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Spatial and Temporal Variability in Nutritional Physiology of Pyropia perforata

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SPATIAL AND TEMPORAL VARIABILITY IN NUTRITIONAL PHYSIOLOGY OF *PYROPIA PERFORATA*

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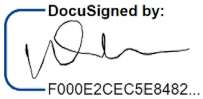

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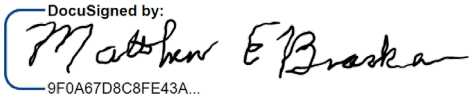

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Dedication

To Shawn M. Hannah.

ABSTRACT

Spatial and Temporal Variability in Nutritional Physiology of *Pyropia perforata*

by
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The red alga, *Pyropia perforata,* commonly referred to as nori, is ubiquitous in intertidal communities throughout coastal California and offers high nutritional value to marine herbivores. However, California rocky shores are dynamic ecosystems, with dramatic abiotic and biotic shifts resulting in environmental heterogeneity. Research has shown that seaweed nutrient physiology is impacted by environmental heterogeneity, yet there is sparse data on how internal nitrogen and total protein content change as a result of environmental heterogeneity. Here, I studied the impacts of seasonality, geography (spanning 4° of latitude from Northern to Southern California), and the onset of environmental heterogeneity on internal nitrogen percentages and total protein content in the nutrient rich, broadly distributed red alga *P. perforata.* Percent nitrogen and total protein in *P. perforata* were found to be higher in northern sites compared to southern sites, suggesting a spatial influence on nutrient physiology. Moreover, the winter and spring seasons had higher % nitrogen and total protein compared to summer and fall, further suggesting a temporal effect on nutrient physiology. Cultured *P. perforata* also exhibited an increase in % nitrogen and total protein as nutrient concentration increased, an effect that was consistent whether the nitrogen source provided was nitrate (NO_3^-) or ammonium (NH_4^+). There was little evidence, however, of a correlation between abiotic factors (including temperature, solar radiation, day length, chl *a* , wind speed, Coastal Upwelling Transport Index, Biologically Effective Upwelling Transport Index , and estimated nitrate) with % nitrogen and total protein. However, solar radiation, daylength, and sea surface nitrogen concentration were significantly correlated with % nitrogen. Overall, there is evidence from this study that % nitrogen and total protein in *P. perforata* vary as a function of time and space. Furthermore, environmental nitrogen concentration can be used as a proxy for internal nitrogen content.

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Introduction

Photoautotrophic eukaryotes, or algae, are pivotal to marine ecosystems, primarily because of their ability to convert inorganic materials into organic compounds. Early research showed that photoautotrophic eukaryotes convert carbon, nitrogen, and phosphorous materials at a constant rate of 106C:16N:1P (Redfield 1934). This Redfield ratio illustrates the average rate of uptake and incorporation that defines the elemental composition of photoautotrophic eukaryotes in the ocean (Arrigo 2005). Algae utilize carbon dioxide as a source of carbon for photosynthesis through the direct uptake of aqueous CO_2 in the ocean, or bicarbonate (HCO_3^-) that is converted to CO_2 (Bowen 1915, Hazen et al. 2013). Nitrogen and phosphorus are taken up as essential nutrients for algal growth (Harvey 1940, Auer 1979), which is controlled by nitrogen and phosphorus availability (Fleming 1940, Reynolds 2006), both of which can be limiting factors for algal growth (Kratz and Myers 1955, Fang 1993). Although both nitrogen and phosphorus limit growth, nitrogen is generally considered to be the most limiting factor to growth in coastal marine algae (Ryther and Dunstan 1971).

Like carbon, nitrogen is absorbed into the ocean through a gaseous form (N_2) and modified by the nitrogen cycle (Schloesing and Muntz 1877, Cooper 1937, Falkowski 1997). Through microbial action, the N_2 gets fixed and converted into oxidized forms (nitrogen species) and back into dissolved N_2 (Hellreigel and Wilfrath 1888, Beijerinck 1901, Harvey 1926, Falkowski 1997). Specifically, after nitrogen fixation, ammonium (NH_4^+) is oxidized into nitrite (NO_2^-) and then nitrate (NO_3^-) (Laurent 1889, Winogradsky 1890, Falkowski 1997); nitrate can also be reduced to nitrite and ultimately ammonium. With seaweeds, many studies have investigated the relationship between nitrogen uptake and nitrogen species and reported that ammonium is taken up more rapidly than nitrate, as nitrate must be converted to ammonium internally via the GS-GOGAT pathway before amino acid assimilation (Kishorekumar 2020),

and, partially because ammonium is the more reduced form of nitrogen (D'Elia et al. 1978, Haines and Wheeler 1978, Rosenburg et al. 1984, Ahn et al. 1998, Phillips and Hurd 2004). Additionally, slower uptake rates of the more oxidized nitrate have been associated with an induction period, which is the time the macroalgae needs to activate energy for nitrate metabolism (Robbins 1937, Cramer and Myers 1948, Thomas and Harrison 1988). Although uptake of ammonium by seaweeds is often faster than nitrate uptake, both nitrogen species are important for nutrient physiology (Duffy et al. 1990, Miller and Hay 1998).

Nutrient physiology is the study of living organisms accruing and transforming nutrients into various pathways such as growth, health, storage, and energy production (Lobban et al. 1994). In seaweeds, nutrient uptake, growth rate, critical concentration of tissue nutrients, and nutrient storage are used as metrics for nutrient physiology, and help to explain internal processes that occur while seaweeds accrue and transform nutrients (Harrison et al. 1989, Harrison and Hurd 2001). These processes are often viewed comparatively, with previous research on nutrient physiology showcasing strong correlations with seaweed growth as a function of nutrient uptake, nutrient storage, and assimilation (Rosenburg et al. 1984, Lobban et al. 1994, Viaroli 1996, Harrison and Hurd 2001).

Nutrients are absorbed by seaweeds through either passive or active transport (Hurd 2014). Passive transport occurs via diffusion of nutrients along a concentration gradient (Roleda and Hurd 2019), whereby the small nutrient molecules move freely through the cell wall without using energy (Dainty 1962). Passive diffusion can be estimated by plotting nutrient uptake against concentration yields and modeling the linear relationship. Alternatively, active transport requires energy to fuel membrane support systems, including carrier proteins (Hurd 2014), and usually has a hyperbolic relationship with nutrient concentration. There is evidence that active transport is primarily used by seaweeds for nitrogen and phosphorus uptake when seaweeds are exposed to

ambient light levels (Lobban et al. 1994). Nutrient uptake rates (V = μmol*h^{-1}*g^{-1}) are useful indicators of seaweed health and growth strategies (Claassen and Barber 1974, D'Elia et al. 1978, Ryther et al. 1981, Duke et al. 1989). Once absorbed, nutrients are transported to different ion channels and are either stored or used for growth (Hurd 2014). However, the process of how and when macroalgae assimilate absorbed nutrients is poorly understood (Hurd et al. 1996, Taylor et al. 1998).

Nutrient uptake (absorption) in seaweeds is generally described as a Michaelis-Menten function, with nutrient uptake rate (V) plotted against nutrient concentration (S) (Maclssac and Dugdale 1969, D'Elia et al. 1978). The Michaelis-Menten function is: $V = (V_{max} \times S)/(K_s+S)$, where V_{max} is the maximal uptake rate and K_s is the half-saturation value. K_s is commonly used to measure macroalgal nutrient uptake at lower concentrations, but this is dependent on V_{max} (Bracken and Stachowicz 2006). Macroalgae with similar Michaelis-Menten slopes (α) may have a different K_s and V_{max}, indicating that K_s is reflective of their V_{max} value, and not their ability to uptake nutrients at low concentrations. α represents the non-saturation rate of nitrate uptake (V_{max}/K_s) and gives a more accurate representation of macroalgal uptake rates at low concentrations (Healey 1980, Harrison et al. 1989, Harrison and Hurd 2001). At high concentrations, a high V_{max} indicates a seaweed's ability to continuously uptake nutrients rapidly (Harrison et al. 1986, Taylor et al. 1998, Bracken and Stachowicz 2007).

Although instantaneous uptake rate is key to understanding seaweed growth rates, it has less relevance to survivorship, which is more often impacted by nutrient storage capacity (Pedersen et al. 2010). Seaweed nutrient storage is affected by many factors. For example, intertidal seaweeds with shorter submergence periods have larger storage capacity (Bracken et al. 2011), suggesting that they have adapted (or can adapt) to changes in submergence by increasing their storage capacity (Benes and Bracken 2016). Increased storage capacity further implies that

less submergence time is advantageous to seaweed consumers, by maximizing nutritional value (Benes and Bracken 2016). This has ramifications for seaweed nutrient physiology as abiotic factors vary across broad temporal and spatial scales (Hanisak and Harlin 1978, Taylor et al. 1998, Phillips and Hurd 2003, Jung et al. 2016). Nutrient storage capacity is best estimated by critical nutrient concentration, which is the percentage of a given element (C, N, or P) present in the seaweed tissue (D'Elia et al. 1978, Pedersen et al. 1997, Harrison and Hurd 2001, Lourenco et al. 2006). Critical nutrient concentration is the best indicator of seaweed nutrient status, because it measures the internal nutrient concentration available for growth (Ulrich 1952). For example, the kelp *Macrocystis pyrifera* is able to utilize its nitrogen reserves up to three weeks without signs of negative growth when exposed to a low nutrient period (Haines and Wheeler 1978). Moreover, the kelp species *Laminaria solidungula* experiences dormancy during the low-nutrient summer months, while actively storing carbon. The carbon storage from the summer allows this kelp to grow during complete darkness in the winter months when nutrients are readily available (Chapman et al. 1980, Dunton et al. 1982). The critical nutrient concentration of the kelp allows for prolonged survival despite no additional nutrients. Many other seaweeds are able to persist for prolonged periods utilizing stored nitrogen (Naldi et al. 2002).

Internal nitrogen concentrations in seaweeds can exceed the amount of nitrogen necessary to sustain seaweed growth, after which additional uptake is considered "luxury" (Hanisak 1983, Rosenbug et al. 1984, McGlathery et al. 1996, Naldi and Wheeler 1999, Shpigel et al. 1999, Taylor et al. 2006). Luxury uptake allows seaweeds to maximize the total nitrogen it can store and use when it becomes nutrient deprived (Hanisak 1990). Seaweeds with higher percent total nitrogen often have higher amino acid concentrations, and possibly higher protein concentrations (Angell et al. 2016), which may convey higher nutritional value to their consumers. Red seaweeds have been shown to have higher percent nitrogen, partly due to their abundant phycobiliproteins,

than brown or green seaweeds (Hanisak 1990, Harrison and Hurd 2001, Lourenco et al. 2002) and many organisms prefer to eat red seaweeds instead of browns or greens (Shipgel et al. 1999).

Environmental heterogeneity alters nutrient physiology of intertidal and subtidal seaweeds (Lobban et al. 1994) through irradiance and ambient nutrient concentrations. These factors of environmental heterogeneity play key roles in physiology, and naturally vary on different spatial and temporal scales due to seasonality, geography, upwelling and eutrophication (Ryther et al. 1981, Gerard et al. 1982, Breeman 1990, Archer et al. 1996, Wilkerson et al. 2000, Gilbert and Burkholder 2011, Hurd 2014). In central California, the main source of nitrate is from upwelling (Barth et al. 2007), where nutrient rich cold water is brought up to the ocean surface at concentrations that can exceed 50 μmol (Graham and Largier 1997). Ammonium concentrations are often measured at low amounts (3μmol), but this is a result of other autotrophs rapidly uptaking ammonium, creating a larger flux in concentration (Kirchman 1994). However, ammonium is often found at higher concentrations near estuaries and tide pools, with less flux in concentrations (Bracken et al. 2011, Hughes et al. 2011). Coastal runoff of heavily eutrophic water can lead to very high ammonium concentrations (Hessen et al. 1997), fueling seaweed growth (Heisler et al. 2008).

The globally ubiquitous red seaweed *Pyropia* has been heavily researched for offshore aquaculture (where it is called nori), primarily because of its broad distribution and high internal nitrogen concentrations (Kim et al. 2017). *Pyropia* populations are typically found in the high intertidal zone (Abbott and Hollenberg 1976) and % nitrogen ranges between 3.5-6% (Kang et al. 2014). For comparison, in giant kelp *Macrocystis pyrifera*, % nitrogen ranges from 2-2.5% (Gerard 1982). Seaweed protein content is generally estimated by multiplying % tissue nitrogen by 5 (Angell et al. 2016), with a higher % N value suggesting a higher protein concentration. The broad geographic range and high protein content of *Pyropia* has led to the development of the

massive nori aquaculture industry (Kurokura 2004, O'Connor 2017), yet temporal and spatial variability in internal nitrogen and protein concentration is still poorly understood. *Pyropia* sp. is a global genus that survives in numerous environments. In California, *Pyropia perforata* is ubiquitous in the upper intertidal zone, and grows in large patches that outcompete other seaweeds (Lindstrom et al. 2015**)**, even though it is an annual. *Pyropia perforata* recruits in early spring, which corresponds with the start of the upwelling season and longer daylength in California (Romero 2009). The geological range, high value of %N, and timing of recruitment in concert with increased nutrient concentrations and daylength, make *Pyropia perforata* an excellent species to use to understand how nutrient physiology changes over space and time.

The goal of this nutrient physiology study with *Pyropia perforata* was to understand how % tissue nitrogen and protein concentration vary geographically and seasonally. On the coast of California, *Pyropia perforata* is common throughout the upper intertidal (Abbott and Hollenberg 1976), but abiotic factors and oceanic nutrient concentrations differ significantly throughout its range and among seasons (Horn et al. 1983, Huyer 1983, Taylor et al. 1998). The direct link between ambient nutrient concentrations and macroalgal nutrient physiology also remains poorly understood. Seaweed % tissue nitrogen is often used to study protein kinetics and survivorship (Ryther et al. 1981, Hurd 2014). Therefore, internal nitrogen % is an important measurement of nutrient physiology, and is useful in estimating protein concentration, however, not all nitrogen stored in seaweed tissues is utilized to make proteins (Lourenco et al. 2002). Therefore, more study is needed to better understand the factors influencing the relationship between % tissue nitrogen and protein concentrations in *Pyropia perforata*.

Since it is poorly understood how time and space affect nutrient physiology, the aim of my first question was to understand how internal % nitrogen and protein concentration vary across a season and over a large geographic scale. I investigated my first hypothesis that %

nitrogen and protein concentration in *Pyropia perforata* would be significantly higher during high intensity upwelling periods and in northern locations by sampling every month during the *Pyropia perforata* season, at five sites spaning a 400-mile range and over 4° of latitude. The differences in environmental heterogeneity between seasons and geography led me to believe that areas with high, consistent nutrient levels would positively influence % nitrogen and protein concentration. My second hypothesis was derived from my first hypothesis. I wanted to test whether nutrient concentration alone could drive % nitrogen and protein concentration in *Pyropia perforata* and examine how nutrient species influenced that relationship. Previous work had shown that ammonium had a faster uptake rate than nitrate in numerous seaweed species, but the quantitative difference in % nitrogen and protein concentration when cultured in a singular nutrient species was poorly understood. Therefore, I hypothesized that nutrient concentration was significantly positively correlated to % nitrogen and protein concentration. For my last hypothesis, I was excited to investigate how the relationship between % nitrogen and protein concentration derived from the previous hypotheses were correlated to abiotic factors that contribute to nutrient physiology in seaweeds. Nutrient physiology is known to be affected by environmental heterogeneity (Lobban et al. 1994), but correlations between % nitrogen and protein concentration and specific abiotic measures (e.g., nutrient concentration, photoperiod, sea surface temperature, chl *a*, solar radiation, and wind speed) were poorly understood. I addressed the role of abiotic factors on % nitrogen and protein concentration, hypothesizing that abiotic factors significantly modify the association between % nitrogen and protein concentration.

Methods

Sites

Collection of *Pyropia perforata* took place at five locations from southern California to northern California (Gaviota, Cayucos, Fanshell Beach, Pigeon Pt, Bodega Bay), spanning 4°

degrees of latitude, in order to encapsulate the environmental heterogeneity of California oceanographic conditions (Figure 1). Generally, there is a gradient of increasing seawater temperatures, decreasing upwelling intensity, and increasing solar radiation among sites, from north to south (Strickland 1970). Real-time data loggers from the Central & Northern California Ocean Observing System (CeNCOOS) data portal were used to obtain information on sea surface temperature (SST), chl *a*, wind speed, photoperiod, and solar radiation values for the entire season represeneting field collection at each location. Nitrogen concentrations from each site were also estimated using a regression model equation from Goes et al. (2000) that uses SST and chl *a* to estimate nitrogen concentration. These metrics were used to test whether there is an association between % tissue nitrogen, protein concentration, and oceanic conditions.

Sampling

Field sampling of *Pyropia* occurred in 2021 spanning the first appearance of *Pyropia perforata* in California (February) to when it disappeared (November) at the end of its annual cycle. *Pyropia perforata* was collected from the mid to upper-intertidal zone defined by the presence of *Silvetia compressa* (Abbott and Hollenberg 1976) during a low-tide event, in which the *Pyropia perforata* was not submerged in seawater. Samples of *Pyropia perforata* were collected using stratified random sampling along a 100-m horizontal transect at five randomly-selected transect points, in order to properly distribute collection effort across each site. *Pyropia perforata* was collected by picking it from the holdfast, and keeping as much of the alga as possible, without tearing it. Samples were stored in a dark cooler on ice and transported to the MLML aquaculture facility in Moss Landing, California.

Objective 1: To assess spatiotemporal variability in % nitrogen and protein concentration in *Pyropia perforata* along a latitudinal gradient.

In the field, *Pyropia perforata* was collected from each site at the beginning of each month of the *Pyropia* season during a two-day period. Sampling occurred across a 100-m horizontal transect at 5 random sampling locations on the transect selected by a random number generator. At each random-sampling location, 100-200 g FW were collected consisting of multiple thalli proximal to the randomized sampling location. Once *Pyropia perforata* were collected and transported to MLML, the samples were cleaned and rinsed with deionized water, dab-dried with paper towels, weighed into 100-g bags, labeled (site, date, number), and frozen until prepped for CHN and protein analysis. CHN produced values of carbon and nitrogen gas concentrations that were then calculated to determine % nitrogen, and were combined with protein analysis via Bradford Assay, to determine nutrient variability among seasons and sites.

Objective 2: To determine if ambient nutrient concentrations are correlated with % nitrogen and protein concentration in *Pyropia perforata*.

To test if ambient nitrogen concentrations are correlated with % nitrogen and protein concentration in *Pyropia perforata*, live *Pyropia perforata* were cultured at a constant nutrient concentration for 10 days and then taken out and measured for % nitrogen and protein concentration. For this objective, *Pyropia perforata* were collected solely from Fanshell Beach, Pebble Beach, California. Fanshell Beach was chosen as the site for collection for Objective 2 because it is the central site of the sampling range, closest of the sites in proximity to MLML, and closest in similarity of environmental conditions to MLML. Roughly 2,000g of *Pyropia perforata* were collected per trial in order to set each tank with the desired mass, plus extra for initial % nitrogen and protein values. The experiment occurred using standardized outdoor 100-gallon

tanks (4 tanks) with tumble aeration and used heat exchangers to keep the temperature of the tanks at ambient seawater temperatures. These tanks were not flow-through seawater tanks but utilized the incoming seawater to keep the tanks at ambient temperature with the heat exchange hoses. Seaweed takes up carbon (Andersen 2012) and increases pH; thus in order to keep carbon levels and pH constant in a recirculating system, pCO_2 was manually bubbled into each tank daily to maintain a pH of 8. pH was measured and recorded daily with a YSI datalogger. The pH in the system naturally increased from 8 to ~8.3-8.4, therefore daily additions of pCO_2 were critical in keeping the pH of the closed system near ambient levels.

Field-collected *Pyropia perforata* were weighed to 400-g using a 5-min net drying method to standardize wet weight and then added to each tank after being placed in a flow-through tumble-culture for 24 hours to acclimate and be cleaned of marine debris (Bracken and Stachowicz 2006). The 5-min net-dry method was tested against a shake net drying method, and a no wait drying method and this technique was found to have the best coefficient of variation (CV = 0.0282; Figure 2). Tanks were weighted each day, then pruned back to an initial weight (400-g) on a daily basis using the 5-min net drying method. Pruning was required daily so that nutrient concentrations were constant with respect to density related uptake rate. *Pyropia perforata* were cultivated with a set nitrogen species (nitrate [NO_3^-] or ammonium [NH_4^+]), and nutrient concentrations of either 0 µm, 5 µm, 15 µm, and 50 µm. The length of each trial was 10 days, which culminated in a total of 13 trials (7 NO_3^- trials and 6 NH_4^+ trials). These concentrations were used to properly scale nutrient fluctuations experienced by coastal algae. The length of each trial was used to allow *Pyropia perforata* to uptake and store nutrients in tissue (Thomas and Harrison 1988). Additionally, a pilot study performed before the study indicated 10 days was the maximum length of time field *Pyropia perforata* could be held in tumble culture before signs of degradation. Each outdoor tank (n = 4) was a closed system with Instant Ocean as a base, and f/2

media additions to maintain trace metal concentrations. Sodium nitrate ($NaNO_3^-$) and ammonium sulfate ($(NH_4^+)_2SO_4$) were used to set nutrient concentrations in each tank. Each trial used ammonium sulfate and sodium nitrate independently, with one trial of each nutrient occurring each month (every two weeks), for the entire *Pyropia perforata* season. Each nutrient concentration was replicated by blocking in time (1 replicate of each nitrogen species and nutrient concentration per month, 6 temporal replicate (blocks) of NH_4^+ and 7 temporal replicates (blocks) NO_3^-). Tank nutrient additions occurred at 8 am, 12 pm, and 4 pm every day during the experiment to reset specific nutrient concentrations. f/2 media was added to each tank every day of the trial to maintain ambient trace metal concentrations. The tanks were randomly assigned a constant nutrient concentration at the beginning of each trial. Set nutrient concentrations were calculated using stoichiometry in order to precisely add in the amount of nutrients for each concentration based on nutrient species.

YSI and HOBO loggers were used to monitor water quality in the tanks and standardize seawater and irradiance exposure among tanks. The YSI measured real time seawater temperature, conductivity, salinity, pH, and dissolved oxygen. HOBO loggers provide a time series of seawater temperature and irradiance, with measurements every ten seconds for the entirety of the trial length. The HOBO loggers were tumbling in the tanks, allowing for irradiance and seawater temperature measurements of the entire closed system. All remaining *Pyropia perforata* after each trial was rinsed in deionized water, dab dried, labeled, and weighted into 100g bags and frozen until processing for % nitrogen and protein analysis for each nutrient species and nutrient concentration treatment, consistent with methods from objective 1. % nitrogen and protein values for each nutrient species were replicated by nutrient species concentration to test how nutrient species and concentration affects % nitrogen and protein

concentration. At the end of the experiment, tanks were bleached and rinsed between each trial to remove any nutrient residue.

Objective 3: To determine the association between % nitrogen and protein concentration in *Pyropia perforata* and how abiotic and biotic factors correlate to % nitrogen and protein concentration.

Seaweed protein concentration is often estimated by a set calculation, multiplying % nitrogen by 5 (Lourenco et al. 2002, Angell et al. 2016). Although this is a good estimation, it does not explain the amount of %N that is actually converted into protein (Shpigel et al. 1999, Angell et al. 2016). For this objective, protein concentration was calculated using the Pierce BCA protein assay **(**Thermo Scientific 2013) for total protein (TP) (*see next section*). The % nitrogen concentrations were obtained by CHN and UC Santa Cruz Stable Isotope Laboratories. Values of % nitrogen and protein concentration from Objectives 1 and 2 were used to test for the correlation between % tissue nitrogen and protein concentration to examine the impact of seasonality, geography, abiotic factors: temperature, solar radiation, day length, chl *a*, wind speed, Coastal Upwelling Transport Index (CUTI), Biologically Effective Upwelling Transport Index (BEUTI), and estimated nitrate; and nutrient concentration.

CHN and Protein Analysis

A CHN Analyzer measures elemental concentrations of carbon, hydrogen, and nitrogen, with precision and accuracy. The CHN Analyzer was used to determine values of % nitrogen in *Pyropia perforata* samples. Frozen samples of *Pyropia perforata* were dried at 60°C for 48-hrs, then broken down into a powder using a ball mill in order to make the samples homogeneous. Approximately 0.1 to 0.3-mg of the homogenized powder was placed into matrices, then placed

into the instrument. The instrument then used flash combustion of the sample to oxidize the sample instantaneously into simple compounds. These simple compounds are then detectable using thermal conductivity detection, which accurately measures the percentage of carbon, hydrogen, and nitrogen that exists in the sample (Thompson 2008).

In order to measure total protein in *Pyropia perforata*, a three-phase methanol-water-chloroform extraction method was used to separate aqueous solutions of protein cells from *Pyropia*. The three-phase extraction method used Millipore H_2O and MS grade methanol and chloroform to separate cells simultaneously into metabolites and proteins from an aqueous solution (Sapcariu et al. 2014). Dried, homogenized *Pyropia perforata* was weighted to 25-mg, then placed into a 1.5-mL Eppendorf tube. Next, the *Pyropia perforata* cells were quenched with 200-µL of methanol, then vortexed for 30 seconds. After the vortex step the Eppendorf tube was placed into a centrifuge for 5 minutes at high speed. After the centrifuge, 200 µL chloroform was added to the Eppendorf tube, then vortexed and centrifuged like the methanol. Lastly 200 µL of Millipore H_2O was added to the Eppendorf tube, vortexed for 30 seconds, and then centrifuged for 30 minutes. This procedure created three layers, an aqueous layer, an interface layer, and a lipid layer (APPENDIX - A.A.).

After the centrifuge step with all the layers, the bottom layer (lipid, chloroform) was sonicated at 18 power at 10 one second pulses, followed by another short centrifuge (5 min) to re-separate the layers. Next, the polar phase (aqueous upper layer) was pipetted out into a new Eppendorf tube and stored at 4°C until analysis (APPENDIX - A.B.). Once the upper layer was pipetted out, the chloroform layer was pipetted out of the Eppendorf, and pipetted into a hazardous waste container. The Eppendorf with the interface layer was then placed in the fume hood for 24-hrs to dry out the interface layer. After 24hrs, the interface was then rehydrated with 600-µL 6M urea buffer, then sonicated at 18 power for 10 one second pulses. Next, the

Eppendorf was vortexed for 30 seconds and then centrifuged for 5 minutes, which created an aqueous layer. The Eppendorf was then placed onto a beaker shaker at 3 power for 12 hours. Next, the Eppendorf was vortexed for 30 seconds and centrifuged for 5 minutes. The urea buffer layer was then pipetted out and put into a new Eppendorf tube and stored at 4°C. The Eppendorf tube with the *Pyropia perforata* sample was then administered a second round of 200-µL urea buffer in order to pull as much protein out of the *Pyropia perforata* sample as possible (APPENDIX – A.C.). The urea buffer layer created from the second round was then pipetted into a new Eppendorf tube and stored at 4°C until analysis. This method resulted in one methanol aqueous layer and two urea buffer layers, which were used to calculate total protein for each *Pyropia perforata* sample. The layers from this method were analyzed using Pierce BCA protein assay kit with the encompassed methodology (Thermo Scientific 2013). The protein assay kit used two reagents that create a colorimetric value based on levels of amino acid present in the aqueous sample. The colorimetric is then run through a spectrophotometer to relay accurate levels of absorbance. The absorbance values are compared with standard curve, allowing the calculation of total protein µg/mL. Total protein in µg/mL was converted to dry weight %, which was used to represent the value of TP. The values derived from this method were then compared to the method of multiplying % tissue nitrogen (%N) by 5 (Angell et al. 2016) to test the accuracy of the current conversion method, and Pierce BCA protein assay method for *Pyropia perforata*.

Data Analysis

In order to test objective 1, how % nitrogen and protein concentration vary in space and time, a linear mixed model was used to compare variability in % nitrogen and TP among sites (fixed factor; 5 levels) and sampling period (fixed factor; 4 levels), and the interaction between sites x period. In order to test objective 2, whether ambient nitrogen concentration correlates with

% nitrogen and protein concentration, a analysis of covariance (ANCOVA) was used to test whether the relationship between (1) [N] and %N and (2) [N] and TP differs between nutrient species. An ANCOVA was also used for objective 2 to test for significant difference between (1) [N] and %N and (2) [N] and TP. In order to test objective 3 whether % nitrogen and protein concentration are correlated, a Pearson's correlation was used to measure the strength of the relationship between %N and TP. Moreover, multiple regression was used to test whether there is an association between the environmental conditions (SST, chl *a*, nitrate concentration, winds, upwelling indices (CUTI and BEUTI), daylength, solar radiation) and % nitrogen and protein concentration.

Results

How does Pyropia % nitrogen and protein concentration vary in time and space?

Pyropia perforata was collected from all sites starting in February of 2021. None of the sites had harvestable sized *Pyropia perforata* before February, so I established February as the start of the *Pyropia perforata* season. The last collection month for each site before disappearance was deemed the last month of the season. The southernmost sites were the first sites to conclude their seasons, which occurred in August for Gaviota and September for Cayucos. Fanshell Beach and Pigeon Pt both ended their season in October, and Bodega Bay was the last in November (Table 1).

Seasonality had a clear significant impact on both %N and TP, with the highest %N and TP found in the early *Pyropia perforata* season. There was no significant interaction effect in %N between season and site (Table 2; Figure 3A). Yet, %N was highest at Bodega, specifically during the winter and spring, and lowest at Cayucos during fall. %N at Pigeon Pt did not vary significantly between seasons. Moreover, Fanshell and Bodega had their lowest %N during the

fall season. Gaviota had its highest %N during the winter and spring season, and had disappeared by the fall season (Figure 3A) A linear mixed model indicated that seasonality significantly affected %N (Table 2, Figure 3B). Seasonal %N was highest in the winter and spring seasons (4.08 and 4.15 %), respectively, second lowest in the summer (3.89%) and the lowest during the fall (3.68 %) (Figure 3B). *Pyropia perforata* collected from Bodega Bay had the highest average %N at 4.5%, while Cayucos had the lowest average %N at 3.05% throughout the collection period (Figure 7). A linear mixed model indicated that site did have a significant effect on %N (Table 2, Figure 3C).

There was no significant interaction effect in total protein (TP) between season and site (Table 3, Figure 4A), however, a linear mixed model indicated that there was a significant difference in TP among seasons (Table 3, Figure 4B). Seasonal TP was highest in the spring and winter (25.02% and 25.63%), and lowest in the fall (15.61%) (Figure 4B). Fanshell beach had the highest average TP at 24.56%, while Cayucos had the lowest average TP at 20.52% (Figure 4C). Yet, there was no significant difference in TP among sites (Table 3, Figure 4C), Additionally, a linear mixed model indicated that site did not have a significant effect on TP (Table 3, Figure 4C). Moreover, the northern most site had the highest %N, but the southernmost site did not have the lowest %N. However, linear regression indicated that there was a significant association between %N and latitude ($R^2 = 0.38$, $F(4,38) = 7.415$, $p = 0.000162$, Figure 5A). There was no association between TP and latitude $R^2 = -0.0623$, $F(4,38) = 0.3839$, $p = 0.8187$, Figure 5B). The hypothesized trend between site location and TP did not follow the trend of highest in the northernmost site and lowest in the southernmost site.

How does ambient nutrient concentration relate to % nitrogen and protein concentration in Pyropia perforata?

Pyropia perforata was collected from Fanshell beach from February through October 2021, twice a month, and placed in 10-day duration *in situ* tumble cultures. Over the time span, thirteen total trials were completed, seven with NO_3^- as the primary nutrient and six with NH_4^+. The culture trials concluded when *Pyropia perforata* disappeared from Fanshell Beach.

Nutrient Uptake

Nutrient uptake of tumble cultured *Pyropia perforata* indicated that at each nutrient concentration after 4-hours, the nutrient concentration was significantly depleted. Both the 50µm and 15µm treatments for NO_3^- cultures depleted by two-thirds of the initial [NO_3^-], yet the 5µm [NO_3^-] treatment only depleted by 1.3µm. Each [NH_4^+] treatment depleted by two-thirds of the initial [NH_4^+] after 4 hours. Furthermore, the depletion of nutrients between nutrient species were similar (Figure 6). Biomass specific uptake rate was determined after 30 minutes of culturing, and was calculated as the change in nutrient concentration over time times dry weight of seaweed (Bracken et al. 2011). Additionally, uptake rate was highest at 50µm, and lowest at 5µm in both nutrient species (Figure 6).

Pyropia perforata cultured at 50 µM had the highest %N in both the nitrate and ammonium cultures, while it had the lowest %N when grown at 0 µM (Figure 7A). An ANCOVA was used to test for a relationship in %N and nutrient concentration for the two nitrogen species (NO_3^- and NH_4^+). There was a significant interaction effect between nutrient species and [N], such that the slopes of the [N] versus %N relationship depended on whether nitrate or ammonium was provided for growth (Table 4). Additionally, nutrient species and [N] had a significant effect on %N (Table 6). *Pyropia perforata* cultured in ammonium had a higher average %N at each nutrient concentration compared to *Pyropia perforata* cultured in nitrate (Figure 7C). %N in

Pyropia tissue also increased with increasing nutrient concentration [N] for both nitrate and ammonium, however that increase in %N was greater with increasing nitrate concentration than ammonium (i.e., steeper slope, Figure 7A).

TP was also highest in 50 µM for both nitrate and ammonium, however, 5 µM nitrate had its lowest TP whereas *Pyropia perforata* in 0 µM ammonium had its lowest TP (Figure 7B). There was no significant interaction effect between nutrient species and [N], however, nutrient species and [N] both had a significant effect on TP (Table 5). *Pyropia perforata* cultured in ammonium had a higher average TP compared to nitrate cultures (15.54 and 11.69%) (Figure 7D). In contrast, *Pyropia perforata* exhibited a weak positive relationships between [N] and TP for both nitrate and ammonium cultures (R^2 = 0.19 and 0.16, Figure 7B.).

Pigmentation

Differences in Pigment were observed in *Pyropia perforata* when cultured *in situ*. At low [N] cultures, the tissue pigment appeared lighter in color, appearing dark yellow (Appendix – C (A, B). At high [N] cultures, pigment color was more reflective of that found in nature, appearing dark red (Appendix – C (C, D).

Does the correlation between % nitrogen and protein concentration in Pyropia perforata vary as a function of abiotic factors and nitrogen concentration?

To test whether *Pyropia perforata* %N and TP are associated, a Pearson's correlation test was used. There was a significant correlation between %N and TP (Pearson correlation, n = 102, r = 0.33, p = 0.0005), such that samples with higher %N also contained higher total protein levels. Comparatively, when TP is calculated using the 5 x %N assumption (Angell et al. 2016), it is perfectly positively correlated (r = 1). An ANCOVA indicated there was no significant difference

in the slope of the relationship between %N and TP for the experiments versus the theoretical prediction (Table 6, Figure 8). Moreover, dividing the total protein (TP) measured by the Pierce BCA assay by the corresponding %N yielded a ratio of 4.9. This indicates that the Pierce BCA assay for TP accounts for only 33% of the correlation between TP and %N in Pyropia perforata (Figure 8). To further explore this relationship, both abiotic and biotic factors were modeled to determine if specific metrics could serve as proxies for %N and TP.

Environmental parameters including temperature, solar radiation (Figure 9), day length, chl *a* (Figure 10), wind speed (Figure 11), CUTI, BEUTI (Figure 12), and estimated nitrate (Figure 13) were extracted from each site and compared to site specific %N and TP *Pyropia perforata* values. A multiple regression analysis revealed that environmental parameters were significant predictors of %N in *Pyropia perforata* (Table 7). Specifically, solar radiation (Figure 9C), chl α (Figure 10A) and day length (Figure 10C) were negatively associated with %N, indicating that higher levels of solar radiation, chl α and longer day lengths corresponded to lower %N levels. In contrast, estimated nitrate concentration (Figure 13A) was positively associated with %N, suggesting that higher nitrate levels led to increased %N.

However, multiple regression analysis found that environmental parameters were not significant predictors of total protein (TP) (Table 8). Despite this, temperature (Figure 13B) was a significant predictor of TP, showing a negative association. Suggesting that higher temperatures were associated with lower TP levels.

Discussion

Previous work has indicated that nutrient physiology of seaweeds, particularly %N and TP, is associated with abiotic factors, tide height, seasonality, and geography (Harrison and Hurd

2001, Bracken et al. 2011, Benes and Bracken 2016). *Pyropia perforata* occurs over a broad geographical range and wide intertidal range, and it is characterized by an annual life-cycle that can endure all four seasons in specific locations (Lindstrom and Cole 1992). The focus of this study was to address how nutrient physiology is affected by seasonality, geography, and abiotic factors in a species of algae that experiences fluidity in its nutrient physiology. This was primarily addressed by analyzing %N and TP. Results indicated that: (1) seasonality was influential for the physiology in *Pyropia perforata*, (2) geography influenced seasonal patterns of physiology in *Pyropia perforata*, and (3) solar radiation, chl α and day length had a negative association with %N, and estimated nitrate had a positive associated with %N, while SST was the only significant predictor of TP, which was negatively associated with %N. Lab experiments further disentangled these results. From the lab experiments, it was determined that: (4) [N] was positively associate with % tissue nitrogen for both ammonium and nitrate cultures, (5) [N] did not significantly influence TP, and (6) *Pyropia perforata* cultured in ammonium had a higher %N and TP compared to those cultured in nitrate. Furthermore, spatial and temporal variables significantly affect %N in *Pyropia perforata*. Nitrogen concentrations also exhibited a significant interaction with %N, with ammonium having the biggest effect on %N in *Pyropia perforata*. To summarize, fluctuations in %N and TP in *Pyropia perforata* occur as a result of changes in seasonality, geography, and nitrogen.

Time and space were used to quantify the significance each played on %N and TP in *Pyropia perforata.* The results indicated that seasonality significantly affects both %N and TP. There was a clear difference with %N and TP being lowest in the fall season (3.68% and 15.75%), and higher in the rest of the seasons (winter: 4.15% and 25.64%, spring: 4.08% and 25.03%, summer: 3.89% and 20.6%). There are multiple reasons why the fall season had the lowest %N and TP compared to the rest of the seasons. *Pyropia* is an annual species, with a life

cycle that begins in late winter, and ends in the fall (Iwasaki, 1961). Seaweeds in the early parts of their life cycle are adapted to uptaking nutrients more rapidly (Thomas et al. 1985), therefore moving nutrients into storage to build up nitrogen reserves and facilitate health and growth. Moreover, at the end of the life cycle, annual seaweeds tend to senesce, release their spores, and desiccate. When seaweeds desiccate, they no longer are moving nitrogen into storage, yet still do uptake nutrients from seawater (Thomas et al. 1985). These stages in the latter part of their life cycle explain why their nutrient physiology is influenced by seasonal and geographic variation in nutrients and other environmental conditions. The dynamic California Current Ecosystem is characterized by significant changes in seasonality, particularly in oceanic nutrient concentration and abiotic factors, all of which have been previously noted to affect nutrient physiology (Harrison and Hurd 2001). California experiences high upwelling intensity in the spring time (Bakun 1990). High upwelling intensity moves deep, nutrient rich seawater to the surface as a result of westward winds pushing across the ocean, therefore saturating the subtidal and intertidal habitat with nutrient rich seawater (Huyer 1983). In the fall, upwelling intensity is low due to offshore relaxed wind intensity (Huyer 1983), therefore having a reduced amount of nutrient rich seawater interacting with intertidal seaweed. Moreover, it was interesting to note that in spring time, when the "spring transition" of upwelling occurs (Strub et al, 1987; Lentz 1987; Lynn et al. 2003), %N and TP were highest; in contrast these metrics were lowest when upwelling intensity was low. Comparatively, Britton et al. (2020) reported similar trends in %N in red seaweeds in Tasmania, with higher %N values during nutrient rich upwelling, and lower values during poor upwelling conditions. However, their trends in %N may be accredited to other environmental drives, such as irradiance and nitrogen availability. Furthermore, similar trends were found in

Chilean seaweeds, where protein and carbohydrate concentrations were significantly elevated in upwelling zones compared to downwelling zones (Pulgar et al. 2022).

Research from previous studies have indicated that after uptake occurs, nutrients are translocated to other areas in the seaweed for growth, physiology, and maintenance (Harrison and Hurd 2001), but why and how the nutrients are moved is poorly understood. My findings suggest that when *Pyropia perforata* exists in the upwelling-rich water, that excess nutrients not used for maintenance are moved to tissue nitrogen and amino acids, thus increasing the nutritional value of the seaweed. Yet, sites such as Cayucos and Gaviota that did not receive upwelling-rich water had their highest values in the winter and spring time as well. However, the region that occupies the southern sites have their highest nutrient concentrations during the winter and spring. Moreover, movement of nutrients is likely influenced by not just upwelling-intensity, but also the life stage of the seaweed (Thomas et al. 1985, Harrison and Hurd 2001). As stated before, annual seaweeds like *Pyropia perforata* generally move nutrients into storage when they are young thalli, in order to promote health and growth. This is likely an adaptation for annual seaweeds, where seaweeds have modified their nutrient physiology to store nutrients when nutrients are most available, simultaneously when young thalli are reproductive (Thomas et al. 1985). Other studies have shown similar results in algae, and have suggested that the movement of excess nutrients to tissue nitrogen and amino acids is the results of luxury consumption (Kang et al. 2021). This is interesting to note, as in the spring time, photosynthesis is slower than it is in the summer time, since photoperiod and solar radiation are higher. Therefore, it could be possible that when seaweeds are maximizing their photosynthesis, that nutrients are utilized, and prioritized for maintenance and growth.

It was hypothesized that geography would have a significant impact on %N and TP. I found that geography significantly impacted %N, however, it did not have a significant impact on TP. This was surprising, as environmental heterogeneity influences such as nutrient concentration, solar radiation, and temperature would change as a result of latitude. Research has shown the impact that temperature can have on TP, suggesting higher temperatures, like 17°C, enhance the protein concentration of *Devaleraea mollis* (Rizzo 2024). Protein in seaweed is often associated with %N, but there are other variables that contribute to %N but are independent of TP. For example, non-protein nitrogen materials have differing effects on %N and protein (Angell et al. 2016). Moreover, other research in Norway had reported a similar result, such that no significance was found between sites in TP, floridoside, ash, and dry matter (Rødde 2004). Additionally, *Ulva intestinalis* collected around Sweden on both coasts showed no difference in protein as a result of geographic location (Olsson et al. 2020). In my study, one reason for the lack of significance in TP could be the geographic scale of collection. The range of collection was approximately 400 miles or 4-degrees of latitude, and geographically that is a relatively small percentage of the full species range of *Pyropia perforata*. However, it is important to note that the southern-most site, Gaviota, is south of Point Conception, CA. Point Conception is a well-known biogeographic boundary for many marine species because it is where the northern California current meets the southern California current (Wares et al. 2001). North of Point Conception, the coastline is parallel to the northwesterly winds that blow offshore, resulting in increased upwelling intensity. The orientation of Point Conception itself causes a deflection to the winds and oceanic current. The coastline south of Point Conception curves eastward, decreasing the intensity of northwesterly winds. This results in decreased upwelling intensity (Dugdale and Wilkerson 1989, Wang 1997). The concept of having a site south of Point Conception was to promote environmental heterogeneity between the sites. This concept could have provided

potential significance, but more sites south of Point Conception were needed to give a better understanding of the effects of geography on TP.

When looking at the interaction between space and time for %N, there was not a significant effect. Three out of the five sites recorded their lowest %N in their last collection month, (Gaviota - summer; Bodega, Cayucos - fall), correlating with reduced seasonal upwelling intensity in California (Bograd 2009). Pigeon Pt and Fanshell however, did not show significant variation between %N and any season. Pigeon Pt and Fanshell may not have experienced seasonal variability in %N due to its localized environmental heterogeneity. Both sites are located approximately 30 miles from Moss Landing, California (Pigeon Pt: north, Fanshell: south), which is located in the middle of the Monterey Bay, where the Monterey Submarine Canyon is located, one of the largest underwater canyons in the world (Martin and Emery 1967). This canyon offers a plentiful amount of rich nutrients that are moved up and down the nearby coasts during an upwelling event, therefore saturating both sites with ample nutrients for year-round growth. Moreover, the presence of nutrient rich seawater year-round may have influenced the lack of variability in %N between seasons. In this study, a lack of significant interaction between time and space in %N demonstrated the fluidity of nutrient physiology in *Pyropia perforata*. In general, abiotic factors affect nutrient physiology with respect to both time and space (Phillips and Hurd 2003). Yet, a more detailed understanding about the life cycle of specific seaweed species, and their association with environmental conditions are necessary for a better understanding of how space and time affect nutrient physiology in seaweed.

Another interesting result was %N and TP seemed to have a relationship to the *Pyropia perforata* life cycle. *Pyropia perforata* becomes a gametophyte in the early spring time, after conchospores from conchocelis (sporophyte) recruit to a holdfast (i.e, rocks, shells) (Brodie and Irvine 2003, Blouin 2011). The life span of the gametophyte is indicative of photoperiod, and

signals for reproduction when day length is shortened (Iwasaki 1961), occurring in the late summer to fall season. Based on the results above, %N and TP were highest when *Pyropia perforata* were young thalli, and lowest when *Pyropia perforata* were old thalli. Young *Pyropia perforata* experiences rapid growth as it emerges from its dormant phase in the spring, leading to large abundances in the summer (Aquilino 2009). Additionally, the abundance of nutrients from upwelling allows *Pyropia perforata* to allocate nutrients to both storage and nutrients. In the summertime, when nutrient concentration in the seawater has reduced as a result of increased seawater temperature and nutrient uptake by other organisms, *Pyropia perforata* may experience a decrease in nutrient availability, therefore prioritizing nutrients to be stored to ensure there is sufficient nutrients reserved for reproduction in the fall. Fall marks the reproductive phase of *Pyropia perforata*, with new conchocelis recruits present from fall to winter (Zertuche-Gonzalez et al. 2000). During this period, seaweeds often invest most of their energy and resources into reproductive structures such as gametophytes and sporophytes, which are catalyzed from stored nutrients (Roleda and Hurd 2019). The changes in %N due to seasonality correlate to the life cycle of *Pyropia perforata*, giving a better understanding of the fluidity in nutrient physiology.

When looking at how ambient nutrient concentration affects %N and TP, there was a significant positive correlation between [N] and %N in both ammonium and nitrate cultures. This was expected, it is intuitive that when seaweed have more available nutrients, they will have a better chance at putting nutrients into tissue nitrogen and amino acids. Nutrient uptake is often a valued parameter when addressing %N in algae, as it is linked to storage, tissue nitrogen, amino acids, and compartmentalization (Harrison and Hurd 2001, Chung et al. 2002). However, it is important to note that nitrogen uptake rate is not always correlated to %N (Duke et al. 1989), as %N can only increase when growth is nitrogen-saturated (Gerard 1982). It was interesting to note that *Pyropia perforata* grown at 0 μM nitrogen had an average %N value of 2.45, which is higher

than the suggested %N value of 2% for nitrogen limitation (Hanisak 1979, 1983 and O'Brien & Wheeler 1987). The initial values of %N for *Pyropia perforata* before entering *in situ* cultures varied between 3-5%. Therefore, cultures with no nutrient additions did indeed lose some % tissue nitrogen over the course of the 10-day trial. However, even after a 10-day trial of zero nutrient additions, *Pyropia perforata* did not become nutrient-limited. It is likely that a 10-day trial was not enough time to allow *Pyropia perforata* to be fully depleted of nutrients. This result is further supported from Hanisak (1993), where *Gracilaria* and *Ulva* were put into outdoor mesocosm systems that forced the seaweeds to decompose and reduce %N. The result from Hanisak (1993) indicated that seaweeds have the ability to lose %N and recover rapidly, suggesting that seaweed can go long periods of time without nutrients whilst remaining nutrient saturated. This is further supported by Gerard (1982), when giant kelp showed positive growth rates during long periods of nitrogen limitation. However, nitrogenous protein in seaweeds may not react in the same manner.

There were strong positive correlations seen between [N] and %N, but this was not observed between [N] and TP. TP in *Pyropia perforata* had a slight positive correlation to [nitrate], but an insignificant correlation to [ammonium]. However, the results from this study showed that TP was highest in the high [N] cultures, and lowest in low [N] cultures in both nutrient species. The expected result was that the correlation between [N] and TP would be positively significant like [N] and %N, however the movement of nutrients to amino acids in *in-situ* cultures could be the cause for a lack of a strong correlation. There are few studies that have cultured *Pyropia perforata* in onshore tank cultures, and even fewer studies that have cultured the species for longer than 4 days (Thomas and Harrison 1985). Another alternative could be that seaweed has significant sources of non-protein nitrogenous material (Amano and Noda 1992). Research has shown that all plant materials including algae have large sources of non-protein

nitrogenous materials such as chlorophyll, nucleic acids, free amino acids and inorganic nitrogen (Lourenco et al. 1998, Naldi and Wheeler 1999, Lourenco et al. 2004). Therefore, the relationship between [N] and TP should be independent of %N, especially if there is not a proper estimate of non-protein nitrogenous materials in algal tissues. Yet, the effect of [N] and TP may have a different relationship when broken down between nitrogen species. It is also possible that specific proteins may take longer to break down in response to nutrient deprivation compared to %N.

When looking at the relationship between [N] and %N and TP, there was a significant effect of nutrient species on the relationship. *Pyropia perforata* cultured in ammonium had higher %N values in all set nutrient concentrations compared to *Pyropia perforata* in nitrate cultures. Research has shown that seaweed species exhibit uptake and storage preferences for either nitrate or ammonium (Pedersen and Borum 1997, Naldi and Wheeler 1999), resulting from seaweed morphology, physiological capabilities, and life histories that affect their utilization of nitrate vs. ammonium (Wallentinus 1984, Pedersen and Borum 1997, Naldi and Wheeler 1999). However, Bracken et al. (2011) reported that there was very little preference in nitrogen species via uptake in *Pyropia perforata*. Through the results of this study, *Pyropia perforata* cultured in ammonium was able to move nutrients to tissue nitrogen at a faster rate, even when uptake preference wasn't significant. This is likely the result of assimilation and nitrate reductase; assimilation being the process of nitrate converting to ammonium, and the other where an enzyme must be activated for synthesis (Boyd and Hurd 2009). In addition, it was suggested by Thomas and Harrison (1989) that an induction period occurs during nitrate uptake within 30-45 minutes of *Pyropia perforata* being submerged in nitrate rich water. The results from this study indicate that uptake induction still may occur, but *Pyropia perforata* in nitrate cultures for 10 days could still be converting the nitrate in itself and that the diffusion process may be different versus ammonium. Moreover, TP between nitrate and ammonium cultures was significantly higher in ammonium cultures

compared to nitrate cultures. Generally, in seaweed, ammonium is not stored in large concentrations, and is rapidly metabolized to amino acids (Taylor and Rees 1999). Nitrate on the other hand can be stored in higher concentrations within the cells, and does not necessarily need to be rapidly metabolized into amino acids. Moreover, the results from this study further the understanding that nitrogen species have different utilization and transportation properties when uptaken by *Pyropia perforata*.

To further understand how nutrient physiology is correlated, the relationship between %N and TP was compared to each other to see if they could be a proxy for each other. It was expected that %N and TP could be proxy for each other, since TP is often calculated by multiplying %N by a factor of 5 (Angell et al. 2016) or 6.25 (Lourenco et al. 2002). In this study, TP was calculated using Pierce BCA protein assay, in which absorbance level was measured from each sample then plugged into a standard curve equation. Based on the results from this study, only 33% of the variance could be explained by the relationship between %N and TP. There was lots of variance from each TP values, even though the conversion factor of TP (TP/%N = conversion factor) to %N averaged 4.9, very close to Angell et al. (2016) value of 5. Yet, the Pierce BCA protein assay may over/under estimate total protein (Niemi et al. 2022). Moreover, the results from this study suggest that the relationship between %N and TP is linear. The differences in linearity between this study and Angell et al. (2016) may be associated with the methodology of the protein assay. When performing the spectrophotometer analysis, the albumin standard used to create the standard curve had to be diluted by a factor of 100:1 in order to be properly detected. Therefore, all the samples before spectrophotometer measurement had to be diluted by 100:1 to match the dilution by the standard curve, increasing the variability between all the TP values. Overall, the closeness in estimation values between this study and Angell et al. (2016) provides strong evidence that %N is a proxy for TP.

An unexpected discovery was the difference in pigment color in *Pyropia perforata* between [N] cultures. At low [N] cultures, pigment looked lighter in color, appearing dark yellow, while at high [N] cultures, pigment color was more comparable to nature, looking dark red. Comparatively, Gao et al. (2019) demonstrated that *Pyropia yezoensis* thalli had deeper color when cultured at higher nitrate compared to lower nitrate. Also, thalli grown at higher pCO_2 had a deeper color compared to those grown at lower pCO_2. The cause of this result was from enhanced phycoerythrin and phycocyanin, which was earlier determined as the main color determinant in *Pyropia yezoensis* (Niwa and Harada, 2013). In my study, *Pyropia perforata* was cultured in both nitrate and ammonium, and both of which showed lighter color in low [N] and deeper color in higher [N]. Therefore, there is reasonable evidence to suggest that pigment color changes as a result of nitrogen availability.

Abiotic factors are often terms used to describe ecology pertaining to a certain environment. In seaweeds, nutrient physiology is largely affected by abiotic factors: photoperiod, solar radiation, sea surface temperature, and physical factors: chl α, nutrients, upwelling and tidal height (Bracken et al. 2011, Harrison and Hurd 2014). Sea surface temperature (SST) is a very common metric that is used to evaluate a variety of influences on seaweed physiology. In coastal waters in California, temperature and nitrogen availability are inversely correlated (Parnell et al. 2010). Therefore, it was assumed that correlations of %N and TP with SST would exist. Moreover, the results from this study showed that there was a significant inverse correlation between SST and %N, such that %N was higher in locations and time periods characterized by cooler water. Furthermore, a significant inverse correlation was observed between SST and TP, with higher protein content observed in samples collection from sites and seasons with colder water. In contrast, other research has indicated positive increases in growth rate with increases in

temperature and photon flux density in other species of seaweed (Bulboa 2008, Eggert 2012). However, the importance of temperature effects on nutrient delivery through upwelling is likely the overriding factor influencing seaweed nutrient content in my study. The range in temperature between sites and seasons in my study was 10-17°C, while previous research has assessed the temperature tolerance in *Pyropia sp.* to be optimal between 10 – 20 °C (Pereira et al. 2006, Kim et al. 2007). Interestingly, the species remains positively photosynthetic at up to 30 °C (Smith and Barry 1986). The observed correlation between %N and TP in this study may have been influenced by the temperature range examined. Extending the temperature range studied could potentially strengthen this correlation. Both laboratory and natural settings indicate that temperature dynamics significantly impact nutrient physiology in Pyropia perforata. This highlights the need for a deeper understanding of temperature as a critical factor in nutrient uptake and utilization.

Chlorophyll α is often used to measure productivity because it is essential to photosynthesis. Other research, including Goes et al. (2000), indicate that when chl α is modeled with SST, predicted sea surface nitrogen (SSN) is accurately measured in the Pacific Ocean (r^2=0.83). My study used the Goes et al. (2000) model to predict SSN, highlighting that predicted SSN is a significant indicator of %N in *Pyropia perforata* (r^2 = 0.44). Comparatively, lab cultured *Pyropia perforata* in this study exhibited similar correlations between [N] and %N ([NO_3^-] r^2=0.559 and [NH_4^+] r^2 =0.389). Southern California can reach nitrogen-limited bounds (< 5 μM), and low nitrogen values have been recorded from SSN in previous studies in California (Wheeler and North 1981, Gerard 1982). The combination of lab and field results on the correlation between [N] and %N provides evidence that [N] may be a proxy for %N, with other studies indicating urea as a proxy for %N as well (Lees et al. 2024). However, there was no relationship

between SSN and TP in this study. The absence of a similar relationship like SSN and TP suggests a divergence in the factors influencing nitrogen metabolism, and the factors that influence protein synthesis. One potential explanation for these results is the differential utilization and regulation of nitrogen, which likely changes due to life cycle phase and impacts of seasonality. My study revealed a positive correlation between SSN and %N, as a result of excess nitrogen being stored in *Pyropia perforata* tissue. However, non-protein nitrogenous material such as chlorophyll, nucleic acids, free amino acids and inorganic nitrogen significantly alter protein content in seaweeds (Lourenço et al. 1998; Naldi and Wheeler 1999; Lourenço et al. 2004, Angell et al. 2016). The specific metabolic pathways and regulatory mechanisms involved in nitrogen assimilation and protein accumulation are dynamic, and therefore may not be explained by a single factor. Furthermore, TP in *Pyropia perforata* from the *in situ* cultures did not correlate with [N] in the same way as %N, thus furthering the notion that the dynamics of protein synthesis, accumulation, and metabolism are not simply explained by [N] alone. In addition, using the Pierce BCA protein assay kit for BCA assay may not have been the best extraction method for total protein. Niemi et al. (2022) alluded that the BCA assay for estimating total protein is unreliable and over/under estimates TP in seaweeds, especially brown seaweeds (Class Phaeophyceae). Moreover, whether it is estimating total protein by a conversion factor, or using an extraction method, it is unclear what happens to nutrients once they have been converted to ammonium by their allocated enzyme. Future studies should focus on non-protein nitrogenous materials in an attempt to quantify what fraction of assimilated nutrients are allocated to protein and nonprotein compartments.

Multiple regression analysis was used to determine whether %N and TP could be predicted by environmental parameters. Only %N was significantly associated with

environmental parameters, specifically with solar radiation, chl α and day length, and estimated nitrate. Interestingly, the Coastal Upwelling Transport Index (CUTI) and Biologically Effective Upwelling Transport Index (BEUTI) did not serve as strong proxies for %N in *Pyropia perforata*. However, it is worth noting that these indices were still positively correlated with %N, which aligns with the expectation that upwelling influences nutrient physiology. CUTI and BEUTI are two upwelling indices that leverage state-of-the-art ocean models as well as satellite and in situ data for the U.S. West Coast (Jacox et al. 2018). CUTI provides estimates of vertical transport near the coast whereas BEUTI provides estimates of vertical nitrate flux near the coast. BEUTI may be more relevant than upwelling strength when considering some biological responses. Increased CUTI and BEUTI correlated with decreased temperature and corresponding increases in nitrate exposure (Navarrete et al. 2021). Yet, BEUTI has been demonstrated to be poorly associated with kelp dissolved inorganic nitrogen (DIN) (Spiecker and Menge 2022). Seaweeds like kelp have displayed strong annual cycles of nitrogen, specifically fluctuations of internal nitrogen through seasons (Brzezinski et al. 2013), yet the findings from this study concludes that increased upwelling index and nitrate fluxes are not coupled with nitrogen content in seaweed. Wind speed is another upwelling variable that is used to further analyze upwelling intensity in coastal California. Even though wind speed was not a significant predictor for %N or TP, a larger timescale and geographical range are necessary to fully understand the impact of wind speed, as well as the timing of uptake rate, nutrient storage, and protein accumulation in *Pyropia perforata*. From this study the impact of upwelling does not couple with nitrogen, even though nitrogen is the limiting factor for growth in seaweed (D'Elia and DeBoer 1978).

Day length (photoperiod) is the amount of time in which an organism is illuminated. Photoperiod is an abiotic variable that changes with respect to time and space, with light lengths that exceeded 14 hours in at multiple sites of this study. The results from this study showed that

photoperiod exhibited a negative association with %N, and no correlation to TP. Other research highlighting the genus *Pyropia* demonstrated growth rate is positively influenced by increasing photoperiod (Green and Neefus 2016), yet this study revealed that photoperiod does not positively influence %N and TP in *Pyropia perforata*. The overlying result was not expected as increased light would increase photosynthetic activity, therefore increase protein concentration. It is likely that the range of photoperiod was limited, given the geographical range of sites, length of season, and not controlling light length in laboratory. Day lengths ranged from 10.1 – 14.7 hours in this study, comparatively when other studies have looked at photoperiod as a variable influencing nutrient physiology in *Pyropia*, using photoperiods from 8 – 16 hours (Green and Neefus 2015, 2016).

Conclusion

Nutrient physiology in seaweeds is fluid and varies as a result of numerous factors. Understanding which factors quantitatively affect nutrient physiology in seaweeds is critically important as oceanic conditions continue to change with climate change. Tissue nitrogen and total protein in *Pyropia perforata* were significantly affected by seasonality in this study, further highlighting that differences in oceanic and environmental conditions will change nutritional value in seaweeds. However, there was not a significant effect seen with space on tissue nitrogen and total protein in *Pyropia perforata*. It is possible that the range of sites did not sufficiently represent the environmental heterogeneity of California, but it is worth noting that the highest values of internal nitrogen and total protein content were found in the northern sites. Laboratory trials from this study showed strong positive correlations to nutrient concentration and internal nitrogen in *Pyropia perforata,* suggesting that when seaweeds are exposed to high and low levels of nutrients for long periods of time, internal nitrogen levels will change. Moreover, when

environmental parameters were modeled against % nitrogen and total protein, estimated sea surface nitrate exhibited a positive correlation with %N. This result furthers this study's findings that nitrogen concentration in controlled and ambient settings does act as a proxy for tissue protein in *Pyropia perforata*. Unfortunately, I did not find significant evidence that nitrogen concentration can act as a proxy for total protein. Moreover, the Pierce BCA protein assay method to calculate total protein revealed a 4.9 conversion factor to tissue nitrogen, very close to Angell et al. (2016) conversion factor of 5. Yet, the Pierce BCA protein assay method for total protein could only account for 33% of the association between total protein and internal nitrogen content, indicating that % nitrogen may not be a reliable proxy for total protein. To further understand nutrient physiology in seaweed, and the link between total protein and % nitrogen, future research should include a wider geographical range, and a more in depth look at the role of non-nitrogenous protein in seaweed. Encapsulating the full bounds of environmental heterogeneity and understanding how nitrogen becomes non-protein or protein will close the gap on the link between environmental parameters and nutrient physiology, and between nitrogen and protein in seaweeds.

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Tables

Table 1: Collection period for each site

Collection Site	First Month Collected	Last Month Collected	Collection period (months)
Bodega	February	November	10
Pigeon Pit	February	October	9
Fanshell	February	October	9
Cayucos	February	September	8
Gaviota	February	August	7

Table 2: Linear mixed model ANOVA (Season and Site). Results for %N variation over the course of time and space.

Source	Df	SS	MS	F	P
Site	4	10.5	2.6	10.8	3.6e-5***
Season	3	2.38	0.79	3.27	0.0385*
Site: Season	12	3.5	0.32	1.33	0.27
Error	24	5.8	0.24		
Total	43				

Table 3: Linear mixed model ANOVA (Season and Site). Results for total protein variation over the course of time and space.

Source	Df	SS	MS	F	P
Site	4	61.8	15.4	0.83	0.519
Season	3	607.7	202.6	10.87	1e-4***
Site: Season	12	234.3	21.3	1.14	0.373
Error	24	447.1	18.6		

Total	43				

Table 4: Analysis of Variance (ANCOVA) for ([N] and Nutrient species). Results for %N variation between [N] and Nutrient species

Source	Df	SS	MS	F	P
[N]	3	49.99	16.663	29.863	4.98e-10***
Nutrient Species	1	6.11	6.115	9.87	0.003**
[N]: Nutrient Species	3	5.31	1.77	2.854	0.048*
Error	44	27.29	0.620		
Total	51				

Table 5: Analysis of Variance (ANCOVA) for ([N] and Nutrient species). Results for total protein variation between [N] and Nutrient species.

Source	Df	SS	MS	F	P
[N]	3	189.4	63.12	3.379	0.026*
Nutrient Species	1	191.5	191.48	10.252	0.0025**
[N]: Nutrient Species	3	16.6	5.53	0.296	0.83
Error	44	821.8	18.68		
Total	51				

Table 6: Multiple regression for Theoretical Total Protein (TP-T) and Experimental Total Protein (TP-E) as Predictors of %N and Observed %N.

Predictor	Estimate	Standard Error	t-value	p-value	Adjusted R-squared	F-value	P-value
TP-T	0.2	1.7e-16	1.2e+15	2e-16***			
TP-E	1.4e-17	1.7e-16	0.1	0.9			
%N: TP-T	1.1e-17	3.8e-17	0.37	0.71			
%N: TP-E	-5.6e-18	-3.8e-17	-0.08	0.88			
Model Statistics							
Adjusted R-squared					1		
F- value						1.1 e+31	
P- value							2.2 e-16

Table 7: Multiple regression for Environmental Predictors of % N and Observed %N

Predictor	Estimate	Standard Error	t-value	p-value	Adjusted R-squared	F- value	P-value
Temperature	-0.13	0.11	-1.26	0.21			
Solar Radiation	-0.0065	0.0026	-2.43	0.02 *			
Day length	0.33	0.12	2.73	0.009 **			
Wind speed	0.05	0.05	1.02	0.31			
Chl α	0.14	0.41	3.44	0.0016**			
CUTI	1.13	0.90	1.25	0.22			
BEAUTI	-0.06	0.05	1.36	0.18			
Estimated Nitrate	0.06	0.02	3.01	0.005 **			
Model Statistics							
Adjusted R-squared					0.59		
F-value						8.594	
P-value							2.57e-05

Table 8: Multiple regression for Environmental Predictors of Total Protein % and Observed Total Protein %

Predictor	Estimate	Standard Error	t-value	P-value	Adjusted R-squared	F- value	P-value
Temperature	-2.05	0.93	-2.18	0.03 *			
Solar Radiation	0.01	0.02	0.65	0.52			
Day length	-0.51	1.05	-0.48	0.63			
Chl α	0.4	0.4	1.0	0.32			
Wind speed	-0.03	0.42	-0.07	0.94			
CUTI	4.25	7.76	0.54	0.58			

BEAUTI	-0.22	0.42	-0.51	0.61			
Estimated Nitrate	-0.11	0.18	-0.6	0.55			
Model Statistics							
Adjusted R-squared					0.15		
F-value						1.928	
P-value							0.08

Figures

Figure 1: Collection map.

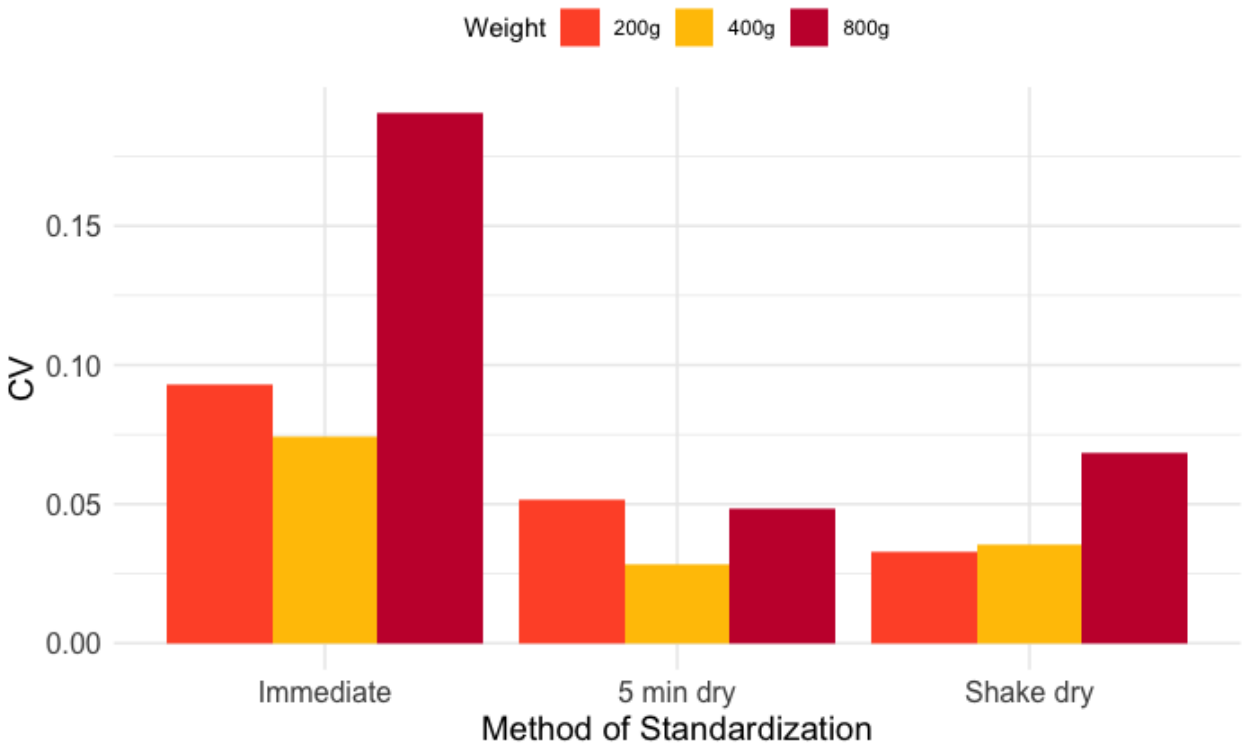


Figure 2: Coefficient of variation (CV) was used to determine the lowest variance of *Pyropia perforata* wet weight (g) compared to three different methods of standardizing wet weight: Immediately weighted, sun dried for 5-minutes, and shake dry. CV was calculated by dividing the standard deviation over the mean. Methods were experimented at three different *Pyropia perforata* biomasses: 200g, 400g, and 800g. CV experiment occurred on 02/01/2021.

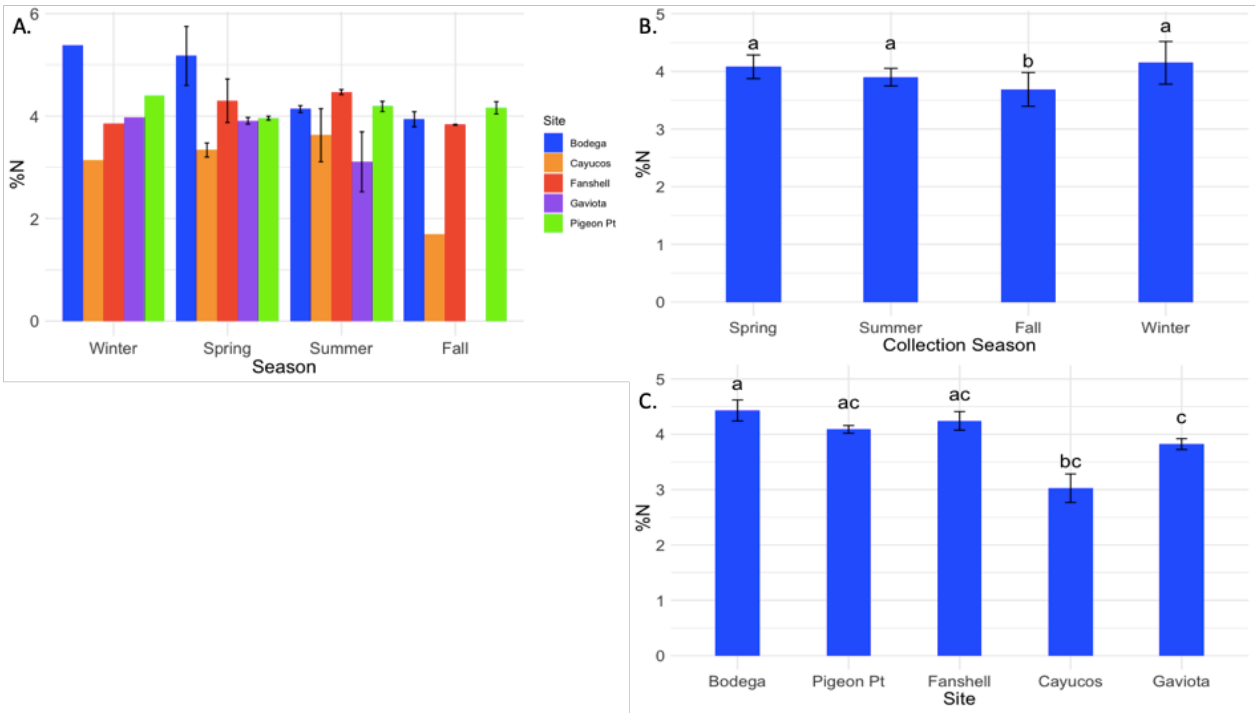


Figure 3: *Pyropia perforata* collection season and site compared to % nitrogen of *Pyropia perforata*. Spring season included the months of March, April, May; Summer season included June, July, August; Fall season included September, October, November; Winter season included December, January, February. All sites were collected from during their *Pyropia perforata* season on the first weekend of each month during the same tidal sequence. Letters above bars indicate significant differences between %N and site. Error bars are ± standard error.

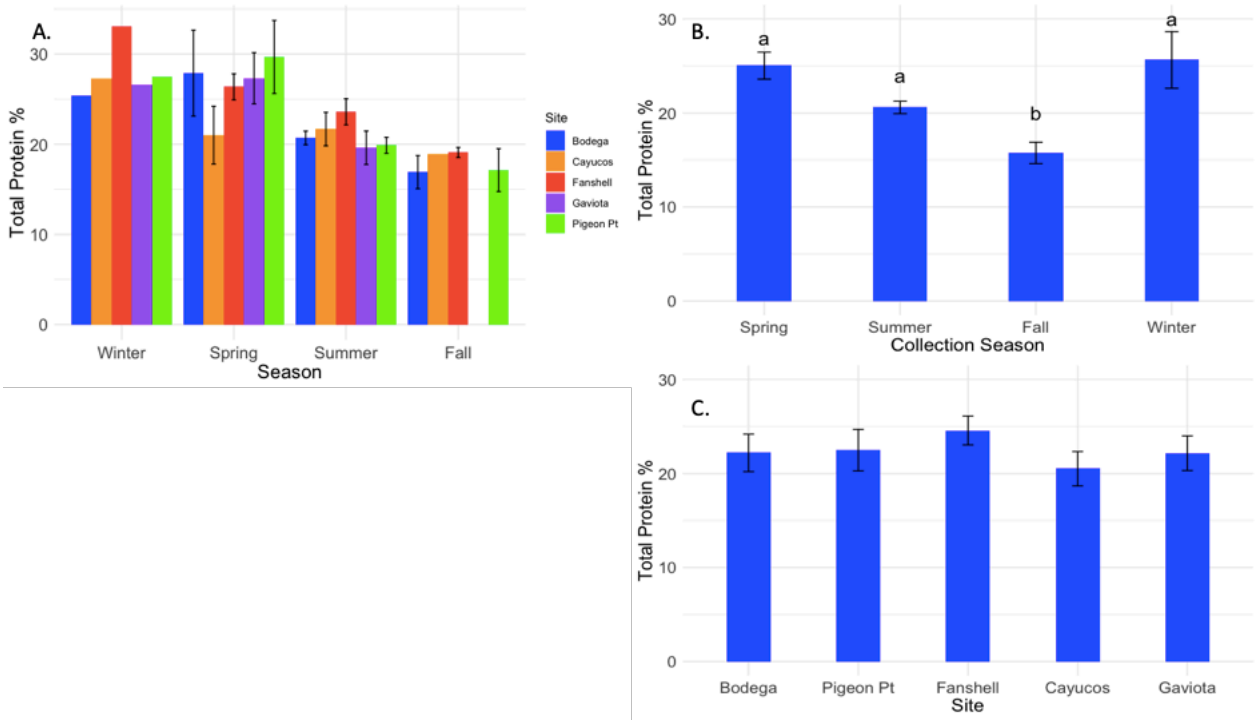


Figure 4: *Pyropia perforata* collection season and site compared to Total Protein % of *Pyropia perforata*. Spring season included the months of March, April, May; Summer season included June, July, August; Fall season included September, October, November; Winter season included December, January, February. All sites were collected from during their *Pyropia perforata* season on the first weekend of each month during the same tidal sequence. Letters above bars indicate significant differences between total protein and season. Error bars are ± standard error.

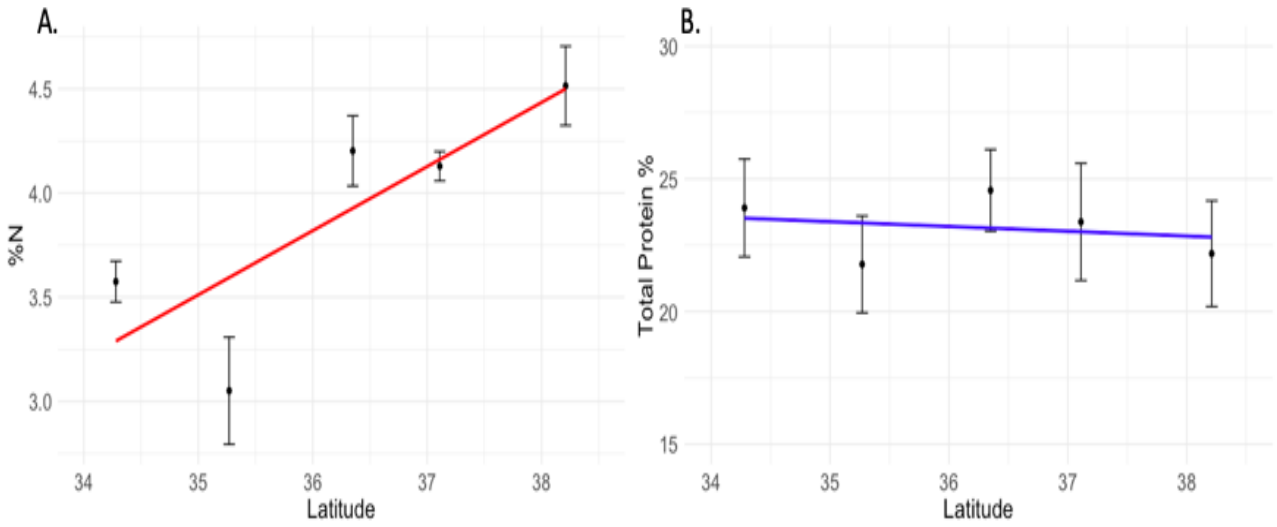


Figure 5: Multiple panel comparison of A. Regression analysis comparing the relationship between % nitrogen and latitude in *Pyropia perforata* (y = -7.55 + 0.32x). B. Regression analysis comparing the relationship between total protein % and latitude in *Pyropia perforata* (y = 30.29 - 0.20x). % nitrogen was measured using CHN analysis. Collections were made on the first weekend of each month from all sites during the same tidal sequence. Error bars are ± standard error.

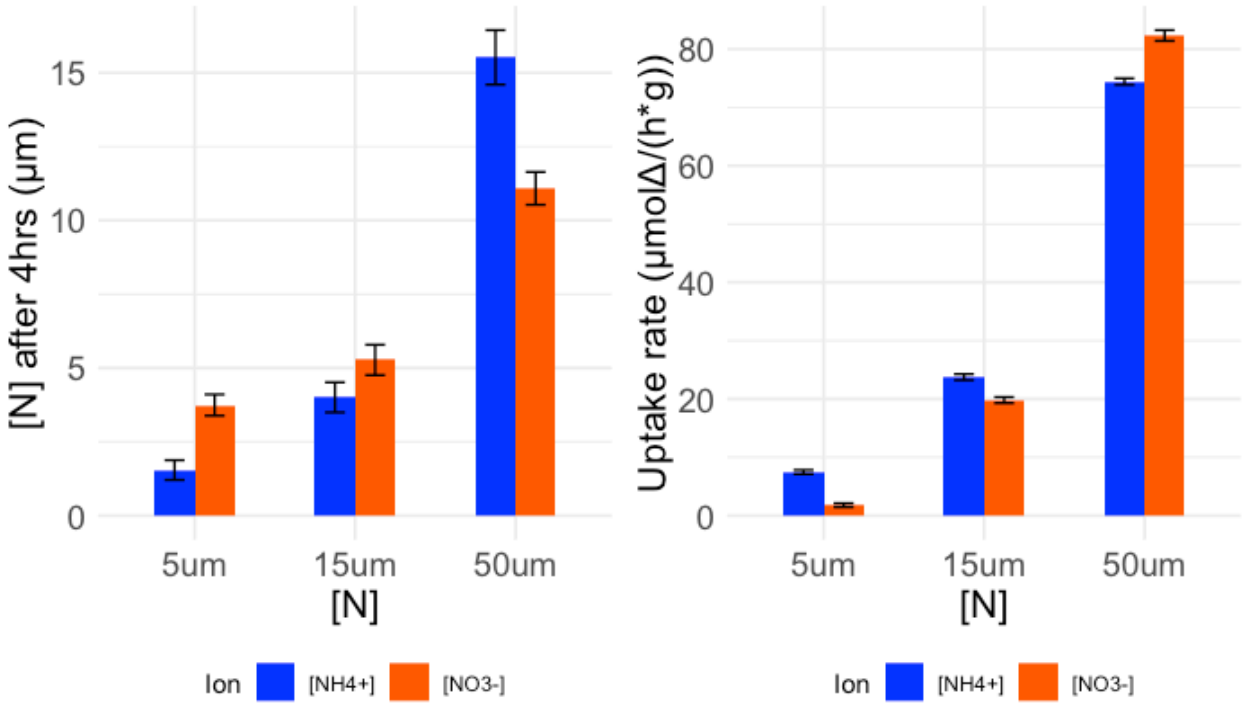


Figure 6: Nitrogen concentration of tank culture after 4 hours compared to uptake rate of *Pyropia perforata.* Tank biomass was set to 400g and uptake rates were compared to three different nitrogen concentrations: 5µm, 15µm, and 50µm. Nitrogen species NO_3^- and NH_4^+ were used separately to compare nitrogen concentration after 4 hours and uptake rates between nitrogen species. Uptake rate experiments were conducted each culturing trial. Error bars are ± standard error.

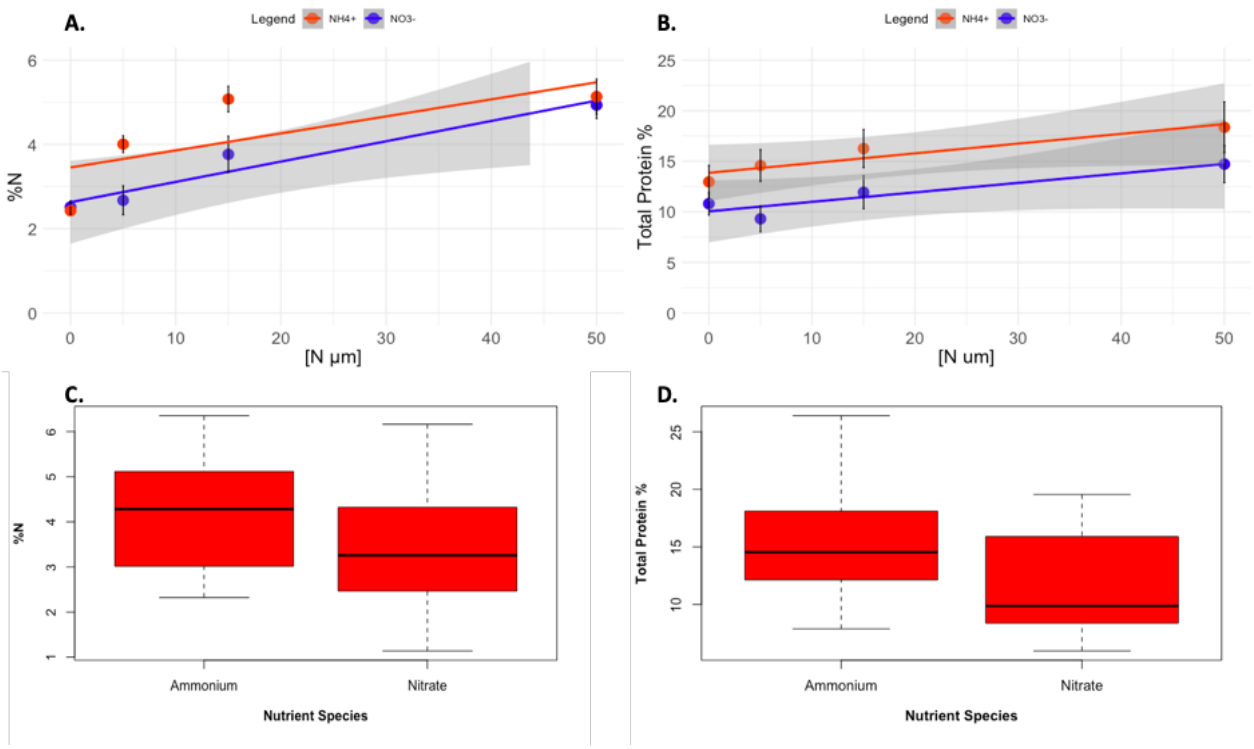


Figure 7: Multi-panel comparison of A. Regression analysis of the relationship between nitrogen concentration and % nitrogen in *Pyropia perforata* (NO_3^-: n = 28, y = 2.6287 + 0.0482*x; NH_4^+: n = 24, y = 3.4529 + 0.0404*x). B. Regression analysis of the relationship between nitrogen concentration and total protein in *Pyropia perforata* (NO_3^-: n = 28, y = 10.05 + 0.0938*x; NH_4^+: n = 24, y = 13.853 + 0.0964*x). C. Box and whisker plot for % nitrogen in *Pyropia perforata* compared to nitrogen species. D. Box and whisker plot for Total Protein in *Pyropia perforata* compared to nitrogen species. Nitrogen species NO_3^- and NH_4^+ were used as the nitrogen source and were used independently of each other. % nitrogen was estimated using CHN analysis. Total protein was estimated using Pierce BCA Protein Assay, and then calculated to percentage total protein per dry wt in grams.

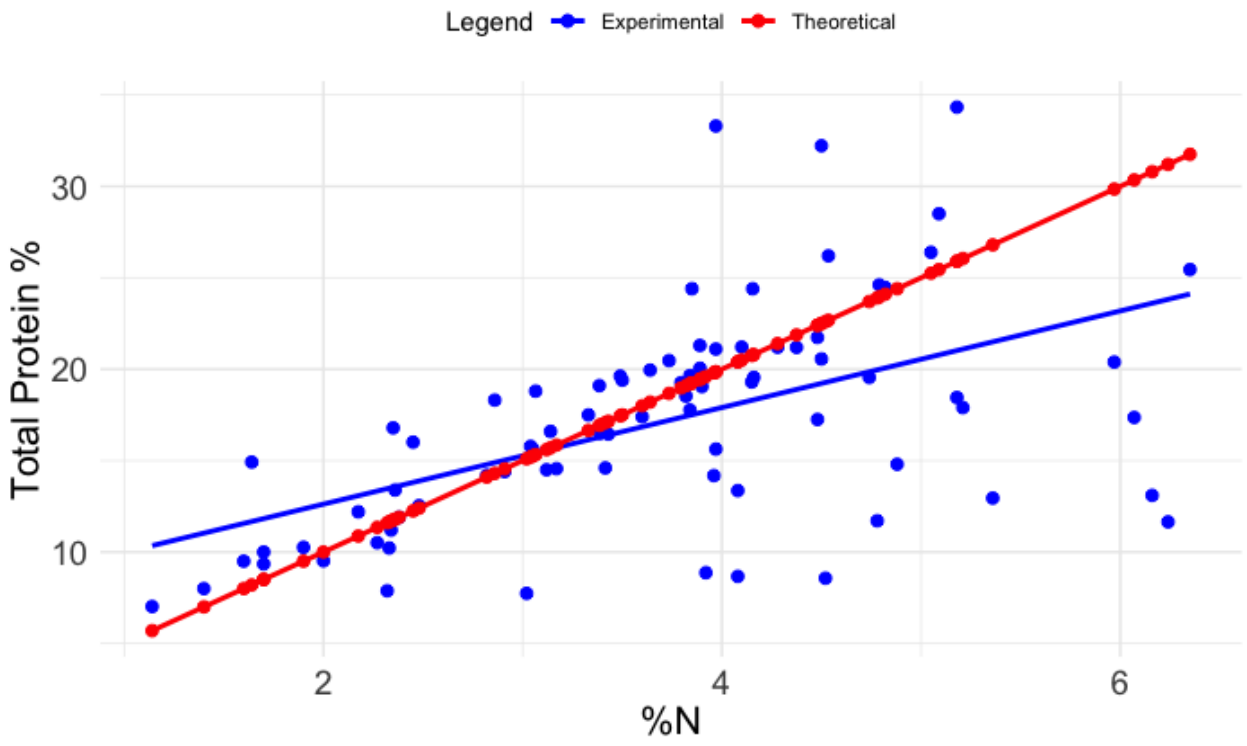


Figure 8: Regression analysis comparing internal nitrogen content (%N) in *Pyropia perforata* to total protein in *Pyropia perforata* (n = 102, y = 6.38 + 3.03*x). Theoretical total protein was calculated by multiplying % tissue nitrogen by a factor of 5 (Angell et al. 2016).

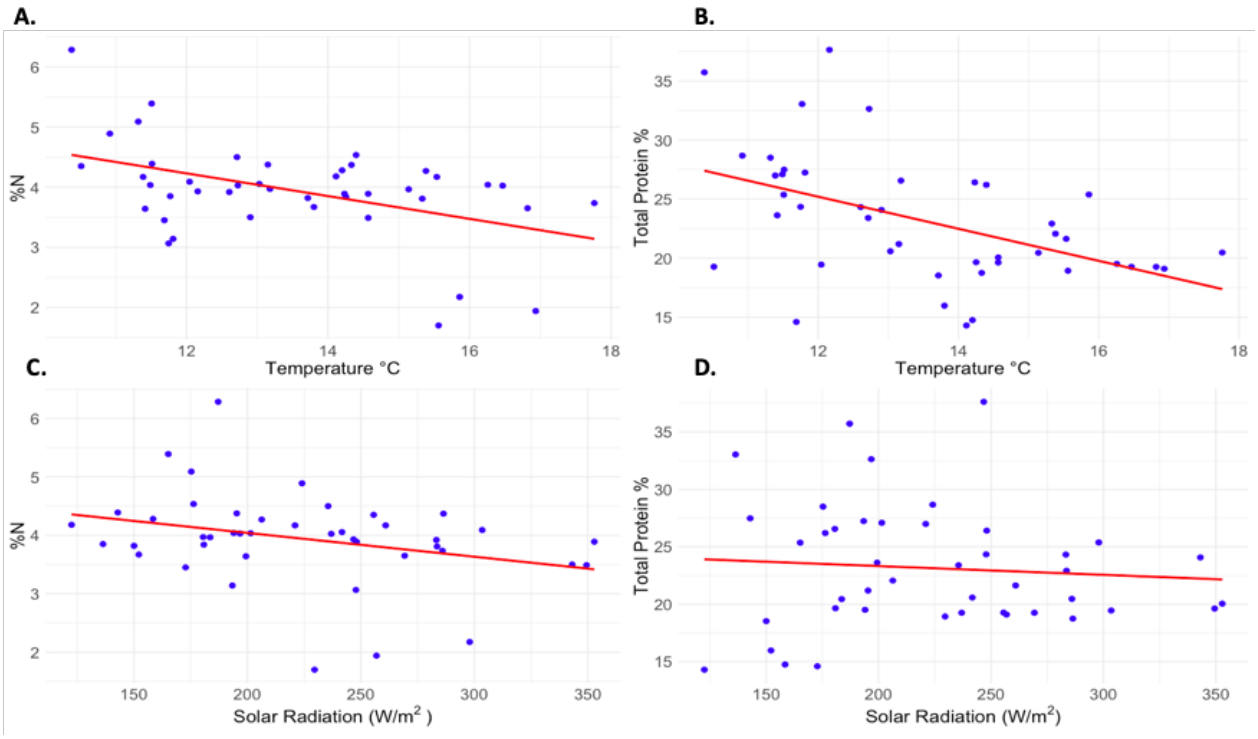


Figure 9: Multi-panel comparison of A. Regression analysis comparing sea surface temperature (SST) to % nitrogen in *Pyropia perforata* (n = 41, y = 6.4989 – 0.1891*x). B. Regression analysis comparing sea surface temperature (SST) to total protein in *Pyropia perforata* (n = 41, y = 41.477 – 01.3568*x). C. Regression analysis comparing solar radiation to % nitrogen in *Pyropia perforata* (n = 41, y = 4.8566 – 0.0041*x). D. Regression analysis comparing solar radiation to total protein in *Pyropia perforata* (n = 41, y = 24.841 – 0.0076*x). SST data from 2021 was downloaded from CeNCOOs data portal. Solar radiation data from 2021 was downloaded from CeNCOOs data portal.

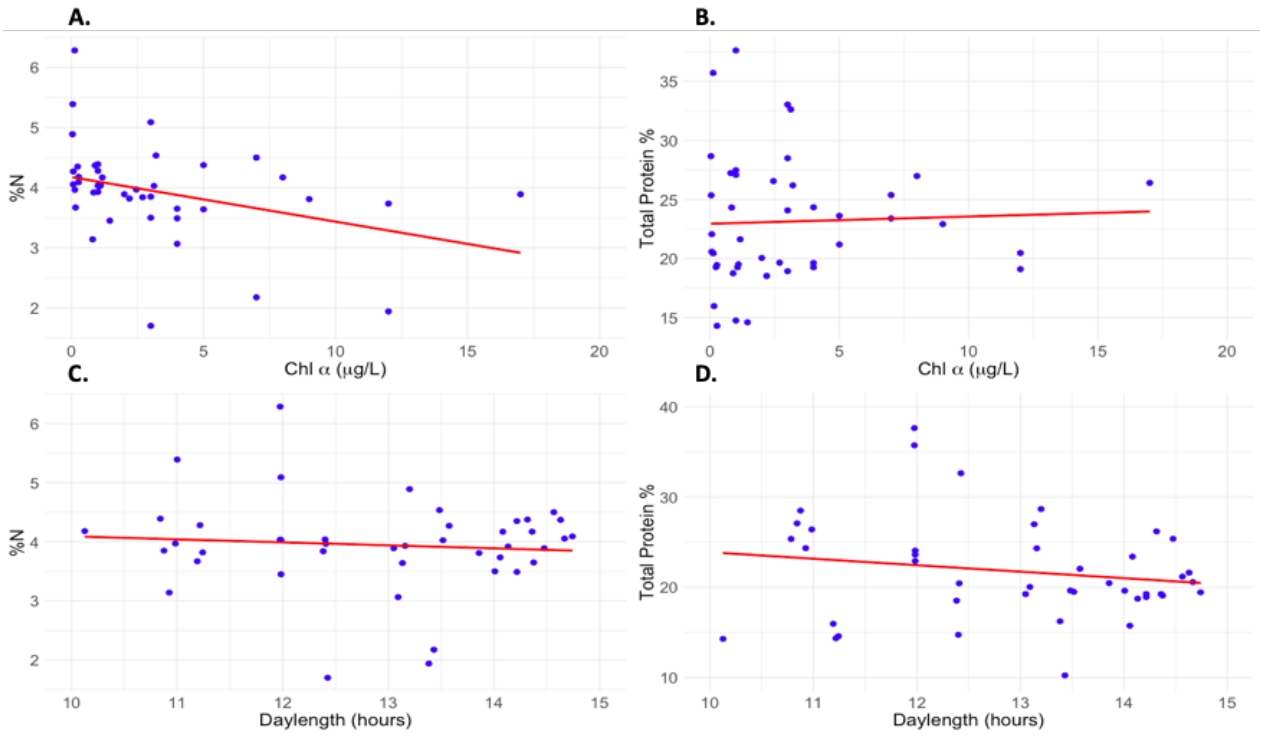


Figure 10: Multi-panel comparison of A. Regression analysis comparing Chlorophyll α concentration to % nitrogen in *Pyropia perforata* (n = 41, y = 4.1747 – 0.0741*x). B. Regression analysis comparing Chlorophyll α concentration to total protein in *Pyropia perforata* (n = 41, y = 22.948 + 0.061*x). C. Regression analysis comparing daylength to % nitrogen in *Pyropia perforata* (n = 41, y = 4.645 – 0.0543*x). D. Regression analysis comparing daylength to total protein in *Pyropia perforata* (n = 41, y = 31.053 -0.7166*x). Chlorophyll α data from 2021 was downloaded from CeNCOOs data portal. % nitrogen was estimated using CHN analysis. Daylength data from 2021 was downloaded from National weather service data portal.

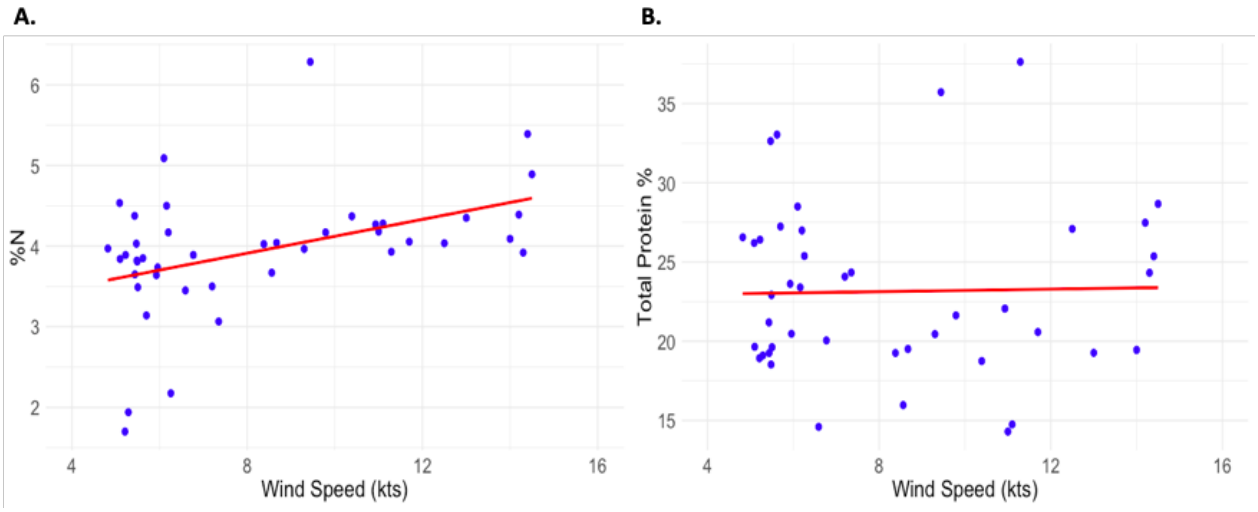


Figure 11: Multi-panel comparison of A. Regression analysis comparing wind speed to % nitrogen in *Pyropia perforata* (n = 41, y = 3.0782 + 0.104*x), and B. Regression analysis comparing wind speed to total protein in *Pyropia perforata* (n = 41, y = 22.727 + 0.0495*x). Wind speed data from 2021 was downloaded from CeNCOOs data portal.

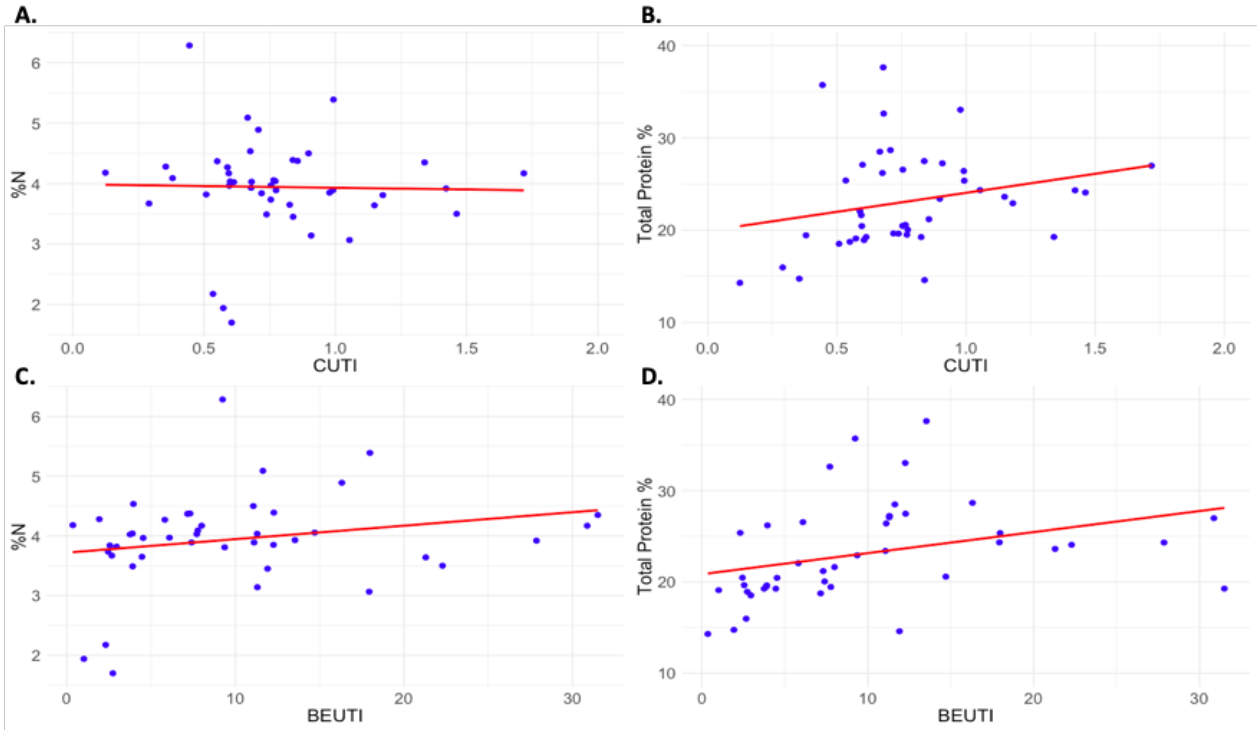


Figure 12: Multi-panel comparison of A. Regression analysis comparing Coastal Upwelling Transport Index (CUTI) to % nitrogen in *Pyropia perforata* (n = 41, y = 3.9871 – 0.0564*x). B. Regression analysis comparing Coastal Upwelling Transport Index (CUTI) to total protein in *Pyropia perforata* (n = 41, y = 19.932 + 4.1173*x). C. Regression analysis comparing Biologically Effective Upwelling Transport Index (BEUTI) to % nitrogen in *Pyropia perforata* (n = 41, y = 3.7195 + 0.0226*x). D. Regression analysis comparing Biologically Effective Upwelling Transport Index (BEUTI) to total protein in *Pyropia perforata* (n = 41, y = 20.851 + 0.2306*x). CUTI and BEUTI data from 2021 was downloaded from NOAA's Southwest Fisheries Science Center data portal.

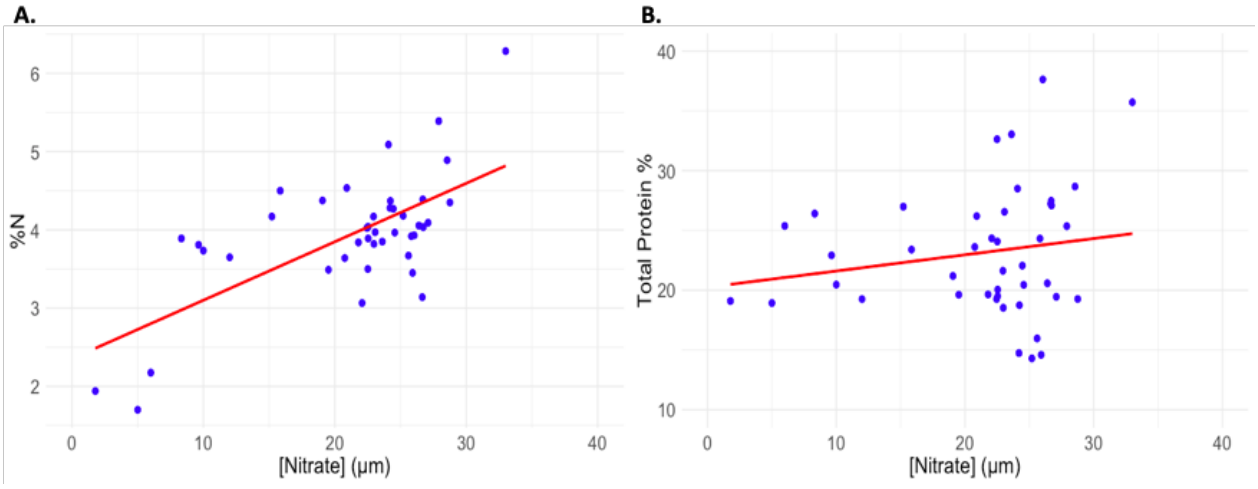
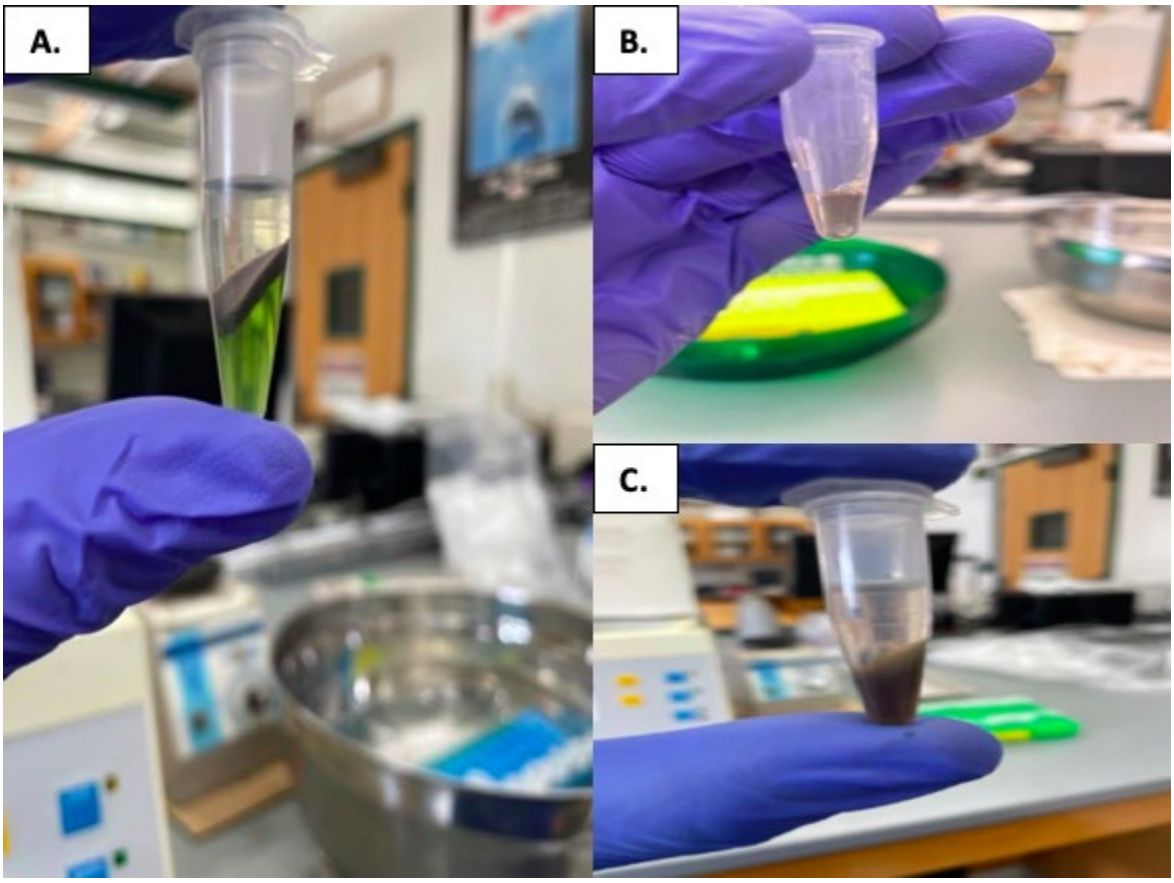


Figure 13: Multi-panel comparison of A. Regression analysis comparing estimated nitrate concentration to % nitrogen in *Pyropia perforata* (n = 41, y = 2.353 – 0.0747*x), and B. Regression analysis comparing estimated nitrate concentration to total protein in *Pyropia perforata* (n = 41, y = 20.249 – 0.135*x). Nitrate concentration from 2021 was estimated using the equation: $NO_3^- = 25.22 – 1.96(T) + 0.04(T)^2 - 1.21(Chl\ \alpha) – 0.05(Chl\ \alpha)^2$.

Appendix



Appendix - A: Multi-panel comparison of A. Protein layers after centrifuge; aqueous (top), interface (middle), and lipid (bottom). B. Aqueous extract. C. 600µL addition of urea buffer to the dried pellet.

Appendix - B: Multi-panel comparison of A. Low [Nitrate] Pigment (0 and 5µm), B. Low [ammonium] Pigment (0 and 5µm), C. High [Nitrate] Pigment (15 and 50µm), and D. High [Ammonium] Pigment (15 an 50µm).