Individual spores of *P. leucosticta* were isolated using sterile Pasteur pipettes and placed into 12- or 24-well culture plates containing sterile Von Stosch Enriched Seawater (VSES) at 10ºC and 10-50 μmol photons m^{-2} s^{-1} under a neutral day photoperiod (12:12 L:D). VSES was based on Ott (1966) with NH_4Cl used as the source of nitrogen. Media was changed weekly. Spores that germinated into blades were transferred to flasks with VSE and held at 15ºC, 10-50 μmol photons m^{-2} s^{-1}, and 12:12 L:D while spores that germinated into conchocelis were maintained at 10ºC, 10-30 μmol photons m^{-2} s^{-1}, and 12:12 L:D.

Genetic Identification of Cultured Material

The genus *Pyropia* contains many species that look morphologically similar, making them difficult to identify without the use of genetic markers. Therefore, DNA barcoding was used to confirm the identity of all specimens. DNA was extracted using a Puregene™ Isolation Kit per manufacturer's instructions. A 298 bp 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) and the reverse primer RBCSPC (5'-CACTATTCTATGCTCCTTATTKTTAT-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 (20mM) of each buffer, 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., Madison, Wisconsin). Following alignment, sequences were compared to a previously published sequence of *P. leucosticta* from the Northwest Atlantic (**AF271078**) on GenBank using MegaAlign v. 7.1 (DNA Star Inc.) in order to confirm species identification.

Microcosm Description and Experimental Design for Foliose Blade Phase Experiments

To determine the optimum conditions for growth, photosynthetic efficiency of photosystem II (PSII), and pigment and protein content of *Pyropia leucosticta*, blades (1-2 cm) cultured from neutral spores were placed in individual 125 mL Erlenmeyer flasks and grown under a matrix of temperatures, light levels, and photoperiods for four weeks. Blades were grown in sterile VSES (30-32 ppt, 125 mL) and supplied constant filter-sterilized air (1 µM, Pall® Life Sciences) using aquarium air pumps (Tetra Whisper® 300).

Photoperiods (8:16, 12:12, and 16:8 L:D) were controlled using three separate growth chambers (Percival Scientific, Model E-30B). In each chamber, six independently controlled water baths were used to maintain temperature (two each at 10º, 15º, and 20ºC). Each water bath had a submersible aquarium heater (Marineland, 25 W) connected to a digital heater temperature controller (Finnex, HC-810M, ±1.1°C). Four flasks were placed in each water bath and individually wrapped with neutral density filter to achieve light levels of 250, 110, 60, and 30 µmol photons m^{-2} s^{-1}. Light was supplied by 8 cool white fluorescent bulbs in each chamber (Philips Alto II^{TM} 17 W, F17T8/TL741). Trials were repeated a total of three times using two cultures for each trial.

Growth and Photosynthetic Efficiency. The blotted-dry fresh weight (FW) of each blade was determined weekly and specific growth rate (SGR) was calculated as: 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. At the end of the 4-week trial period, blades were split into two; one half was used for phycobilin and protein analysis, while the other half was used for chlorophyll and carotenoid analysis.

Photosynthetic efficiency of PSII (F_v/F_m) was determined weekly starting one week after the blades were placed in the microcosm. 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.

Phycobilins. Phycobilins (R-phycoerythrin, R-PE, & R-phycocyanin, R-PC) were extracted from samples (5-50 mg fresh weight) and determined per the methods of Sampath-Wiley and Neefus (2007) using a dual beam UV-visible spectrophotometer (Helios Alpha).

Soluble and Structural Protein. To measure soluble protein, 1.5 mL of Coomassie Reagent (Bradford 1976) was added to 0.3 mL of supernatant from the phycobilin extraction and incubated at room temperature for 10 minutes. Absorbance was read at 595 nm using a dual-beam UV-visible spectrophotometer (Helios Alpha) and concentrations were calculated by means of standards made with bovine serum albumin (G-Biosciences 786-006).

To measure structural protein, 1.0 mL of 1.0 M NaOH was added to the pellet from the phycobilin extraction and samples were incubated at 4°C for 24 hours. Following incubation, 48 µL of concentrated HCl (12 N) was added to each sample to correct the pH (Korbee et al. 2005a). The above protein assay (Bradford 1976) was then performed on all samples.

Microcosm Description and Experimental Design for Conchocelis Phase Experiments

To determine the optimum conditions for growth, development of mature conchosporangial branches, and conchospore release and germination of *Pyropia leucosticta*, free-living balls of conchocelis filaments visible to the unaided eye were placed in individual 125 mL Erlenmeyer flasks and grown under 9 combinations of temperatures and photoperiods for 8-12 weeks with VSES containing either 20 or 500 µM NH_4^+. Filter-sterilized air (1 µM, Pall® Life Sciences) was supplied to each flask using aquarium air pumps (Tetra Whisper® 300).

Photoperiods (8:16, 12:12, and 16:8 L:D) were controlled using three separate growth chambers (Percival Scientific, Model E-30B). In each chamber, six independently controlled

water baths were used to maintain temperature (two each at 10º, 15º, and 20ºC). Each water bath had a submersible aquarium heater (Marineland, 25 W) connected to a digital heater temperature controller (Finnex, HC-810M, ±2°F). Four flasks were placed in each water bath and light levels were kept at a constant level of 30 µmol photons m^{-2} s^{-1} in each chamber by installing a sheet of neutral density Plexiglas below the light bank. Light was supplied by 8 cool white fluorescent bulbs in each chamber (Philips Alto II™ 17 W, F17T8/TL741). All flasks in one water bath representing each temperature were enriched with either 20 or 500 µM NH_4^+. Trials were repeated a total of 3 times, using conchocelis derived from 2 separate parents in each trial, except the first trial which had conchocelis from a single parent.

Measuring Growth and Assessing Reproductive Development. On a weekly basis, individual balls of conchocelis filaments were photographed using a digital camera (MacroFire, Optronics®) mounted on a dissecting microscope (Olympus SZH10 Research Stereo, Olympus Corporation of America®) and visually assessed for conchosporangial branch development, spore release, and conchospore germination using both a dissecting and compound microscope. At the start of the experiment, all samples had visible signs of mature conchosporangial branch formation, appearing as distinctive square shaped cells with a central stellate chloroplast (Conway and Cole 1977). Conchospore germination was verified by the presence of foliose blades (typically 3-6 cells). Growth of the conchocelis was measured as an increase in surface area (increase in mm^2 day^{-1}; Varela-Alvarez et al. 2004) determined from photographs using the software program ImageJ (1.43u, National Institute of Health, U.S.A.). Growth was measured from the beginning of the experiment until conchospore release and germination occurred, as conchocelis growth stops after conchospore release and germination (Sidirelli-Wolff 1992).

Statistical Analyses

Foliose Blade Phase Experiments. Growth rate and F_v/F_m were each analyzed as a split-split-split plot analysis of variance (ANOVA) with photoperiod as the main plot, temperature as the sub plot, light level as the sub-sub plot, and week as the sub-sub-sub plot (Federer and King 2007). Phycobilin and protein results were each analyzed using a split-split plot ANOVA with photoperiod as the main plot, temperature as the sub plot, and light level as the sub-sub plot (Federer and King 2007). The response variables 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 for (Systat, Inc.).

Conchocelis Phase Experiments. Growth and time until blades were visible were each analyzed as a split-split plot ANOVA with photoperiod as the main plot, temperature as the sub plot, and NH_4^+ concentration as the sub-sub plot (Federer and King 2007). The growth data did not conform to the assumptions of normality and was rank transformed prior to analysis (Conover and Iman 1981). All post-hoc comparisons were made using the Tukey's HSD test. All analyses were performed in SYSTAT 13.00.05 (Systat, Inc.).

Results

Foliose Blade Phase Experiments

Growth Rate. The growth rate (% growth day^{-1}) of *Pyropia leucosticta* blades was significantly affected by light level ($F_{3,108}$= 56.69, p<0.001). Growth rate increased with increasing light level up to 110 μmol photons m^{-2} s^{-1}. Post-hoc analysis revealed that growth was significantly higher at 250 (12.0 ±0.45 % day^{-1}) and 110 (11.18 ±0.46 % day^{-1}) than at 60 (8.1 ±0.44 % day^{-1}) and 30 μmol photons m^{-2} s^{-1} (3.72 ±0.45 % day^{-1}).

The effect of photoperiod on growth rate was dependent on the week ($F_{6,557}$=7.06, p<0.001). At all photoperiods, growth decreased over time, although the magnitude of the decrease differed (Fig. 1). Significant differences in growth under different photoperiods were observed only for the first week, where growth under long day (16:8; 18.63 ±0.76% day^{-1}) conditions was significantly higher than those at neutral day conditions (12:12; 12.33 ±0.76% day^{-1}). Growth under both long and neutral day conditions was significantly higher than that under short day conditions (8:16; 8.67 ±0.76% day^{-1}) during the first week. The highest growth rate was recorded after one week under long day conditions (18.63 ±0.76% day^{-1}) and the slowest growth rate after week four under short day conditions (4.11 ±0.77% day^{-1}; Fig. 1).

Photosynthetic Efficiency. Photosynthetic efficiency of PSII (F_v/F_m) was significantly affected by light level ($F_{3,108}$=32.94, p<0.001) and decreased with increasing light level up to 110 µmol photons m^{-2} s^{-1}. The lowest F_v/F_m was observed in blades grown at 110 (0.51 ±0.01) and 250 µmol photons m^{-2} s^{-1} (0.51 ±0.01), while the highest was recorded in blades grown at 60 (0.55 ±0.01) and 30 µmol photons m^{-2} s^{-1} (0.56 ±0.01).

The effect of temperature on F_v/F_m was dependent on the week ($F_{6,557}$=3.85, p=0.001). There were no significant differences between the F_v/F_m at 10°C across the four weeks (Fig. 2). Blades grown at 15°C had similar F_v/F_m during weeks 1, 3, and 4 but week 1 had a significantly higher F_v/F_m than week 2 (p=0.032). Blades grown at 20°C showed the highest F_v/F_m following the first week of growth, which was followed by a significant reduction in F_v/F_m in week 2; the F_v/F_m remained at the same level from weeks 2-4 (Fig. 2).

Phycobilin Content. The effect of light level on R-phycoerythrin (R-PE) content of blades of *P. leucosticta* was dependent on photoperiod ($F_{6,141}$=3.37, p=0.004). There was no significant difference in R-PE content of blades grown under short or neutral day conditions across the

tested light levels (Table 1). Blades grown under long day conditions showed a unique pattern, with no difference between R-PE content in blades grown at 30 and 60, 110 and 250, or 60 and 250 µmol photons m^{-2} s^{-1}. However, blades grown under long day conditions at 30 and 60 µmol photons m^{-2} s^{-1} had a significantly higher R-PE content than those grown at 110 µmol photons m^{-2} s^{-1} (30: $p<0.001$, 60: $p=0.004$; Table 1).

There was no difference in R-PE content between the three photoperiods at 30 or 60 µmol photons m^{-2} s^{-1} (Table 1). At 110 µmol photons m^{-2} s^{-1}, blades grown under short and neutral day conditions had significantly higher R-PE content than those grown under long day conditions (8:16: $p<0.001$, 12:12: $p=0.003$). At 250 µmol photons m^{-2} s^{-1} blades grown under short day conditions had significantly higher R-PE content than those grown under neutral ($p=0.003$) and long day conditions ($p<0.001$; Table 1).

The effect of light level on R-phycocyanin (R-PC) content was again dependent on photoperiod ($F_{6,141}=3.2$, $p=0.006$). Similar to the pattern observed in R-PE content, there was no difference in the content of R-PC of blades grown under short day conditions across all light levels tested (Table 1). In blades grown under neutral day conditions, R-PC content was significantly higher at 30 than at 250 µmol photons m^{-2} s^{-1} ($p=0.006$). Similarly, the R-PC content of blades grown under long day conditions was significantly higher in blades exposed to 30 µmol photons m^{-2} s^{-1} than in blades grown at 110 ($p<0.001$) and 250 µmol photons m^{-2} s^{-1} ($p<0.001$; Table 1).

There was no significant difference between the R-PC content of blades across the three photoperiod at 30 µmol photons m^{-2} s^{-1} (Table 1). However, at 60 and 110 µmol photons m^{-2} s^{-1} blades grown under short day conditions had significantly higher R-PC content than blades grown under long day conditions (60: $p=0.008$, 110: $p<0.001$). At the highest light level tested,

250 µmol photons m^{-2} s^{-1}, blades grown under short day conditions had significantly higher R-PC content than those grown under both neutral (p<0.001) and long day conditions (p<0.001; Table 1).

Soluble and Structural Protein Content. Light level significantly affected soluble protein content ($F_{3,136}$=5.28, p=0.002), with content generally decreasing with increasing light level. In this case, the effect of light level was independent of photoperiod. There was no significant difference between the soluble protein content of blades grown at 30 and 60 µmol photons m^{-2} s^{-1}. However, blades grown at 30 µmol photons m^{-2} s^{-1} had significantly higher soluble protein content (29.78 ±6.13 mg g^{-1} FW) compared to those grown at 110 (19.04 ±6.05 mg g^{-1} FW; p=0.005) and 250 µmol photons m^{-2} s^{-1} (19.57 ±5.96 mg g^{-1} FW; p=0.004). There was no significant difference in the soluble protein content between blades grown at 60 µmol photons m^{-2} s^{-1} (30.87 ±6.04 mg g^{-1} FW) and those either at 110 or 250 µmol photons m^{-2} s^{-1}.

Similarly, light level significantly affected the structural protein content ($F_{3,141}$=8.87, p<0.001) of *Pyropia leucosticta*, although the pattern differed from that detailed above. Like the pattern observed in soluble protein content, there was no significant difference between the soluble protein content of blades grown at 30 (33.94 ±4.06 mg g^{-1} FW) and 60 µmol photons m^{-2} s^{-1} (33.24 ±4.06 mg g^{-1} FW). However, blades grown at 30 µmol photons m^{-2} s^{-1} had significantly higher soluble protein content compared to those grown at 110 (24.10 ±4.06 mg g^{-1} FW; p<0.001) and 250 µmol photons m^{-2} s^{-1} (30.30 ±4.01 mg g^{-1} FW; p=0.002). Blades grown at 60 µmol photons m^{-2} s^{-1} had significantly higher structural protein compared to those grown at 110 (p=0.021), but they did not differ from blades grown at 250 µmol photons m^{-2} s^{-1}.

Ratio of Phycobilin to Soluble Protein Content. The effect of light level on the ratio between phycobilin (PB) and soluble protein (SP) content was dependent on photoperiod

($F_{6,135}$=2.23, p=0.044). In blades grown under both short and neutral day photoperiods, the PB:SP was constant across all light levels. Blades grown under long day conditions had the highest PB:SP at 30 µmol photons m^{-2} s^{-1} (0.63 ± 0.06), which was significantly higher than in blades grown at 110 (0.28 ± 0.06, p<0.001) and 250 µmol photons m^{-2} s^{-1} (0.32 ± 0.06, p=0.018; Table 1).

Conchocelis Phase Experiments

Growth. Growth of free-living conchocelis tufts was significantly affected by photoperiod ($F_{2,4}$=16.55, p=0.012), with highest growth under long day conditions (0.06 ±0.01 mm^2 day^{-1}). Conchocelis tufts grown under long and neutral day (0.04 ±0.01 mm^2 day^{-1}) conditions grew significantly faster than those under short day conditions (0.02 ±0.01 mm^2 day^{-1}; 16:8: p=0.011, 12:12: p=0.034).

Conchospore Germination. The appearance of developing blades was used as an indication of conchospore release and germination. Conchocelis tufts cultured at 10°C took an average of 36.97 ±3.21 days until conchospore germination occurred, while tufts at 15°C and 20°C took an average of 41.55 ±3.48 and 48.63 ±22.27 days, respectively. Conchospore germination in cultured tufts under long day conditions took an average of 39.63 ±6.57 days, while tufts cultured under neutral and short day conditions took 42.55 ±7.04 and 44.97 ±9.17 days, respectively. Nitrogen concentrations had no effect on the days until conchospore release and germination with tufts cultured with 20 µM NH_4^+ taking 42.84 ±4.89 days, while those cultured with 500 µM NH_4^+ took 41.93 ±15.06 days. None of the differences were statistically significant.

Discussion

The results of this study provide some of the background information required to initiate aquaculture of *Pyropia leucosticta* in the Northwest Atlantic. It is important to note that the effect of one factor, such as photoperiod, on growth rate may depend on the level of another factor, such as time. The interaction we found between the effects of photoperiod and week was also found by Sidirelli-Wolff (1992) who reported that blades grown under long day conditions grew quickly for the first week and then experienced a rapid decrease in growth thereafter. Such results illustrate the importance of examining multiple factors simultaneously in elucidating the optimal conditions for growing seaweeds as aquaculture crops. Further, while different combinations of conditions can produce statistically significant differences in a response, some of these differences may not be of biological or practical significance. For example, we found statistically significant differences in F_v/F_m measurements across light levels, but the overall range was very small (0.5-0.56).

Some of our results were similar to those found in previous studies (Sidirelli-Wolff 1992; Orfanidis 2001; He and Yarish 2006) while others were not. For example, previous studies reported a broad range of tolerance to temperature in *P. leucosticta* from the North Atlantic (Sidirelli-Wolff 1992; He and Yarish 2006) and Mediterranean (Orfanidis 2001), but our results indicate temperatures above 15°C may be sub-optimal. This and other differences between studies might actually reflect species differences. Many species of *Pyropia* are morphologically similar and difficult to distinguish without molecular tools. Historically, there has been some confusion regarding the identity of *P. leucosticta*. It is possible that species used for physiological studies from the Mediterranean, where higher optimal temperatures were reported, were actually *P. koreana* (M.S. Hwang & I.K. Lee) M.S. Hwang, H.G. Choi, Y.S. Oh & I.K. Lee

(Brodie et al. 2007; Vergés et al. 2013). Similarly, molecular analysis of cultures from the He and Yarish (2006) *P. leucosticta* study suggests that they were actually using *P. yezoensis*, an Asian species introduced to Long Island Sound sometime after 1970 (Neefus et al. 2008).

Studies on diverse seaweeds have shown that red algae can adjust both the size of photosynthetic antennae and the number of photosynthetic units (PSU) depending on the light level (Waaland et al. 1974; Mishkind and Mauzerall 1980). Due to the high energetic cost of PSU adjustments, it is generally regarded that seaweeds optimize rather than maximize photosynthesis (Ramus 1981). Our results indicate that blades of *P. leucosticta* grown under short day conditions had higher phycobilin (R-PE and R-PC) content than blades grown under neutral or long day conditions at high light levels. There are two explanations for this pattern. First, blades grown under neutral and long day conditions had higher growth rates and could have effectively diluted the photosynthetic pigment concentration as they expanded rapidly. Second, blades grown under short day conditions may have been light limited by daylength. Therefore, these blades may have been practicing PSU adjustment (both in size of antennae and number of PSUs) to optimize light capture and photosynthesis at all light levels. Our data support this hypothesis since phycobilin content was independent of light level in blades grown under short day conditions (Table 1).

Phycobilins form structures on the surface of the thylakoid membrane, held together by covalent bonds in phycobilin-protein complexes known as phycobiliproteins (Lobban and Harrison 1994), which, unlike chlorophylls and carotenoids, are water-soluble. Hence, a relationship between phycobilin and soluble protein content has been reported in several red seaweeds (Hernández et al. 1993; Korbee et al. 2005b). The ratio between PB: SP in this study increased with decreasing light level under long day conditions, which indicates that phycobilins

were serving as nitrogen storage compounds under low light, long day conditions (Table 1). Phycobilins accounted for up to 76% of soluble protein depending on the treatment conditions. We also found that soluble protein content (i.e. proteins that dissolve in water including phycobiliproteins) was highest at low light levels, coinciding with the highest pigment content. Furthermore, seaweed blades exposed to low light typically have more chloroplasts or larger chloroplast with more thylakoid membranes per unit area (Talarico and Maranzana 2000). An increase in thylakoid membranes would explain the observed increase in structural protein content in blades of *P. leucosticta* grown under low light.

Previous work has indicated that the conchocelis of *P. leucosticta* requires short day conditions for the release of conchospores (Gargiulo et al. 1994; Orfanidis 2001). Contrary to these previous reports, conchospores in this study were released and germinated under all photoperiods, temperatures, and NH_4^+ levels tested. However, earlier studies have been conducted with material from the Mediterranean, which as previously mentioned, may well be a different species. Since many foliose Bangiales are difficult to distinguish morphologically, molecular verification is essential when conducting experiments on this group.

Successful cultivation of any crop requires an understanding of its physiology and environmental requirements. A goal of this study was to provide some of the knowledge required to grow *Pyropia leucosticta* as an aquaculture crop in the Northwest Atlantic. Optimum conditions for the production of *P. leucosticta* will ultimately be based on the intended use. It could be utilized as a sea vegetable, source of pigments (namely, R-phycoerythrin which is used as a fluorescent tag), protein substitute for fish meal, and countless other applications (Mumford and Miura 1988). For example, if *P. leucosticta* were used as a sea vegetable, production should

365 focus on producing highly pigmented biomass and optimum conditions would range from 10-
366 15°C, 30-110 µmol photons m^2 s^{-1}, with ≥12 hours of light in the day.

367 Understanding and controlling the life history of a potential aquaculture crop is also
368 paramount to future success. While we were successful in completing the life history of *Pyropia*
369 *leucosticta*, further work is still required to successfully control its conchocelis phase. We were
370 able to successfully induce conchospore release under a wide range of factors, but were not able
371 to identify environmental conditions that would suppress release and allow vegetative
372 proliferation of the conchocelis phase. In this study, we found that conchospore release occurred
373 promptly after the formation of conchosporangial branches and previous studies have shown that
374 growth of the conchocelis stops after conchospore release (Sidirelli-Wolff 1992). Future research
375 should focus on determining parameters (photoperiod, light level, light quality, temperature) that
376 will suppress the formation of conchosporangial branches to enhance vegetative growth of the
377 conchocelis phase. Mass quantities of conchocelis are required prior to conchosporangial branch
378 formation and subsequent conchospore release in order to support a commercial-scale
379 aquaculture operation.

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387 **Literature Cited**

388 Bradford MM (1976) A rapid and sensitive method for the quantification of microgram
389 quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:
390 248-254
391 Bray TL, Neefus CD, Mathieson AC (2006) Morphological and molecular variability of
392 *Porphyra purpurea* (Roth) C. Agardh (Rhodophyta, Bangiales) from the Northwest
393 Atlantic. Nova Hedwigia 82: 1-22
394 Brodie J, Bartsch I, Neefus C, Orfanidis S, Bray T, Mathieson AC (2007) New insights into the
395 cryptic diversity of the North Atlantic-Mediterranean '*Porphyra leucosticta*' complex: *P.*
396 *olivii* sp. nov. and *P. rosengurtii* (Bangiales, Rhodophyta). Eur. J. Phycol. 42(1): 3-28
397 Brodie J, Irvine LM (2003) Seaweeds of the British Isles, Volume 1 Rhodophyta, Part 3B
398 Bangiophycidae. The National History Museum, London
399 Brodie J, Mols-Mortensen A, Ramirez ME, Russell S, Rinkel B (2008) Making the links:
400 towards a global taxonomy for the red algal genus *Porphyra* (Bangiales, Rhodophyta). J.
401 Appl. Phycol. 20: 939-949
402 Broom JES, Farr TJ, Nelson WA (2004) Phylogeny of the *Bangia* flora of New Zealand suggests
403 a southern origin for *Porphyra* and *Bangia* (Bangiales, Rhodophyta). Mol. Phylogenet.
404 Evol. 31: 1197-1207
405 Clokie JJP, Boney AD (1980) *Conchocelis* distribution in the Firth of Clyde: estimates of the
406 lower limits of the photic zone. J. Exp. Mar. Biol. Ecol. 46: 111-125
407 Cole K, Conway E (1980) Studies in the Bangiaceae: reproductive modes. Bot. Mar. 23: 545-
408 553
409 Conover WJ, Iman RL (1981) Rank transformation as a bridge between parametric and
410 nonparametric statistics. Am. Stat. 35(3): 124-129

411 Conway E, Cole K (1977) Studies in the Bangiaceae: structure and reproduction of the
412 conchocelis of *Porphyra* and *Bangia* in culture (Bangiales, Rhodophyceae). Phycologia
413 16(2): 205-216
414 Drew KM (1949) Conchocelis-phase in the life-history of *Porphyra umbilicalis* (L.) Kütz.
415 Nature 164: 748-749
416 Federer WT, King F (2007) Variations on split plot and split block experimental designs. Wiley-
417 Interscience, Hoboken
418 FAO, Food and Agricultural Organization of the United Nations (2014) Cultured aquatic species
419 information programme: *Porphyra* spp. FAO Fisheries and Aquaculture Department,
420 Rome
421 Figueroa FL, Salles S, Aguilera J, Jimenez C, Mercado J, Vinegla B, Flores-Moya A, Altamirano
422 M (1997) Effects of solar radiation of photoinhibition and pigmentation in the red alga
423 *Porphyra leucosticta*. Mar. Ecol. Prog. Ser. 151: 81-90
424 Gargiulo GM, Masi FD, Genovese G, Tripodi G (1994) Karyology and effects of temperature
425 and photoperiod on the life-cycle of *Porphyra leucosticta* Thuret in Le Jolis (Bangiales,
426 Rhodophyta) from the Mediterranean Sea. Jpn. J. Phycol. 42: 271-280
427 He P, Yarish C (2006) The developmental regulation of mass cultures of free-living conchocelis
428 for commercial net seeding of *Porphyra leucosticta* from Northeast America.
429 Aquaculture 257: 373-381
430 Hernández I, Corzo A, Gordillo FJ, Robles MD, Saez E, Fernández JA, Niell FX (1993)
431 Seasonal cycle of the gametophytic form of *Porphyra umbilicalis*: nitrogen and carbon.
432 Mar. Ecol. Prog. Ser. 99: 301-311

Israel A (2010) The extreme environments of Porphyra, a fast growing and edible red marine macroalga. In: Seckbach J, Chapman DJ (eds) Red Algae in the Genomic Age, Cellular Origins, Life in Extreme Habitats and Astrobiology. Springer Science and Business Media, New York, pp 61-75

Kim JK, Kraemer GP, Neefus CD, Chung IK, Yarish C (2007) Effects of temperature and ammonium on growth, pigment production and nitrogen uptake by four species of *Porphyra* (Bangiales, Rhodophyta) native to the New England coast. J. Appl. Phycol. 19: 431-440

Klein AS, Mathieson AC, Neefus CD, Clain DF, Taylor HA, Teasdale BW, West AL, Hehre EJ, Brodie J, Yarish C, Wallace AL (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

Korbee N, Figueroa FL, Aguilera J (2005a) Effect of light quality on the accumulation of photosynthetic pigments, proteins and mycosporine-like amino acids in the red algae *Porphyra leucosticta* (Bangiales, Rhodophyta). J. Photoch. Photobio. B. 80: 71-78

Korbee N, Huovinen P, Figueroa FL, Aguilera J, Karsten U (2005b) Availability of ammonium influences photosynthesis and the accumulation of mycosporine-like amino acids in two *Porphyra* species (Bangiales, Rhodophyta). Mar. Biol. 146: 645-654

Krishnamurthy V (1969) The conchocelis phase of three species of *Porphyra* in culture. J. Phycol. 5: 42-47

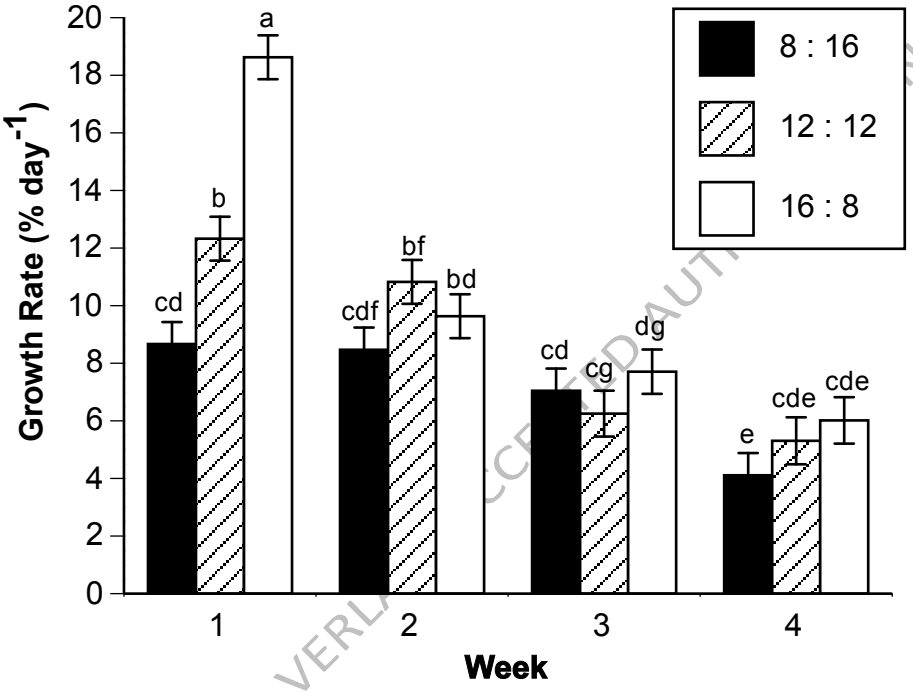
Lobban CS, Harrison PJ (1994) Seaweed ecology and physiology. Cambridge University Press, New York

455 Mishkind M, Mauzerall D (1980) Kinetic evidence for common photosynthetic step in diverse
456 seaweeds. Mar. Biol. 56(4): 264-265
457 Mols-Mortensen A, Neefus CD, Nielsen R, Gunnarsson K, Egilsdóttir S, Pedersen PM, Brodie J
458 (2012) New insights into the biodiversity and generic relationships of foliose Bangiales
459 (Rhodophyta) in Iceland and the Faroe Islands. Eur. J. Phycol. 47(2): 146-159
460 Mumford TF, Miura A (1988) *Porphyra* as food: cultivation and economics. In: Lembi CA,
461 Waaland JR (eds) Algae and Human Affairs. Cambridge University Press, New York, pp
462 87-117
463 Neefus CD, Mathieson AC, Bray TL, Yarish C (2008) The distribution, morphology, and
464 ecology of three introduced Asiatic species of *Porphyra* (Bangiales, Rhodophyta) in the
465 Northwestern Atlantic. J. Phycol. 44: 1399-1414
466 Orfanidis S (2001) Culture studies of *Porphyra leucosticta* (Bangiales, Rhodophyta) from the
467 Gulf of Thessaloniki, Greece. Bot. Mar. 44: 533-539
468 Ott FD (1966) A selected listing of xenic cultures. *Systematics-Ecology Program* No. 72 (Mar.
469 Biol. Lab., Woods Hole, MA), pp 1-45
470 Pereira R, Yarish C (2010) The role of *Porphyra* in sustainable culture systems: physiology and
471 applications. In: Israel A, Einav R (eds) Role of Seaweeds in a Globally Changing
472 Environment. Springer, New York, pp 339-354
473 Ramus J (1981) The capture and transduction of light energy. In: Lobban CS, Wynne MJ (eds)
474 The Biology of Seaweeds. Blackwell Scientific Publications, Boston, pp 458-492
475 Sampath-Wiley P, Neefus CD (2007) An improved method from estimating R-phycoerythrin and
476 R-phycocyanin contents from crude aqueous extracts of *Porphyra* (Bangiales,
477 Rhodophyta). J. Appl. Phycol. 19: 123-129

478 Sidirelli-Wolff M (1992) The influence of temperature, irradiance and photoperiod on the
479 reproductive life history of *Porphyra leucosticta* (Bangiales, Rhodophyta) in laboratory
480 culture. Bot. Mar. 35: 251-257
481 Schmitt C (2013) Maine's kelp highway. Maine Boats, Homes & Harbors 122: 30-35.
482 Sutherland J, Lindstrom SC, Nelson W, Brodie J, Lynch M, Hwang MS, Choi HG, Miyata M,
483 Kikuchi N, Oliveira M, Farr T, Neefus C, Mols-Mortensen A, Milstein D, Müller K
484 (2011) A new look at an ancient order: generic revision of the Bangiales (Rhodophyta). J.
485 Phycol. 47: 1131-1151
486 Talarico L, Maranzana G (2000) Light and adaptive responses in red macroalgae: an overview. J.
487 Photoch. Photobio. B 56: 1-11
488 Teasdale B, West A, Taylor H, Klein A (2002) A simple restriction length polymorphism
489 (RFLP) assay to discriminate common *Porphyra* (Bangiophyceae, Rhodophyta) taxa
490 from the Northwest Atlantic. J. Appl. Phycol. 14: 293-298
491 Varela-Alvarez E, Stengel DB, Guiry MD (2004) The use of image processing in assessing
492 conchocelis growth and conchospore production in *Porphyra linearis*. Phycologia 43(3):
493 282-287
494 Vergés A, Comalada N, Sánchez N, Brodie J (2013) A reassessment of the foliose Bangiales
495 (Rhodophyta) in the Balearic Islands including a proposed synonymy of *Pyropia olivii*
496 with *Pyropia koreana*. Bot. Mar. 56(3): 229-240
497 Waaland JR, Waaland SD, Bates G (1974) Chloroplast structure and pigment composition in the
498 red alga *Griffithsia pacifica*: regulation by light intensity. J. Phycol. 10: 193-199

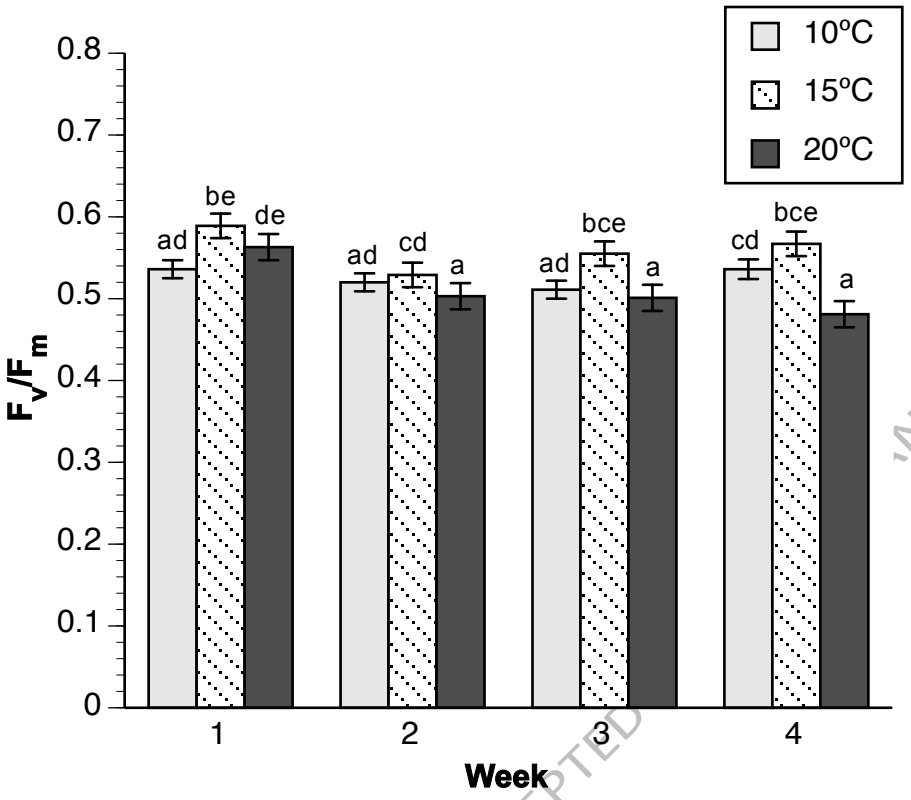
499 Yarish C, Wilkes R, Chopin T, Fei XG, Mathieson AC, Klein AS, Neefus CD, Mitman GC,
500 Levine I (1998) Domestication of indigenous *Porphyra* (nori) species for commercial
501 cultivation in Northeast America. World Aquac. 29: 26-29

502 **Figure Captions**



503

504 Fig. 1: Growth rate (% growth day^{-1}) of *Pyropia leucosticta* at three different photoperiods (8:16,
505 12:12, and 16:8 L:D) over a period of four weeks (mean ± SE). Bars with a letter in common are
506 not significantly different (α=0.05). Although analysis was performed on rank transformed data,
507 original data and standard errors are graphed with letters derived from post-hoc analysis of the
508 rank transformed data.



509

510 Fig. 2: Photosynthetic efficiency of PSII (F_v/F_m) of *Pyropia leucosticta* at three different

511 temperatures (10°, 15°, and 20°C) over a period of four weeks (mean ± SE). Bars with a letter in

512 common are not significantly different (α=0.05). Although analysis was performed on rank

513 transformed data, original data and standard errors are graphed with letters derived from post-hoc

514 analysis of the rank transformed data.

515

Table 1: R-phycoerythrin (R-PE), R-phycocyanin (R-PC), and The ratio of phycobilin (PB) to soluble protein (SP) content in blades of *Pyropia leucosticta* grown under a combination of three photoperiods (8:16, 12:12, and 16:8 L:D) and four light levels (30, 60, 110, and 250 µmol photons m^{-2} s^{-1}; mean ± SE). Boxes with a letter in common *within each column* are not significantly different (α=0.05). Although analysis was performed on rank transformed data, original data and standard errors are presented with letters derived from post-hoc analysis of the rank transformed data.

Photoperiod (L:D)	Light Level (µmol photons m^{-2} s^{-1})	R-PE (mg g^{-1} FW)	R-PC (mg g^{-1} FW)	PB:SP
8:16	30	6.38 ± 0.61^{a}	3.61 ± 0.39^{a}	0.49 ± 0.06^{ac}
	60	7.11 ± 0.61^{a}	3.64 ± 0.39^{a}	0.50 ± 0.06^{ac}
	110	5.96 ± 0.61^{ah}	2.84 ± 0.39^{ad}	0.46 ± 0.06^{ac}
	250	7.33 ± 0.61^{a}	4.06 ± 0.39^{a}	0.44 ± 0.06^{abc}
12:12	30	5.92 ± 0.61^{af}	3.14 ± 0.39^{ad}	0.36 ± 0.06^{bc}
	60	5.75 ± 0.64^{af}	2.76 ± 0.41^{adf}	0.40 ± 0.06^{abc}
	110	4.60 ± 0.64^{aef}	2.10 ± 0.41^{de}	0.35 ± 0.06^{abc}
	250	3.98 ± 0.61^{efgh}	1.71 ± 0.39^{bcef}	0.33 ± 0.06^{ab}
16:8	30	5.69 ± 0.64^{af}	3.04 ± 0.41^{ad}	0.63 ± 0.06^{c}
	60	4.59 ± 0.61^{acf}	2.14 ± 0.39^{bd}	0.42 ± 0.06^{ac}
	110	2.18 ± 0.61^{bdg}	0.94 ± 0.39^{c}	0.28 ± 0.06^{b}
	250	2.94 ± 0.61^{bce}	1.30 ± 0.39^{bce}	0.32 ± 0.06^{ab}