

**EFFECT OF NITROGEN SOURCE ON THE THERMAL TOLERANCE OF
DIFFERENT STRAINS OF THE SYMBIOTIC DINOFLAGELLATE
BREVIOLUM MINUTUM IN CULTURE AND WITHIN A CNIDARIAN HOST**

by

Jessica Capista

A thesis submitted to the Faculty of the University of Delaware in partial
fulfillment of the requirements for the degree of Master of Science in Marine Studies

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ABSTRACT

Symbiotic cnidarians often occupy oligotrophic environments but are able to thrive due to their dinoflagellate symbionts, however, rising temperatures and changing nutrient regimes put this symbiosis at risk of bleaching. Bleaching response is highly dependent on nutrient concentration, nitrogen form, and stoichiometry, as moderate NO_3^- enrichments, especially in the absence of balanced phosphorus additions have been linked to increased bleaching while moderate NH_4^+ enrichments are associated with increased thermal tolerance. Despite these differential responses, the underlying mechanisms driving these outcomes are not well understood. Since algal photosystem II (PSII) is a central target of bleaching, I hypothesized the additional energy requirement of reducing NO_3^- to NH_4^+ in the chloroplast was driving electrons away from photosynthesis and slowing PSII protein repair rates, thus leading to increased damage. I investigated how NO_3^- and NH_4^+ in tandem with heating effected *Breviolum minutum* in culture and within the cnidarian host, *Exaiptasia diaphana*. Contrary to previous work on coral hosts, I did not find a clear differential response based on nitrogen form in culture or within a sea anemone host. Additionally, a bleaching response was observed as a loss of algal cells in culture and in hospite but PSII did not appear to be damaged. My findings highlight the importance of assessing multiple cnidarian hosts and closer examining how both the algae and the host respond to better predict how these organisms will respond to future climate conditions.

Chapter 1

EFFECTS OF NITROGEN FORM ON THERMAL TOLERANCE OF TWO STRAINS OF *BREVIOLUM MINUTUM* IN CULTURED ALGAE

1.1 Abstract

Symbiotic cnidarians often occupy oligotrophic environments but are able to thrive due to their dinoflagellate symbionts; however, anthropogenic induced climate change puts this symbiosis at risk of bleaching, with algal photosystem II (PSII) being a central target. Nitrogen form could further affect bleaching response and has been found to impact symbiont performance within a cnidarian host. NO_3^- is generally associated with increased bleaching and NH_4^+ is associated with increased thermal tolerance, but the mechanisms underlying this response are not well understood. Here I investigated how two strains of *Breviolum minutum*, one thermally sensitive and one thermally tolerant, respond physiologically and photochemically to heating when grown on nutrient limited NO_3^- or NH_4^+ media. Findings show nitrogen form did not have a significant impact on thermal tolerance of symbionts in culture. When heated, photochemical efficiency and symbiont growth declined for all cultures; however, cultures maintained carbon fixation. Lastly, despite previous characterization of these *B. minutum* strains as thermally tolerant and thermally weak, thermal tolerance was similar when cultures were grown semi-continuously with nutrient limited media. These findings highlight the importance of assessing the response of algae alone and under different growth media that is more representative of N-limited states they typically inhabit *in hospite* to determine how cultures respond to environmental stress.

1.2 Introduction

Coral reefs are highly productive ecosystems that provide a suite of ecosystem services and economic benefits including pharmaceuticals, food and fisheries jobs, tourism, coastal protection, and more (Moberg & Folke, 1999). Corals often occupy oligotrophic environments but are able to thrive due to their symbiosis with dinoflagellates of the family Symbiodiniaceae (Muscatine & Porter, 1977). However, these organisms are particularly sensitive to the effects of climate change and coral reef health has declined rapidly in recent years. Rising temperatures increase stress and lead to bleaching, the expulsion of algal symbionts. However, it is important to note that extreme genetic and physiological diversity in algal symbionts leads to different tolerances to environmental stress and damage (Gabay et al., 2019; LaJeunesse et al., 2003; Leal et al., 2015; Rådecker et al., 2018; Suggett et al., 2017). While thermally resistant strains may provide some refuge under future ocean conditions, it is vital to understand algal dynamics under multistressor scenarios common with climate change, such as temperature and nutrient stress to predict the success of symbiotic-cnidarians.

Widespread bleaching is triggered by the combination of high light and temperature, often inducing damage to photosystem II (PSII) in the algal symbionts (Brown, 1997; Hoegh-Guldberg & Jones, 1999; Hoegh-Guldberg & Smith, 1989). Light and temperature damage decrease photosynthetic electron transport and reduce PSII photochemical efficiency (Warner et al., 1999). One central target of thermal stress is the D1 protein, which along with the D2 protein makes up the central core of the PSII reaction center. The D1 protein is responsible for binding primary quinone molecules that are central to the initial photosynthetic electron transport from PSII to PSI. Under ambient conditions, this protein is constantly broken down and repaired,

and this repair rate is directly proportional to the amount of incident light reaching the chloroplast (Ohad et al., 1984; Takahashi et al., 2004).

During thermal stress, D1 protein repair is inhibited in some cnidarian symbionts (Takahashi et al., 2004). When the D1 repair rate is unable to keep pace with the rate of photo-damage, PSII becomes photoinactivated, leading to a decline in photochemical efficiency and further cell damage. Rising temperatures may increase the susceptibility to photoinhibition even if light intensity declines (Takahashi et al., 2004). During PSII photoinactivation, excess energy that cannot be used for photochemistry may form singlet oxygen species or other reactive oxygen species (ROS) capable of damaging both the host and symbionts and leading to increased photoinhibition and oxidative stress (Lesser, 1996). Both hosts and symbionts have several enzymatic pathways that scavenge ROS; however, under thermal stress, high ROS concentrations can overwhelm these pathways and accumulate, eventually leading to algal symbiont removal via self-degradation, host digestion, or expulsion of cells (Lesser, 1996; Weis, 2008).

Algal symbionts employ a variety of strategies to lower over-excitation and prevent photoinactivation. For example, many algae may decrease the functional absorption cross section of PSII (σ_{PSII}) and limit the amount of excitation energy reaching the PSII reaction center (Falkowski & Raven, 2007; Gorbunov et al., 2001; Lesser & Gorbunov, 2001). Cells can also increase photoprotective pigments to dissipate excess light energy (Falkowski & Raven, 2007; Warner & Berry-Lowe, 2006). Additionally, alternative electron sinks, such as the water-water cycle, can also be used for photoprotection. The water-water cycle is an electron bleed pathway that can be used to protect PSII from increased excitation energy, by reducing oxygen at

PSI to a superoxide radical, converting superoxide into hydrogen peroxide (H_2O_2), and then reducing H_2O_2 back to water. However, under high stress this is an imperfect solution as the pathway can contribute to photodamage by generating excess ROS (Asada, 2000).

In addition to warming waters, climate change and anthropogenic activity affects local and global nutrient regimes, with increased storm frequency, sedimentation, and development leading to higher nutrient levels in the oligotrophic waters cnidarian-algal symbioses typically occupy (Brown, 1997; Fabricius, 2005; Hoegh-Guldberg et al., 2007). While high levels of nutrient enrichments are bad for reef environments, leading to macroalgal overgrowth and bleaching (Fabricius, 2005), moderate nutrient additions could benefit cnidarian-algal symbioses. Moderate ammonium (NH_4^+) additions help maintain stable symbioses in times of thermal stress, whereas moderate nitrate (NO_3^-) enrichments, especially in the absence of balanced phosphorus enrichments can exacerbate thermal stress and increase and prolong bleaching (Béraud et al., 2013; Burkepile et al., 2020; Ezzat et al., 2016; Fernandes de Barros Marangoni et al., 2020; Ezzat et al., 2015; Wiedenmann et al., 2013). Specific mechanisms explaining why one nutrient leads to success and thermal tolerance and another exacerbates stress are not well understood and have not been heavily investigated in algal symbionts, despite PSII being a central target of bleaching.

Nitrogen type could play a critical role in D1 protein repair and prevention of photoinactivation. As NH_4^+ is already reduced, it can be used for direct assimilation into the glutamine synthetase and glutamate synthase (GS-GOGAT) cycle where it could then be used to assimilate proteins, possibly helping with general protein repair

(Glibert et al., 2016). Conversely, NO_3^- reduction requires eight electrons before it can enter the GS-GOGAT cycle. Under heat stress, this additional energy requirement could slow protein production and repair, and slow photosynthesis as electrons are used to reduce NO_3^- , leading to increased photodamage and decreased photochemical efficiency. Alternatively, NO_3^- assimilation could benefit the symbionts under thermal stress by serving as an electron bleed pathway that could maintain linear electron flow in the chloroplast and prevent possible photodamage of PSII.

The role of nitrogen form has been largely overlooked when studying symbionts in isolation. When investigating Symbiodiniaceae response to environmental stress, cultures are typically grown in nutrient replete media, such as f/2, which only contains NO_3^- . If grown in another medium that contains NO_3^- and NH_4^+ , NH_4^+ concentrations are much lower than NO_3^- . F/2 medium has ~5880x higher concentrations of N than what is seen in the symbiont's natural environment (den Haan et al., 2016). Although NO_3^- tends to be the dominant form of nitrogen in marine environments, anthropogenic activity, such as coastal development, increases availability of NH_4^+ (Glibert et al., 2016). While nutrient replete media is beneficial for growing cultures fast, and maintaining log-phase growth longer, the disparity between growth media and environmental conditions of the algae could be a confounding factor when investigating questions related to thermal stress or light stress. Thus, it is important to understand how these algae would respond to stress under conditions more similar to those of their natural environment to better understand and anticipate the effects of climate change.

The goal of this study was to investigate how inorganic nitrogen sources (NH_4^+ and NO_3^-) may affect the photochemical and physiological response to heating within

two different strains of the symbiotic dinoflagellate *Breviolum minutum* that are previously documented as having different levels of thermal tolerance (Mansour et al., 2018). I grew cultures semi-continuously on either 25 μM NO_3^- or NH_4^+ media and heated them for 28 days, monitoring PSII stability over time. I hypothesized that photochemical efficiency would decline under heating, with NO_3^- treatments having lower photochemical efficiency, lower carbon fixation, and increased D1 protein damage, as inferred by measuring photoinactivation rates, than NH_4^+ treatments due to the higher energetic demand of converting NO_3^- to NH_4^+ for protein assimilation. Additionally, I expected the thermally sensitive strain would show signs of photochemical stress significantly earlier than the thermally tolerant strain. Surprisingly, nutrient type did not affect thermal tolerance of either algal strain in culture. When heated, algal growth and photochemical efficiency declined for all cultures; however, cultures maintained carbon fixation rates, indicating that heat did not cause a breakdown in PSII. Additionally, I saw no increased signs of thermal stress in thermally susceptible cultures when grown semi-continuously on nutrient limited media. My findings show that the relationship between nitrogen and heat on algae in culture is more complex than previously thought, as PSII does not appear to be a major target of damage and algae show different trends than previous work on studying symbionts *in hospite*.

1.3 Materials and Methods

1.3.1 Experimental Design

Two strains of *Breviolum minutum* were obtained from the Penn State culture collection of T. LaJeunesse. RT002, hereafter referred to as Strain 1, was previously

identified as thermally sensitive using rapid acute heating assays, while B7, Strain 2, was characterized as thermally tolerant (Mansour et al., 2018). Cultures were transitioned from nutrient replete f/2 (Guillard & Ryther, 1962) that contained 882 μM NO_3^- to nutrient limited media over eight months, initially transitioning to 100 μM N (NO_3^- or NH_4^+), then 50 μM , and eventually to 25 μM . Cultures were grown at 28° C in sterilized seawater media containing either 25 μM NO_3^- or NH_4^+ , and 1.5 μM PO_4^{3-} , and f/2 concentrations of trace elements and vitamins. Light was provided on a 12:12 light:dark cycle with cool white fluorescent bulbs set to 95 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Li-Cor, LI250A). Cell growth was first recorded while cultures were held in batch culture under 25 μM N-limited media for 14 days and were used to calculate μ_{max} values for each culture and N-type (Strain 1 NH_4^+ = 0.282, Strain 1 NO_3^- = 0.231, Strain 2 NH_4^+ = 0.277, Strain 2 NO_3^- = 0.201). These μ_{max} values were then used to determine necessary dilution rates for semi-continuous growth.

Growth was monitored by measuring *in vivo* chlorophyll *a* fluorescence on a Turner10AU fluorometer to ensure that cell density remained stable during the semi-continuous acclimation phase. Cultures that would undergo heating began acclimation to semi-continuous growth earlier than those that would be maintained at ambient temperatures and thus were grown semi-continuously for 32 days prior to implementing different temperature regimes. Cultures that would remain at ambient temperatures were acclimated to semi-continuous growth for 14 days prior to implementing different temperature regimes. Cultures were split into control and heated treatments (n = 4 per treatment). Heated cultures were shifted from 28° C to 32° C over 4 days (1° C day⁻¹ ramping rate), and were maintained semi-continuously at 32° C for 28 days. Heated cells were monitored by *in vivo* chlorophyll *a*

fluorescence measurements and active chlorophyll *a* fluorescence every other day. Control cells were monitored by *in vivo* chlorophyll *a* fluorescence every other day; however, due to time constraints, active chlorophyll *a* fluorescence was monitored less frequently (dark acclimated fluorescence was measured days 0, 5, 9, 13, 17, 19, and 21; light acclimated fluorescence was measured days 0, 5, 9, and 21). After 28 days, cultures were subsampled to measure carbon fixation, cell counts, and photosystem II (PSII) photoinactivation.

1.3.2 Active Chlorophyll *a* Fluorescence

A fluorescence induction and relaxation (FIRE) fluorometer (Satlantic Inc., Halifax, Canada) was used to generate single turnover and multiple turnover fluorescent transients to characterize various aspects of PSII photochemistry, including the maximum quantum yield of PSII (F_v/F_m), maximum quantum yield of PSII in the light acclimated state at the growth light level or effective quantum yield (F_q'/F_m'), functional absorption cross section in the dark and light acclimated state (σ_{PSII} and σ_{PSII}' respectively), and rate of PSII reoxidation in the dark and light acclimated state (τ and τ'). Excitation was provided by a high-luminosity blue light emitting diode (peak λ 455 nm). The sampling protocol consisted of a single turnover (ST) excitation from a 120 μ s saturating light pulse and ST relaxation phase over 60 μ s with 40 samples for each ST relaxation interval. Ten iterations of each sequence were averaged for each measurement. Samples were dark acclimated for 15 minutes prior to measuring fluorescence kinetics in the dark, followed by a four minute exposure to 95 μ mol quanta $m^{-2} s^{-1}$ provided by a blue LED (peak λ 455 nm). Culture filtrate (0.2 μ m) was used for blank signal correction. Fluorescence curves were fitted with the biophysical model of Kolber et al. (1998) using the FIREPRO software.

1.3.3 Carbon fixation

Cultures were sampled after 28 days of heating ($n = 4$ per treatment). 500 μL of culture was subsampled for cell counts and preserved with 20 μL 8% glutaraldehyde. Depending on cell density, 1 or 3 mL of cell culture were spiked with 5 μL ^{14}C labeled sodium bicarbonate (8.4 mCi mmol^{-1}) and placed in a temperature controlled photosynthetron (set to either 28 or 32°C) with PFD of 95 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ for 60 minutes. After incubation, 250 μL of 6 N HCl was added to each vial, and samples were allowed to off gas overnight. Samples were then combined with 5 mL scintillation cocktail (Ultima Gold, Perkin Elmer) and read on a scintillation counter (Beckman LS-6500). Cell were manually counted on a light microscope under 100x magnification using a Neubauer hemocytometer (eight independent replicates). Total carbon fixation was normalized to cell number and incubation duration.

1.3.4 Rate constant for photoinactivation

After 28 days of heating, cultures were subsampled to measure the rate of photoinactivation under high light while exposed to the protein inhibitor lincomycin. Lincomycin specifically blocks chloroplast protein repair, thereby allowing one to measure the true rate of PSII photoinactivation (Campbell & Tyystjärvi, 2012). For each culture and treatment condition, lincomycin was added to 24 tubes to a final concentration of 200 $\mu\text{g ml}^{-1}$ and an additional 24 tubes contained culture alone. All samples were dark acclimated with lincomycin for 15 minutes and then placed in a temperature controlled photosynthetron connected to a water bath set to either 28 or 32° C. Light was provided under each photosynthetron well by a single LED set to 450 $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ (Cree Cool White XP-G3). Samples were removed for active chlorophyll *a* fluorescence at the start of the time course and then after 10, 20, 30, 45,

and 95 minutes of light exposure. After removal, samples were dark acclimated for 15 minutes prior to measuring chlorophyll *a* fluorescence induction curves in a FIRE fluorometer as described above. Fluorescence traces were processed using the FIREPRO Software to calculate minimum fluorescence (F_o), maximum fluorescence (F_m) and F_v/F_m . For each experimental condition, the decay in F_v/F_m in lincomycin treated samples was plotted against time and the rate constant for photoinactivation (k_{PI}) was calculated by fitting a single exponential decay to each curve.

1.3.5 Statistical Analyses

Fluorometry datasets were visually checked for assumptions of homogeneity of variance and normal distribution. Linear mixed models were used to test significant main effects and interactive effects of heating, time, and nitrogen type in fluorometric data. Post hoc tests were used to distinguish specific drivers of significance. All other data sets were tested for assumptions of homogeneity of variance and normal distribution using the Levene and Shapiro-Wilk tests. If either test invalidated these assumptions the data were log transformed and retested to ensure homoscedacity and normality prior to further analysis. Two-way analysis of variance (ANOVA) was used to test for significant differences among heating and nutrient type in carbon fixation and rates of photoinactivation. Photoinactivation rates were first calculated by fitting a single exponential decay to each lincomycin treatment curve in GraphPad (Prism GraphPad Software). A three-way ANOVA was also used to test for significant differences among cultures, heating, and nitrogen type for photoinactivation data. A Tukey post hoc test was used to further detect significant differences among samples. All ANOVAs and linear mixed model analyses were performed using R software (R Core Team, 2022) with the "car", "lmer", and "emmeans" packages.

1.4 Results

1.4.1 Cell Abundance and Photochemistry

In vivo chl fluorescence, which was used as a proxy for cell density, significantly declined in heated treatments over time (Fig. 1.1; $p < 0.0001$ time), while cell density in ambient (28°C) treatments remained stable. Active chlorophyll *a* fluorescence analysis results are constrained to a 21 day timeline to account for when both heated and control samples were measured. Active chlorophyll *a* fluorescence analysis revealed Strain 1 heated NO_3^- and NH_4^+ cultures had significantly lower maximum quantum yield of PSII (Fv/Fm) than ambient NO_3^- and NH_4^+ cultures respectively, wherein Fv/Fm of heated NH_4^+ treatments continually declined over time to be lower than heated NO_3^- (Fig 1.2 a, b; $p < 0.0001$ time x heat). In addition, under ambient conditions, Fv/Fm was lower in NH_4^+ cultures than NO_3^- cultures throughout most of the time course ($p < 0.0001$ nutrient). Fv/Fm was also significantly lower in Strain 2 heated NH_4^+ and NO_3^- treatments than respective ambient treatments, starting on day 5 ($p < 0.0001$ time x heat). Unlike Strain 1, nitrogen type had no significant effect on Fv/Fm in Strain 2 heated treatments; however, under ambient conditions Fv/Fm significantly increased in NH_4^+ cultures, while Fv/Fm of NO_3^- cultures remained constant throughout the experiment (Fig. 1.2 c, d, $p = 0.022$ nutrient x heat).

The functional absorption cross section (σ_{PSII}) of Strain 1 heated cultures varied slightly, albeit significantly, from ambient cultures (Fig. 1.2 e, f; $p < 0.001$ time x heat); however, after 21 days, there was no difference between heated and ambient treatments. In contrast, Strain 2 σ_{PSII} was significantly larger in heated treatments than ambient treatments, and σ_{PSII} increased over time in both heated NO_3^- and NH_4^+ treatments (Fig 1.2 g, h; $p < 0.0001$ time x heat), despite the significant decline in

Fv/Fm due to heating. Throughout most of the experiment, σ_{PSII} was significantly larger in heated NO_3^- cultures than in heated NH_4^+ samples ($p < 0.0001$ nutrient x heat), while ambient treatments remained constant through time regardless of nitrogen form.

Photosystem II reoxidation (τ) was significantly slower in heated Strain 1 cultures than ambient cultures; however, fluctuations in ambient treatments over time led to only certain days being significant (Fig. 1.2 i, j; $p < 0.0001$ time x heat). τ oscillated for both ambient NO_3^- and NH_4^+ treatments, but after 21 days there was no significant difference in τ relative to the start of the experiment for either nitrogen treatment. Strain 2 cultures followed a similar pattern to Strain 1, wherein τ was significantly slower in heated treatments than ambient treatments, and τ of ambient treatments fluctuated over time (Fig. 1.2 k, l; $p < 0.01$ time x heat).

When cultures were exposed to the growth light, the effective quantum yield of PSII (F_q'/F_m') was significantly lower in Strain 1 heated treatments than the respective ambient treatments; however the decline was much smaller than what was seen in the dark acclimated state. F_q'/F_m' of heated NH_4^+ treatments remained lower than ambient NH_4^+ treatments throughout the experiment, while F_q'/F_m' of heated NO_3^- varied with time but was also significantly lower than ambient NO_3^- (Fig. 1.3 a, b; $p < 0.01$ time x heat). In contrast to F_q'/F_m' for Strain 1 cultures, Strain 2 F_q'/F_m' was significantly higher in heated treatments than ambient treatments, and F_q'/F_m' increased over time in both heated NO_3^- and NH_4^+ cultures (Fig. 1.3 c, d; $p < 0.0001$ time x heat).

The PSII functional absorption cross section in the light (σ_{PSII}') did not change in heated Strain 1 NH_4^+ cultures despite the decline in F_q'/F_m' . σ_{PSII}' of heated NO_3^-

cultures slightly increased through time, and was significantly, larger than σ_{PSII} ' of the ambient NO_3^- cultures on day 21 (Fig. 1.3 e, f; $p = 0.048$ time x heat). σ_{PSII} ' remained constant through time for all other treatments. Strain 2 heated cultures had significantly larger σ_{PSII} ' than ambient treatments, and σ_{PSII} ' increased through time (Fig 1.3 g, h; $p < 0.0001$ time x heat). Heated NO_3^- treatments had significantly larger σ_{PSII} ' than heated NH_4^+ cultures throughout most of the experiment ($p < 0.001$ nutrient x heat), while σ_{PSII} ' of Strain 2 ambient cultures was the same through time regardless of nitrogen type.

PSII reoxidation in the light (τ') was significantly slower for Strain 1 heated NO_3^- ; however, after day 5 τ' decreased and returned to starting rate (Fig. 1. 3 i, j; $p = 0.048$ time x heat). After day 5 there was no effect of heat or nitrogen type on τ' for any treatment. Strain 2 τ' was significantly faster in heated cultures than ambient treatments and rates decreased over time for both heated NO_3^- and NH_4^+ treatments (Fig. 1.3 k, l; $p < 0.001$ time x heat). In contrast to dark acclimated samples, τ' remained constant for ambient treatments in Strain 1 and 2.

1.4.2 Carbon Fixation

After 28 days of heating, carbon fixation ($\mu\text{g C cell}^{-1} \text{ hr}^{-1}$) was significantly higher in Strain 1 heated NH_4^+ cultures than heated NO_3^- cultures (Fig 1.4; $p = 0.011$ nutrient x heat), while ambient NO_3^- and NH_4^+ treatments had equivalent carbon fixation rates. Carbon fixation was significantly higher for Strain 2 heated NH_4^+ cultures than all other treatments (Fig 1.4; $p < 0.0001$ nutrient x heat). Carbon fixation did not differ depending on nutrient type for Strain 2 ambient treatments. Strain 2 heated NO_3^- treatments fixed significantly more carbon than ambient NH_4^+ cultures (Tukey $p = 0.04101$), but rates were equivalent to ambient NO_3^- cultures.

1.4.3 Photoinactivation

The rate of photoinactivation was measured using the chloroplast protein synthesis inhibitor, lincomycin. NH_4^+ treatments had significantly faster rates of photoinactivation than NO_3^- treatments in Strain 1 cultures (Fig 1.5; $p < 0.001$ nutrient), while photoinactivation rates were not significantly affected by nutrient type or heat for Strain 2 cultures. Photoinactivation rates of Strain 1 NH_4^+ cultures were also significantly faster than Strain 2 NO_3^- and NH_4^+ treatments ($p = 0.023$ nutrient x culture).

1.5 Discussion

The goal of this study was to assess if nitrogen type affected photochemistry and physiology of two different strains of the symbiotic dinoflagellate, *B. minutum*, grown semi-continuously under nutrient limited media in ambient conditions and under chronic thermal stress. Strain 1 was previously identified as thermally sensitive, while Strain 2 was thermally tolerant. Despite nitrogen type having markedly different responses on cnidarian-algal holobionts under thermal stress (as reviewed by Morris et al., 2019), with NO_3^- being associated with increased bleaching (Burkepile et al., 2020; Fernandes de Barros Marangoni et al., 2020; Wiedenmann et al., 2013) and NH_4^+ linked to thermal resilience (Béraud et al., 2013; Fernandes de Barros Marangoni et al., 2020), I did not observe a clear effect of nitrogen type on heating in cultured algae. Thermal stress led to decreased growth and PSII photochemical efficiency in all treatments, regardless of nutrient. Additionally, my findings show no clear difference in thermal tolerance of algal strains, despite previous characterizations as tolerant and susceptible.

1.5.1 Physiological response to nitrogen form and thermal stress

Within a cnidarian host, NO_3^- enrichments can exacerbate thermal stress and increase bleaching due to decreased photochemical efficiency and carbon translocation (Burkepile et al., 2020; Fernandes de Barros Marangoni et al., 2020), while NH_4^+ can mitigate thermal stress by allowing symbionts to maintain photochemical efficiency and carbon fixation and translocation (Béraud et al., 2013; Fernandes de Barros Marangoni et al., 2020). However, findings show the effect of nitrogen type on thermal tolerance in cultured algae is less clear. Upon heating, cells density declined for all treatments, with NH_4^+ grown cultures losing cells at a faster rate than NO_3^- treatments for Strain 1 alga. Additionally, heating led to a partial loss of PSII activity; however, declines in photochemical efficiency were small, and did not have downstream effects on carbon fixation. Strain 1 heated treatments had slight but significant differences in Fv/Fm based on nitrogen form with NH_4^+ treatments having continually lower Fv/Fm than NO_3^- treatments while Strain 2 alga had no difference in Fv/Fm based on nitrogen. All heated treatments had slower rates of PSII reoxidation than ambient treatments, regardless of algal strain or nutrient type; however, these rates were only slightly slower than ambient cultures, and further demonstrate that heat did not have detrimental effects of PSII physiology.

As photochemical efficiency declined due to heating, I expected σPSII would also decrease to limit the amount of excitation energy entering PSII and prevent photoinhibition. However findings show that , σPSII did not change for Strain 1 alga. Additionally, Strain 2 alga increased σPSII upon heating, with NO_3^- treatments having larger σPSII than NH_4^+ treatments. Increased σPSII with declining Fv/Fm could indicate reaction centers are damaged (Falkowski & Raven, 2007). Alternatively, under sub-saturating light and nutrient limited conditions cells might increase σPSII in

order to increase NO_3^- assimilation or increase ATP production so NH_4^+ can be converted into amino acids (Falkowski & Raven, 2007). This could explain why σPSII is larger for Strain 2 heated NO_3^- treatments than heated NH_4^+ treatments. While Strain 2 cultures do not appear to be affected by photoinactivation, the potential tactic of increasing σPSII to increase nutrient assimilation and amino acid/ protein production could explain why σPSII is increasing for these cultures in both the dark acclimated state and under actinic light.

Upon heating, cultures maintained or increased carbon fixation rates, further emphasizing that heat did not have detrimental effects on PSII physiology despite slight declines in photochemical efficiency. Carbon fixation was significantly higher in heated NH_4^+ treatments than heated NO_3^- treatments, and there were strain specific responses for heated NO_3^- cultures. Strain 1 NH_4^+ cultures were able to maintain carbon fixation, while NO_3^- cultures decreased carbon fixation when heated. Strain 2 NH_4^+ cultures had significantly higher carbon fixation rates upon heating, while heated NO_3^- treatments maintained carbon fixation rates upon heating. Interestingly, heated Strain 2 cultures showed no difference in photochemical efficiency depending on nitrogen form, however my findings demonstrate differential effects of nitrogen downstream of PSII. Higher temperatures could have increased electron flow and Rubisco activity, thus leading to the higher rates of carbon fixation for heated NH_4^+ treatments, while NO_3^- reduction in the chloroplast could have diverted electrons away from photochemistry, and thus explain why carbon fixation rates are lower in heated NO_3^- treatments than heated NH_4^+ .

Under ambient conditions, Ezzat et al. (2015) found that NH_4^+ additions led to higher carbon fixation rates of *Stylophora pistillata* symbionts, while NO_3^- additions

lowered carbon fixation rates. Although a similar pattern was observed for Strain 1 algae at elevated temperatures, at ambient temperatures NO_3^- and NH_4^+ cultures had the same carbon fixation rates. For Strain 2 cultures, NO_3^- had higher carbon fixation rates than NH_4^+ cultures at ambient temperatures. Fernandes de Barros Marangoni et al. (2020) measured carbon translocation in the coral host *S. pistillata* and found symbionts of NH_4^+ enriched corals maintained carbon translocation rates, while symbionts of NO_3^- enriched corals lowered carbon translocation rates under elevated temperature. While carbon fixation rates are not directly synonymous with carbon translocation to the host since symbionts could be increasing carbon fixation but not translocating this carbon to the host, my Strain 1 cultures indicate similar findings as Fernandes de Barros Marangoni et al. (2020), with NH_4^+ having higher translocation and NO_3^- trending lower. However, my Strain 2 NO_3^- cultures maintained carbon fixation upon heating. Therefore, the assertion that NH_4^+ and NO_3^- differentially impact carbon translocation is not universally true, although differences between algae in culture vs within a cnidarian host could also be contributing to these findings.

I hypothesized that the differential response of NO_3^- and NH_4^+ was driven by PSII protein repair rates, with NO_3^- reduction slowing protein repair while NH_4^+ would be readily assimilated into proteins for PSII repair. However, when measuring photoinactivation to infer protein repair, I found different algal strains had different nitrogen requirements for PSII repair. Strain 1 NH_4^+ cultures had higher rates of photoinactivation than NO_3^- treatments, regardless of heat, hence NH_4^+ treatments relied more heavily on PSII repair to maintain Fv/Fm than NO_3^- cultures. In Strain 1, heated NH_4^+ cultures downregulated PSII activity slightly more than heated NO_3^- cultures but were able to maintain carbon fixation rates similar to ambient

temperature. Conversely, PSII protein repair does not seem to play an important role for Strain 2 cultures, as rates of photoinactivation were the same regardless of heat or nitrogen form. Since PSII repair does not appear to inhibit photochemical efficiency, this further explains why carbon fixation rates of heated NH_4^+ treatments were so high and why heated NO_3^- treatments maintained carbon fixation rates upon heating. Contrary to what I had assumed, nitrogen form only appeared to have an effect on PSII repair for Strain 1 cultures, and there was no difference based on heating. Although NH_4^+ can be more readily assimilated into proteins, and thus be used to repair PSII and the D1 protein, my findings showed that PSII was not the point of damage or stress among these cultures because they maintained photochemical efficiency and carbon fixation.

1.5.2 *In vivo vs in hospite* algae

Within a cnidarian host, moderate nitrogen enrichments have distinct responses depending on nitrogen form; however, impacts of nitrogen are not analogous for cultures. My findings showed nitrogen form had some impacts on photophysiology, carbon fixation, and PSII repair, but these were also strain dependent. Despite seeing some differences between NO_3^- and NH_4^+ , NO_3^- was not particularly detrimental to the algae as it is within coral hosts. One reason I might not be seeing negative effects of NO_3^- is because NO_3^- reduction could serve as an electron bleed pathway to maintain linear electron flow in the chloroplast and prevent photodamage and oxidative stress. Alternatively, the fundamental differences between cultured algae and algae within a cnidarian host could explain why my study with cultures does not replicate previous findings within corals.

It is not possible to truly replicate the symbiosis in culture alone. Hosts are believed to keep symbionts in a nitrogen limited state to maintain symbiosis (Dubinsky & Jokiel, 1994; Davy et al., 2012; Ezzat et al., 2015, Räddecker et al., 2021). Although I grew algae on media which I knew to be nutrient limiting since it decreased algal growth rate, a nitrogen supply of 25 μM is still higher than nitrogen concentrations of the oligotrophic environments these symbioses occupy. However, semi-continuous or continuous culturing is the only way to keep algae N-limited and remove the confounding factor of constantly changing nutrient concentrations that happens in batch growth. By growing my algae semi-continuously I maintained steady state and balanced growth; however, algae *in hospite* are in unbalanced growth as cnidarian hosts regulate growth by limiting nutrients (Dubinsky & Jokiel, 1994; Davy et al., 2012, Ezzat et al., 2015, Räddecker et al., 2021). *In hospite*, cellular constituents grow at different rates and typically have thinner cell walls, more lipid droplets, and smaller chloroplasts than cells in culture (Pasaribu et al., 2015; Wang et al., 2015). Algal growth rates are also much slower within a cnidarian host, with doubling time ranging from 6-74 days (Muscatine et al. 1984; Wilkerson et al., 1988) vs approximately 3 days in culture (Fitt & Trench, 1983). Additionally, light availability and microbiomes differ between cultured algae and symbionts residing in a cnidarian host (Maruyama & Weis, 2020). Along with fundamental differences between algae in culture vs in hospite, my study was designed as a nutrient limitation experiment, while studies looking at holobiont response to nitrogen form are designed as nutrient enrichment experiments. Although all of these factors could have contributed to the different findings I observed, it is still important to study cultured algae to better understand the symbiosis. Studying algae in culture allows researchers to use different

methodologies to further investigate parts of the algae that they would not be able to when studying the host and symbionts together.

1.5.3 Culture methods and heating assays alter susceptibility

Unexpectedly, I found that Strain 1 cultures were not more thermally sensitive than Strain 2 cultures when grown semi-continuously under nutrient limited media. Mansour et al. (2018) established that the Strain 1 alga was thermally sensitive and the Strain 2 alga was thermally tolerant by growing algae in batch culture and exposing them to acute stress to identify thylakoid membrane melting points. Additional work (personal communication with Tim Bateman and Mark Warner) has also shown similar findings as Mansour et al., (2018), with Strain 1 being sensitive and Strain 2 being thermally tolerant when grown in batch and acutely heated. The decline in Fv/Fm upon heating in my study was no different for Strain 1 or Strain 2 cultures. However, the rate of photoinactivation was higher for Strain 1 NH₄⁺ treatments than all Strain 2 treatments but this difference was driven by nutrients rather than heat. As with Strain 2 cultures, heated Strain 1 cultures still had high carbon fixation rates. While my results show clear differences between Strain 1 and Strain 2 algae, including different photoacclimation tactics and responses to nitrogen form, these differences are not driven by heat alone. These findings further imply that lab based experiments may not be representative of how algae will respond in the natural environment, and specific algal strains may be more thermally tolerant than previously thought. To understand if this response is driven by media or growth regimes, further research needs to be done. One could grow nutrient replete cultures semi-continuously and then expose the algae to chronic thermal stress to distinguish if semi-continuous growth is affecting the algae. Alternatively, one could grow nutrient limited cultures in batch to

assess thermal tolerance; however, it is critical to ensure that cultures do not become nutrient starved.

1.5.4 Conclusion

Although nutrient types have been found to have markedly different effects on cnidarian-algal symbioses when coupled with heat stress, nitrogen form does not have a clear effect on cultured algae in my experimental growth conditions. Therefore, the mechanisms driving differential responses of nitrogen form on cnidarian-algal symbioses are still not clearly understood. Heat alone decreases photochemical efficiency of cultures but does not negatively affect carbon fixation rates. Additionally, laboratory culture methods and thermal stress regimes could skew our understanding of thermal susceptibility of algal symbionts.

FIGURES

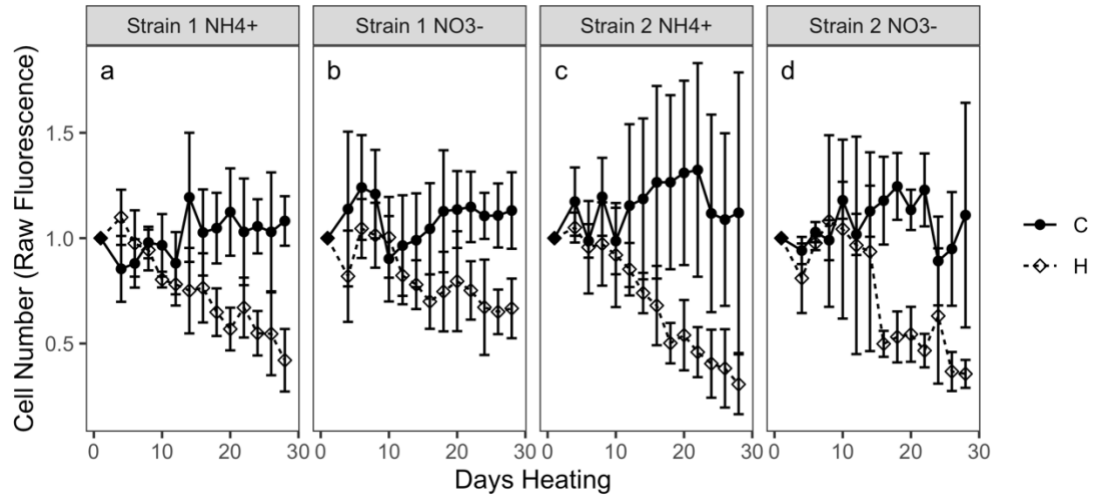


Figure 1.1. Change in cell number as measured by *in vivo* chlorophyll fluorescence over 28 days. Mean \pm SD are shown for ambient and heated treatments (ambient = C closed circles, heated = H open circles), $n=4$ per treatment. All heated treatments showed a significant decline in cell number over time ($p < 0.0001$), while ambient treatments did not change over time.

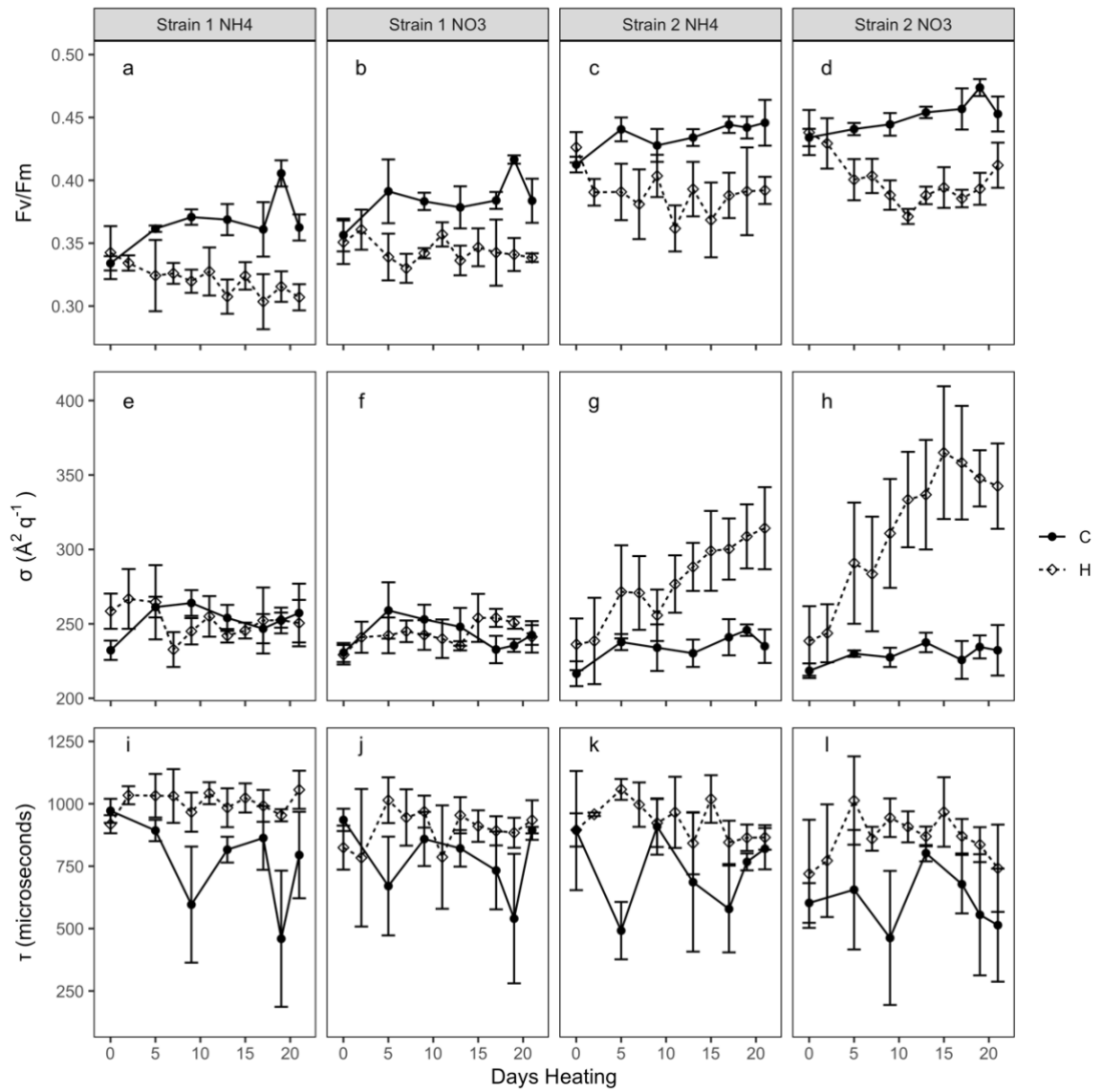


Figure 1.2. Maximum quantum yield of PSII (Fv/Fm) under ambient and heated conditions (a-d) Functional absorption cross section of PSII (σ) under ambient and heated conditions (e-h) Reoxidation rate of PSII (τ) (i-l) under ambient and heated conditions. Each column is a different algal strain-nutrient pair. Mean \pm SD are shown for ambient and heated treatments (ambient = C closed circles, heated = H open circles). Fv/Fm of all heated treatments significantly declined over time (a-d; $p < 0.0001$ time \times heat) and heated Strain 1 NH_4^+ declined more than heated NO_3^- ($p < 0.0001$ nutrient). σ significantly increased for Strain 2 heated cultures with NO_3^- cultures having larger σ than NH_4^+ treatments (g,h; $p < 0.0001$ time \times heat; $p < 0.0001$ nutrient \times heat). τ was significantly slower in heated treatments (Strain 1 $p < 0.0001$; Strain 2 $p < 0.01$ time \times heat).

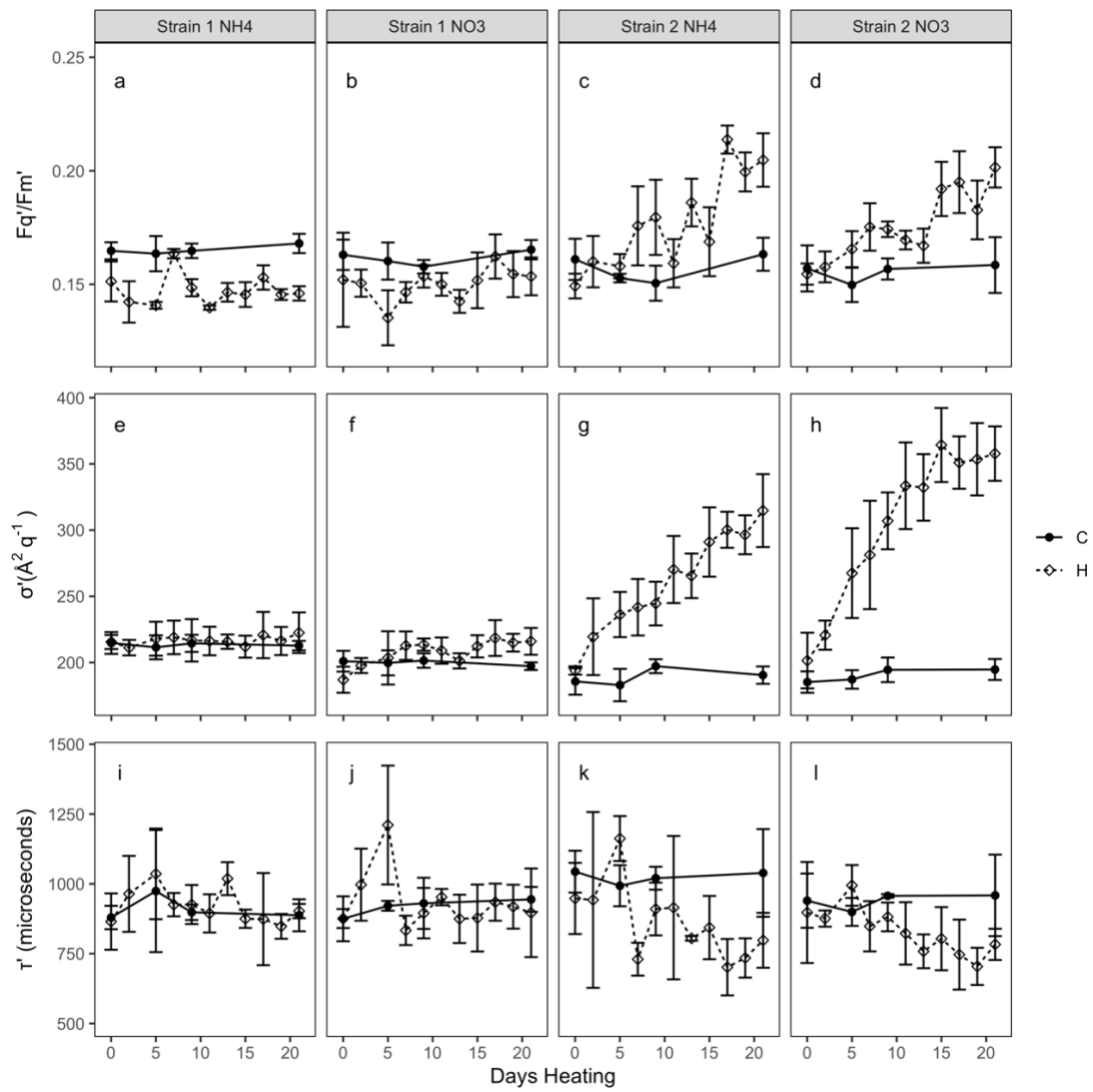


Figure 1.3. Effective quantum yield of PSII (F_q'/F_m') under ambient and heated conditions (a-d) Light acclimated functional absorption cross section of PSII (σ') under ambient and heated conditions (e-h) Light acclimated reoxidation rate of PSII (τ') (i-l) under ambient and heated conditions. Each column is a different algal strain-nutrient pair. Mean \pm SD are shown for ambient and heated treatments (ambient = C closed circles, heated = H open circles), n = 4 per treatment. Effective quantum yield was significantly slower in Strain 1 heated treatments than ambient (a,b; $p < 0.01$ time x heat), and significantly faster in Strain 2 heated treatments than ambient (c,d; $p < 0.0001$ time x heat). σ' was significantly larger in Strain 2 heated treatments than ambient, and larger for NO_3^- treatments than NH_4^+ treatments (g, h; $p < 0.0001$ time x heat; $p < 0.001$ nutrient x heat). τ' was significantly slower in heated Strain 2 cultures than ambient Strain 2 cultures (k,l; $p < 0.001$ time x heat).

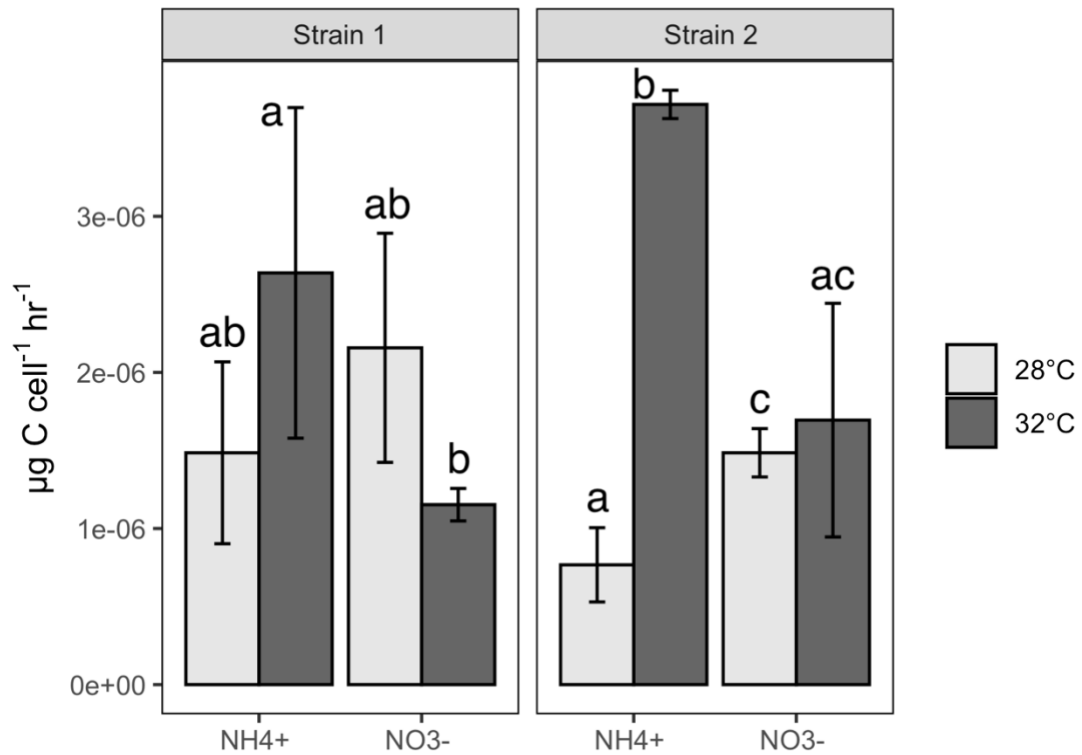


Figure 1.4. Carbon fixation rates of Strain 1 and Strain 2 cultures after 28 days of heating while grown under different forms of N. $N=4$, Mean \pm SD are shown for ambient and heated treatments. Strain 1 cultures showed a significant interactive effect of nitrogen form and heating, wherein heated NH_4^+ had higher carbon fixation rates than heated NO_3^- , and carbon fixation rates of heated NO_3^- cultures trended lower than ambient NO_3^- cultures ($p = 0.011$ nutrient \times heat). Strain 2 heated NH_4^+ cultures also had significantly greater carbon fixation rates than all other treatments ($p < 0.0001$ nutrient \times heat).

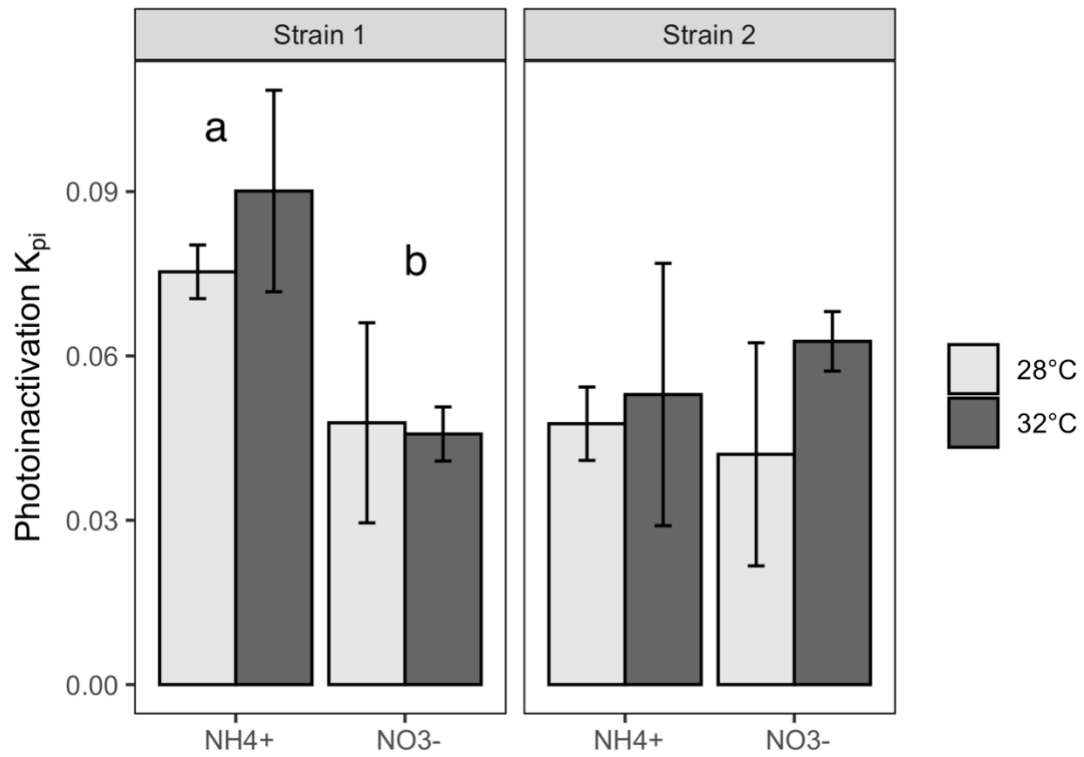


Figure 1.5. Rate of photoinactivation (K_{pi}) of Strain 1 and Strain 2 cultures after 28 days of heating and with two nitrogen forms. Mean \pm SD are shown for ambient and heated treatments. Strain 1 NH_4^+ cultures had significantly higher rates of photoinactivation than NO_3^- cultures ($p < 0.001$ nutrient).

Chapter 2

PHYSIOLOGICAL RESPONSE OF THERMALLY SUSCEPTIBLE BREVIOLUM MINUTUM TO NUTRIENT ENRICHMENTS AND THERMAL STRESS WITHIN ANEMONE HOST

2.1 Abstract

Rising temperatures and changing nutrient regimes from local and global stressors are often associated with cnidarian bleaching, however this response is highly dependent on the nutrient concentration, nitrogen form, and stoichiometry. Moderate NO_3^- enrichments have been linked to enhanced thermal stress, while moderate NH_4^+ enrichments have been characterized as beneficial for the symbiosis. Here I investigated the effects of NO_3^- and NH_4^+ enrichments in tandem with thermal stress on physiological and photochemical health of algal symbionts and the sea anemone *Exaiptasia diaphana*. Findings show nutrient additions had no effect on symbiont response, at ambient or high temperatures. Heated treatments showed signs of bleaching, as loss in symbiont density over time; however, symbionts maintained photochemistry and carbon demands of the host, regardless of nutrient status. These findings highlight the importance of assessing the response of other cnidarian hosts to environmental stress, to better predict how nutrient fluxes and thermal stress will affect cnidarians. I predicted heated NO_3^- treatments would have the lowest photochemical efficiency and carbon translocation rates. Moreover, NH_4^+ addition would provide some thermal tolerance, by increasing carbon translocation. I further hypothesized that heated anemones would have higher feeding rates that could

supplement declined photosynthetic efficiency caused by heat stress. Despite my hypotheses, I found that nutrient type did not have a significant impact on thermal tolerance of symbionts within a cnidarian host.

2.2 Introduction

In the past several decades mass coral bleaching events, where reef corals lose a substantial number of their symbiotic dinoflagellates, have increased in frequency and intensity (Hoegh-Guldberg et al., 2017). The effect of rising temperatures on coral bleaching has been heavily investigated; however, the role of nutrients in bleaching remains equivocal, with various results depending on inorganic vs organic, nutrient concentration, form, and stoichiometry. Heterotrophically derived nutrients and moderate levels of ammonium (NH_4^+) tend to benefit the holobiont (cnidarian host + symbiotic algae) and help maintain stable symbioses in times of thermal stress, whereas moderate nitrate (NO_3^-) enrichments, especially in the absence of balanced phosphorus enrichments can exacerbate thermal stress, increasing and prolonging bleaching (Béraud et al., 2013; Burkepile et al., 2020; Ezzat et al., 2015; Ezzat et al., 2016; Fernandes de Barros Marangoni et al., 2020; Grottoli et al., 2006; Palardy et al., 2008; Wiedenmann et al., 2013). With climate change, nutrient regimes will shift in one of two ways. Warmer oceans could lead to more stratified waters, and lower nutrient availability, however this is more commonly seen in open oceans (Falkowski and Raven, 2007). In reef environments, increased storm frequency, sedimentation, coastal development and land clearing all lead to increased nutrients. Typically, eutrophication and anthropogenic pollution events have been associated with increased NO_3^- ; however, fish excretion and aquaculture, as well as wastewater, fertilizer, and coastal development are all sources of increased NH_4^+ (Allgeier et al., 2017; Fabricius,

2011; Glibert et al., 2016; Malone & Newton, 2020; Shantz & Burkepile, 2014). With shifting nutrient dynamics and continually warming waters, it is essential to understand how holobiont health will change as a result of multistressor scenarios common with climate change.

Several studies have investigated the effects of inorganic nutrients on cnidarian algal symbiosis; however, much emphasis has been placed on the physiological response of the animal, typically reef-building corals, rather than the response of the algae (Béraud et al., 2013; Burkepile et al., 2020; Tanaka et al., 2007). While studying the response of the animal is important, bleaching often involves damage to photosystem II (PSII) in symbionts, so it is essential to investigate how the symbionts are changing physiologically as well. High levels of nutrient enrichment (e.g., $\geq 50 \mu\text{M}$ N) can lead to macroalgal overgrowth and coral bleaching (Fabricius, 2005; see also Bruno et al., 2009). However, moderate NH_4^+ enrichments at lower concentrations (3-15 μM) could benefit the holobiont, especially during thermal stress. In the absence of thermal stress, the effects of moderate NH_4^+ enrichments are less clear. 3 μM NH_4^+ additions to the coral *Stylophora pistillata* led to increased chlorophyll *a* production (Fernandes de Barros Marangoni et al., 2020; Ezzat et al., 2015) and algal carbon fixation and translocation to host (Ezzat et al., 2015), whereas moderate NH_4^+ enrichments (3-20 μM) had no significant effect on photosynthesis or holobiont health in scleractenian corals, *Pocillopora damicornis*, *Montipora verrucosa*, and *Turbinaria reniformis* (Béraud et al., 2013; Sakami, 2000; Stambler et al., 1994). Under thermal stress, moderate NH_4^+ enrichments are important for maintaining photosynthesis (Béraud et al., 2013) and carbon translocation to meet host metabolic demands (Fernandes de Barros Marangoni et al., 2020). NO_3^- enrichments typically induce

negative responses in the holobiont when coupled with thermal stress, such as decreased photosynthetic efficiency, increased oxidative stress, and faster and prolonged bleaching (Burkepile et al., 2020; Ezzat et al., 2016; Fernandes de Barros Marangoni et al., 2020). However, N:P stoichiometry also plays an important role, as P limitation has been associated with damage to PSII and thylakoid membrane structure (Ezzat et al., 2016; Rosset et al., 2017; Wiedenmann et al., 2013). Balanced NO_3^- : PO_4^{3-} enrichments had little effect on the reef corals *Acropora pulchra* and *T. reniformis* (Dobson et al., 2021; Tanaka et al., 2007); however, balanced NO_3^- : PO_4^{3-} additions still had deleterious effects on *S. pistillata*, including reduced photosynthetic efficiency and chlorophyll *a* content, with and without thermal stress (Fernandes de Barros Marangoni et al., 2020).

The different holobiont responses to thermal stress and NO_3^- and NH_4^+ additions alone could be due to the algae's cellular demands for using NO_3^- . NO_3^- assimilation and reduction requires eight electrons for successful conversion to NH_4^+ , where it is used for direct assimilation into the glutamine synthetase and glutamate synthase (GS-GOGAT) cycle (Glibert et al., 2016). Under thermal stress, this additional energy requirement could slow protein production and repair thereby negatively affecting photosynthetic rates as well as carbon translocation to the host. Alternatively, NO_3^- assimilation might benefit the symbionts under thermal stress by serving as an additional pathway for excess electron flow that could maintain linear flow in the photosynthetic electron transport chain and prevent possible photodamage to PSII.

The different response to nitrogen form could also be driven by the symbionts as the sole source for NO_3^- assimilation. Conversely, both the host and the algal

symbionts are cable of NH_4^+ assimilation, although the algae can assimilate 14-23 times more than the host (Pernice et al., 2012). It has been suggested that host-induced nitrogen limitation is essential for a stable symbiosis and facilitates photosynthate translocation to the host (Dubinsky & Jokiel, 1994; Ezzat et al., 2015), thus increased NO_3^- that can only be assimilated by the alga could alter this balance by leading to increased symbiont densities and less translocation. Under thermal stress, this disruption in host control could be exacerbated if symbionts retain photosynthates and reduce carbon translocation. This would increase carbon demand of the host and lead to catabolism of host amino acids to meet demands. In breaking down amino acids, more N would be available to symbionts to continue the cycle of photosynthate production and retention (Rädecker et al., 2021). Since NH_4^+ is assimilated by both the host and the symbionts, this increased nutrient availability could be enough to offset the host's needs and disrupt the response to thermal stress. On the other hand, moderate NO_3^- enrichments could increase symbiont densities, retention of photosynthates, and carbon demand of the host, thus contributing to the feedback loop and exacerbating the negative effects of thermal stress.

Trophic plasticity could offer some relief during times of thermal stress, by allowing cnidarians to meet their metabolic demands. Despite having symbiotic algae, all corals rely on feeding to support some of their energetic and nutritional demands. Heterotrophy can account for up to 60% of the hosts metabolic demands in healthy symbioses (Palardy et al., 2008) and up to 100% in some bleached corals (Grottoli et al., 2006). Trophic plasticity increases the resilience to bleaching and expedites recovery against stress (Grottoli et al., 2006; Palardy et al., 2008). However, the degree of trophic plasticity varies depending on symbiont-host pairings and inorganic

nutrient availability. The link between autotrophy and heterotrophy during thermal stress still remains unclear, with some corals increasing heterotrophy in response to decreased autotrophy (Grottoli et al., 2006), while increased heterotrophy is positively correlated with high rates of autotrophy in other symbiotic cnidarians, such as the sea anemone *Exaiptasia* (Leal et al., 2015). In addition, increased heterotrophy might fail to serve as a substitute for autotrophy under environmental stress. For example, inorganic nutrient limitation led to increased feeding in the coral *S. pistillata*; however, heterotrophically derived nitrogen did not meet the demands of the algal symbionts (Rosset et al., 2015). Despite feeding under thermal stress and nutrient limitation, corals could not assimilate heterotrophically derived nutrients and became carbon starved (Ezzat et al., 2019). Clearly heterotrophy plays an extremely variable role depending on treatment conditions, yet several studies will withhold food sources to organisms to see direct effects of inorganic nutrients or heating alone. Since these organisms rely on heterotrophic feeding to some extent, it is beneficial to include in this study to better understand the response of the holobiont to other stressors.

The goal of this study was to investigate the effects of different nitrogen sources on health and response to heating in the symbiotic sea anemone *Exaiptasia diaphana* (Dungan et al., 2020), a widely used cnidarian-algal model system (Bellantuono et al., 2019; Leal et al., 2015; Rädercker et al., 2018; Xiang et al., 2020). Anemones were infected with a thermally sensitive strain of *Breviolum minutum* and fed to repletion once a week. I used a fully factorial experiment to assess the effects of nitrogen form (NO_3^- , NH_4^+ , and no N additions with artificial seawater) under thermal stress on the photochemistry, algal physiology, carbon fixation and translocation, nutrient drawdown, and anemone feeding. I hypothesized NH_4^+ additions in the

absence of heating would not have significant impacts on the health of the holobiont relative to control treated anemones. However, I predicted NO_3^- treated anemones would have lower carbon translocation rates than the other treatments. I did not expect to see differences in photophysiology from nutrient treatments at ambient temperatures. Once heated, I hypothesized that heated treatments would have lower photochemical efficiency than ambient treatments. I predicted heated NO_3^- treatments would have the lowest photochemical efficiency and carbon translocation rates. Moreover, I thought NH_4^+ addition would provide some thermal tolerance, by increasing carbon translocation. I further hypothesized that heated anemones would have higher feeding rates that could supplement declined photosynthetic efficiency caused by heat stress. Despite my hypotheses, I found that nutrient type did not have a strong impact on thermal tolerance of symbionts within a cnidarian host. Heated treatments showed signs of bleaching, as loss in symbiont density over time; however symbionts maintained photochemistry and carbon demands of the host, regardless of nutrient status. Additionally, heated NO_3^- treated anemones had higher heterotrophy rates than ambient NO_3^- treated anemones. This study will improve our understanding of how nutrients, both organic and inorganic, will affect cnidarian algal symbioses in future climate conditions.

2.3 Materials and Methods

2.3.1 Experimental Design

Eight months prior to the experiment, anemones were infected with a thermally sensitive strain of *B. minutum* (culture RT002). Animals were held in artificial seawater (ASW) (Instant Ocean, 33-35 psu, N-free) in clear polycarbonate containers

(Cambro) fitted with false bottoms and magnetic stir bars. Animals were held in Percival incubators (model IL-VL) set to 28° C with 90 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ of light provided by cool white fluorescent bulbs set to a 12:12 light:dark cycle. Animals were acclimated to these conditions for 33 days prior to starting nutrient additions. Throughout the experiment, all animals were fed once each week with freshly hatched *Artemia salina* and containers were cleaned weekly to remove fouling. When cleaned, NO_3^- and NH_4^+ treatments were filled directly from the 5 μM stock carboy.

After the initial acclimation period, three nutrient treatments were used consisting of a control with only ASW, NO_3^- enrichment (5 μM NO_3^- + .3 μM PO_4^{3-}), and NH_4^+ enrichment (5 μM NH_4^+ + .3 μM PO_4^{3-}), with 8 containers treatment⁻¹ each holding 6 anemones. Nutrients were continuously delivered to each container from 5 μM stocks via peristaltic pump (Watson Marlow 205u) at a constant flow rate of 1.2 ml min^{-1} , and each container was gently mixed with constant stirring from magnetic stir bars. After 23 days, containers were transferred from the incubators and placed in continuously circulating water baths for easier measurement access. Each water bath consisted of a central freshwater sump that was heated by 500 volt titanium heaters controlled by a digital aquarium control system (Apex Energy Bar) that continuously monitored the temperature in the sumps, while individual water bath temperatures were monitored with temperature loggers (HOBO, Water Temp Pro V2). Nutrients were delivered to each container with the same pumps described above and the water in each container was mixed by air stones. LED lights (CREE cool-white XP-G3) provided the same irradiance of 90 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (12:12LD cycle). All anemones were fed on the same feeding schedule as described above.

After 31 days of nutrient supply, 5 anemones treatment⁻¹ were randomly sampled to test for any effects of nutrients alone on algal photo-physiology and carbon translocation. Water bath temperatures were then increased 1° C day⁻¹ to 32° C in half of the treatments, while the other baths remained at 28° C. Nutrient flow rate was increased to 2 mL min⁻¹ to account for the rapid drawdown of nutrients and try to keep nitrogen concentrations closer to the experimental target for longer. After 10 days at 32° C, heated NH₄⁺ anemones detached from the bottom of the containers and became contracted. Salinity, temperature, and NH₄⁺ levels of heated NH₄⁺ containers were checked to ensure there was no clear difference driving the animals' response. All metrics were found to be normal. After 14 days at 32° C, minimal changes in photochemistry were noted (described below) and the temperature was increased to 33° C in the heated treatments. All remaining anemones were sampled once more after 28 days of chronic heating in order to document any changes in animal biometrics, photosynthetic activity, and carbon translocation (described below).

2.3.2 Nutrient Assimilation

Nutrient assimilation rates were assessed over 2 h for each container prior to thermal stress. Water in each container was removed and refilled from the 5 µM stock container and nutrients were delivered into each container at 1.2 mL min⁻¹ with continuous stirring provided by magnetic stir bars set to 300 RPM. Samples were collected from each container by syringe and passed through a 0.2 µm filter at the start of the experiment and subsequent water samples were removed and filtered after 5, 10, 20, 30, 45, 60, and 120 minutes. Samples were frozen at -20° C and analyzed the following day using methods described below.

After 21 days of heating nutrient drawdown rates were measured again. However, to eliminate any drawdown from fouling periphyton attached to the false bottoms of each container, individual anemones were used rather than entire containers with multiple anemones. Four individual anemones nitrogen treatment⁻¹ were placed in 50 mL beakers that were fitted with a false bottom made from a rubber washer and nitex mesh over a stir bar spinning at 300 RPM. Beakers were filled with the 5 μ M nutrient stock and nutrients were continually delivered to each beaker at 2 mL min⁻¹, replicating the delivery rate for each container in the experimental system. Water samples were collected from each beaker by syringe and passed through a 0.2 μ m filter at the start of the experiment and then sampled once more after 10, 20, 30, 45, 60, and 120 mins. Samples were frozen at -20° C and analyzed the following day.

Drawdown was determined by measuring the nitrogen concentration of samples at each sampling timepoint using microplate assays. NO₃⁻ concentrations were quantified based on methods described by Ringuelet et al., (2011), using a potassium nitrate standard. A mixed enzyme solution made up of 5.6 mL potassium phosphate buffer (28 mM KH₂PO₄, 25 mM KOH, and 25 mM EDTA, pH = 7.5), 560 μ L nitrate reductase and 280 μ L NADH (NECI superior enzymes) was added to each well containing samples and standards. The microplate was lightly shaken for one hour on a rotator table (Barnstead Lab-Line). After incubation, Reagent 1 (58mM Sulfanilamide in 3.6N HCl) and Reagent 2 (3.9mM NED) were added to each microplate well. The microplate incubated for an additional 10 minutes while shaking before quantifying the absorbance at 540 nm on a FLUOstar Omega Plate Reader (BMG Labtech, Germany). NH₄⁺ concentrations were quantified based on a modified indophenol blue colorimetric method (Ringuelet et al., 2011) using an ammonium

sulphate standard. Reagent 1 (1632 mM citrate enzyme), Reagent 2 (404 mM phenol and 1.3 mM sodium nitroprusside), and Reagent 3 (Sodium hypochlorite and 500 mM NaOH) were added to each well. The microplate was vortexed for 2 minutes at 750 RPM and then incubated in the dark for two hours. Absorbance was quantified at 630 nm on a FLUOstar Omega Plate Reader (BMG Labtech, Germany). Nutrient drawdown was determined by looking at the percent change of nutrients over time. For each nutrient treatment, nutrient concentrations were also plotted against time and the rate constant for nutrient assimilation was calculated by fitting a single exponential decay to each curve in Graphpad (Prism GraphPad Software).

2.3.3 Symbiont Photochemistry

Algal photochemistry was assessed using active chlorophyll *a* fluorescence by multi-PSII turnover (MT), pulse amplitude modulation (PAM) fluorometry, and by single-PSII turnover (ST), fluorescence induction and relaxation (FIRE) fluorometry. For PAM fluorescence measurements, a Diving PAM (Waltz, Germany) was used to record dark acclimated maximum quantum yield of PSII (F_v/F_m^{MT}) of 5 randomly selected anemones treatment⁻¹ every 2–3 days within a 1 hour window after the lights turned off each night. The specific PAM settings were: measuring light = 3, saturation intensity = 12, saturation width = 0.8 s, gain = 3, and damping = 2.

For FIRE fluorometry, animals were measured at the end of the 31 days of nutrient exposure, 10–11 days after heating at 32° C, 20 days after heating at 33° C and on day 27 of heating at 33° C. A FIRE fluorometer (Satlantic Inc., Halifax, Canada) was used to generate single turnover and multiple turnover fluorescent transients to calculate various aspects of PSII photochemistry, including the maximum quantum yield of PSII (F_v/F_m^{ST}), maximum quantum yield of PSII in the light

acclimated state (F_q'/F_m'), and the rate of PSII reoxidation in the dark and light acclimated state (τ and τ' , respectively). Light excitation was provided by a high-luminosity blue light emitting diode (peak λ 455 nm). The sampling protocol consisted of a single turnover (ST) excitation from a 120 μ s pulse and a ST relaxation phase over 60 μ s with a ST relaxation interval consisting of 40 samples. Ten iterations of each sequence were averaged for each measurement. Anemones were dark acclimated 15 minutes prior to measurement, followed by exposure to an actinic light source of 90 μ mol quanta $m^{-2} s^{-1}$ (peak λ 455 nm) for 4 minutes when light acclimated fluorescence transients were recorded once more. A seawater blank taken from one of the containers was measured and used for correction. Fluorescence induction traces were fitted with the biophysical model of Kolber (1998) using the FIREPRO software.

2.3.4 Carbon Uptake and Translocation

Anemones were sampled 31 days after nutrient exposure ($n = 5$ treatment⁻¹) and 28 days after chronic heating ($n = 4$ treatment⁻¹). Individual anemones were placed in a 7 mL scintillation vial with 2 mL of seawater and 15 μ L ¹⁴C- labeled sodium bicarbonate (8.4 mCi/mmol). Vials were placed in a temperature controlled photosynthetron (CHPT Inc., DE, USA) connected to a water bath set to either 28 or 33°C. Light was provided under each photosynthetron well by a single LED set to 255 μ mol quanta $m^{-2} s^{-1}$ (Cree Cool White XP-G3) for 90 minutes. This higher light level was used to record maximum photosynthesis, with previous experiments with these anemones indicating this light level was saturating ($\geq E_K$).

After incubation, anemones were rinsed two times with filtered seawater and transferred to plastic tubes for homogenization. Anemones were homogenized with three stainless steel beads in 1 mL of potassium phosphate buffer (50 mM, 0.1 mM

EDTA, pH 7.8) for 2 minutes at 5 m s^{-1} (FastPrep 24, MP-Biomedical). The homogenate was centrifuged at 10,000 RPM for 5 minutes to pellet the algae. The supernatant was transferred to a new tube and held as the *Host* material. The algal pellet was resuspended in 500 μL buffer, re-homogenized by vortexing, and centrifuged once more to separate remaining host material from the algae, and the second supernatant was transferred to the original *Host* tube. The algal pellet was then resuspended in 800 μL filtered artificial seawater (AFSW, 0.4 μm) and collected as the *Symbiont* material. Subsamples of the *Symbiont* material were taken to quantify symbiont density and chlorophyll *a*. 50 μL of *Host* material was removed to quantify soluble animal protein. 350 μL of the remaining *Symbiont* and 500 μL of *Host* material were added to individual scintillation vials and then acidified with equal parts 1 N HCl to remove any residual inorganic ^{14}C . Samples were off-gassed overnight and then combined with 5 mL scintillation cocktail (Ultima Gold, Perkin Elmer) and read on a scintillation counter (Beckman LS-6500).

Symbiont density samples were preserved with 5 μL 8% glutaraldehyde and manually counted on a light microscope under 100x magnification using a Neubauer hemocytometer (six–eight independent replicate counts). Chlorophyll *a* was extracted by bead beating cells with 0.5 mm glass beads and cold methanol for 2 minutes at 5 m s^{-1} (FastPrep 24, MP-Biomedical). Samples were incubated at -20°C overnight and then centrifuged for 5 minutes at 10,000 G to remove any debris. Absorbance was read at 630, 664, and 750 nm on FLUOstar Omega Plate Reader (BMG Labtech, Germany). Chlorophyll concentrations were calculated using established equations (Ritchie, 2006). Protein was quantified using the Bradford assay, with a bovine serum

albumin standard (Bradford, 1976). Host protein content was measured at 465 and 595 nm on a FLUOstar Omega Plate Reader (BMG Labtech, Germany).

Calculations for net photosynthesis and translocation follow previously established methods for *E. diaphana* (formerly *Aiptasia pallida*) (Davy & Cook, 2001). Photosynthesis and translocation rates were determined by the average specific activity (g C dpm⁻¹) and incubation duration of 90 mins. The fraction of carbon produced from net photosynthesis (P_{net}) was normalized to symbiont density and calculated as:

$$P_{\text{net}} = \text{Host} + \text{Symbiont}$$

The rate of carbon translocation to the host (T_L) was normalized to either symbiont density or host protein content depending on the analysis.

$$T_L = \text{Host}$$

The percent photosynthate translocated to the host was calculated as:

$$T_L / P_{\text{net}} = \text{Host} / (\text{Host} + \text{Symbiont})$$

2.3.5 Heterotrophy

5 animals treatment⁻¹ were selected to measure heterotrophy. Animals had been starved for 6 days prior to feeding trials. Feeding trials were performed in a temperature controlled incubator (Percival Scientific, model IL-VL) set to 28 or 33° C with an irradiance of 90 μmol quanta m⁻² s⁻¹ light. Anemones were placed in 50 mL glass beakers fitted with false bottoms and magnetic stir bars as described above and suspended in 20 mL of seawater directly from the treatment carboys containing 5 μM NO₃⁻, NH₄⁺, or ASW. Feeding chambers were stirred at 300 RPM. 3 mL of freshly hatched *Artemia salina nauplii*, containing 1015 *Artemia*, were added to each feeding chamber. After 60 minutes of feeding, anemones were removed from the feeding

chamber and the remaining *Artemia* were preserved in 0.5 mL formalin. *Artemia* were counted on a dissection microscope with a Bogorov counting chamber. Total heterotrophy was quantified as the number of *Artemia* eaten anemone⁻¹ hour⁻¹.

2.3.6 Statistical Analyses

All datasets were tested for assumptions of homogeneity of variance and normal distribution using the Levene and Shapiro-Wilk tests. If either test invalidated these assumptions the data were log transformed or the reciprocal of the data was taken and retested to ensure homoscedacity and normality prior to further analysis. Linear mixed models were used to test significant main effects and interactive effects of heating, time, and nutrient type, with anemone replicate as a random fixed factor in the fluorometric data. Post hoc tests were used to distinguish specific drivers of significance. Two-way analysis of variance (ANOVA) was used to test for significant differences among heating and nutrient type for all carbon assimilation and translocation data, nutrient assimilation, and heterotrophy experiments. A Tukey post hoc test was used to further detect significant differences among samples. All statistical analyses were performed using R software (R Core Team, 2022) with the “car”, “lmer”, and “emmeans” packages.

2.4 Results

2.4.1 Nutrient Assimilation

Prior to heating, nutrient drawdown was measured in bins, each containing 6 anemones. NO₃⁻ and NH₄⁺ concentrations declined after two hours; however, there was no difference in drawdown based on nitrogen form (data not shown).

Nutrient concentrations were measured again with single anemones and once heating was implemented. Over the course of two hours, 56% of starting concentrations of ambient NO_3^- remained, while heated NO_3^- was at 38% (Fig 2.1). Ambient NH_4^+ dropped to 11% of starting concentration after two hours, and heated NH_4^+ dropped to 13%. Nutrient decay rate was significantly lower in ambient NO_3^- treatments than all other treatments (Fig 2.2).

2.4.2 Photochemistry

After 26 days of thermal stress, F_v/F_m^{MT} measured using PAM fluorometry revealed no difference between heated or ambient treatments (Fig. 2.3). Additionally, nutrient type had no effect on F_v/F_m . Single turnover fluorescence analysis revealed a significant interactive effect between nutrient type and time (Fig. 2.4 a, b, c; $p < 0.0001$) and nutrient type, time, and heating ($p < 0.01$) on maximum quantum yield of PSII, driven by a change in F_v/F_m^{ST} in the heated NH_4^+ treatments on day 10. In particular, the heated NH_4^+ treated anemones detached from the container base and walls and began to contract and withdraw their tentacles. F_v/F_m^{ST} was significantly lower in the heated NH_4^+ treatments than the ambient (28° C) NH_4^+ treated anemones at this time, and lower than both heated NO_3^- and heated control anemones. However, heated NH_4^+ anemones appeared to recover and after 27 days of heating, and there was no significant difference in F_v/F_m^{ST} in heated samples and ambient NH_4^+ treated anemones. Additionally, nutrient treatment had no effect on F_v/F_m by day 27. Despite no change in F_v/F_m after 27 days of heating, the rate of PSII reoxidation (τ) was significantly slower in heated NO_3^- and NH_4^+ treatments than their respective treatments at 28 °C (Fig 2.4 d, e, f; $p = 0.034$ nutrient x heat). Additionally, τ increased significantly through time in all heated treatments and ambient controls ($p <$

0.001 time x heat), while nutrient type had no effect on τ for any treatments. This increase in τ could be an artifact of shifting animals from halogen lights to LED lights in the wet lab.

After animals were exposed to actinic light, the response of heated NH_4^+ treated anemones became more pronounced as the effective quantum yield (F_q'/F_m') dropped significantly lower than all other treatments on day 10 and remained significantly lower than ambient NH_4^+ treatments throughout the duration of the experiment (Fig. 2.5 a, b, c; $p < 0.01$ nutrient x heat). PSII reoxidation in the light activated state was significantly slower in heated NH_4^+ treatments than ambient NH_4^+ starting on day 10 and throughout the remainder of the experiment (Fig. 2.5 d, e, f; $p < 0.01$ nutrients x time). Over the course of 27 days, all heated treatments had significantly slower τ' compared to day 0, while τ' did not change for ambient treatments ($p = 0.022$ time x heat); however, only control treated anemones had significantly slower τ' at 33° C than 28° C. Again, these responses could be due to shifting animals from halogen lights to LED lights.

2.4.3 Photosynthesis and symbiont physiology

After 31 days of nutrient additions and prior to heating, net photosynthesis algal cell⁻¹ remained the same across treatments and hence nutrient type had no effect on algal carbon fixation or translocation (data not shown). However, upon heating, net photosynthesis algal cell⁻¹ significantly differed (Fig 2.6 a; $p < 0.01$ heat), with heated control treatments having significantly higher rates than ambient control treatments. Net photosynthesis rates did not change for NO_3^- and NH_4^+ treated anemones upon heating (Fig 2.6 a). Under ambient conditions, symbiont density did not differ between nutrient treatments, but upon heating density significantly declined in NO_3^- and NH_4^+

treatments (Fig 2.6 b; $p < 0.01$ nutrient; $p < 0.0001$ heat). Chlorophyll *a* content cell⁻¹ was significantly higher in heated control treatments than ambient control treatments (Fig 2.6 c; $p < 0.01$ nutrient x heat); however, heating had no effect on chl *a* cell⁻¹ for NO₃⁻ and NH₄⁺ treatments.

2.4.4 Carbon Fixation and Translocation

After 31 days of nutrient exposure, the rate of carbon translocation and the percentage of photosynthetically fixed carbon translocated to the host anemone were similar across all treatments (data not shown). After an additional 28 days of nutrient treatment conditions, symbionts in ambient control anemones translocated a significantly higher percentage of photosynthate to the host than ambient NO₃⁻ and NH₄⁺ treatments (Fig. 2.7 a; $p < 0.01$ nutrient). Upon heating, the percent carbon translocated to the host significantly increased in NO₃⁻ and NH₄⁺ treatments, while symbionts in heated control anemones maintained the same percent translocated carbon as the ambient control anemones ($p < 0.0001$ nutrient x heat). The increased percent carbon translocated to the host in heated NO₃⁻ and NH₄⁺ treatments was equivalent to that of control and heated control anemones. Meanwhile, algal cell specific carbon translocation rates ($\mu\text{g C cell}^{-1} \text{ hr}^{-1}$) were significantly higher in heated control and heated NO₃⁻ treated anemones than in ambient control treatments (Fig 2.7 b; $p < 0.0001$ heat). When carbon translocation rates were normalized to host protein, heated control treatments translocated significantly more photosynthate than ambient control treated anemones (Fig 2.7 c; $p < 0.01$ nutrient, $p < 0.01$ heat).

2.4.5 Heterotrophy

Under ambient conditions, NO_3^- treated anemones consumed significantly fewer *Artemia* than NH_4^+ treated anemones (Fig. 2.8), while there was no difference in the rate of shrimp eaten between the control and NH_4^+ treatments at 28° C. However, upon heating, NO_3^- treated anemones consumed significantly more *Artemia* than ambient NO_3^- treated anemones ($p < 0.01$ nutrient x heat). The increased feeding in heated NO_3^- treatments was equivalent to *Artemia* consumption of heated control and heated NH_4^+ treated anemones.

2.5 Discussion

2.5.1 Physiological response to nutrient enrichment and thermal stress

My findings show nutrient type did not have a strong impact on the physiological response of algal symbionts within the sea-anemone *E. diaphana*. I observed no notable differences in photochemistry or physiology at ambient temperature and no signs of thermal tolerance or enhanced susceptibility of PSII at elevated temperatures driven by nutrient type alone. Heated treatments showed signs of bleaching, as loss in symbiont density over time, with NO_3^- and NH_4^+ treatments having significantly fewer cells at 33° C than 28° C, and all heated anemones appearing visually lighter than those kept at ambient temperatures. Heated treatments did have slower PSII reoxidation rates in the dark acclimated state, with NO_3^- and NH_4^+ treatments being significantly slower than ambient treatments; however, this did not appear to have any effect on photochemistry and photosynthetic rates of heated symbionts. Heated NO_3^- and NH_4^+ treatments maintained photosynthetic rates, and heated control treatments had higher photosynthetic rates than ambient control treatments. Despite loss in symbiont density upon heating, NO_3^- and NH_4^+ treated

anemones were able to meet metabolic demands of the host by increasing percent photosynthate translocated to the host. Additionally, symbionts of heated control anemones increased carbon translocation rates and were able to maintain percent photosynthate translocation.

Upon heating, I had predicted that symbionts of NH_4^+ enriched anemones would maintain photosynthetic rates while NO_3^- enriched anemones would show signs of bleaching. Two weeks of heating and balanced N:P nutrient enrichments of $3 \mu\text{M}$ NO_3^- led to decreased cell density for the coral *S. pistillata*; however, heated corals exposed to $3 \mu\text{M}$ NH_4^+ enrichments did not have significantly fewer symbionts than ambient treatments (Fernandes de Barros Marangoni et al., 2020). Additionally, NO_3^- enrichments in tandem with thermal stress decreased photosynthetic efficiency of coral symbionts (Burkepile et al., 2020; Fernandes de Barros Marangoni et al., 2020) while NH_4^+ enriched corals had higher photosynthetic efficiency than heated control corals (Béraud et al., 2013). Furthermore, NO_3^- enrichments decreased chlorophyll production and carbon translocation in corals, while NH_4^+ enrichments enhanced carbon translocation, chlorophyll production, and net photosynthesis (Béraud et al., 2013; Fernandes de Barros Marangoni et al., 2020; Ezzat et al., 2015). Balanced N:P additions were not detrimental to corals *A. pulchra* with Tanaka et al. (2007) noting increased chlorophyll concentrations and carbon fixation at ambient temperatures, and under thermal stress and increased $p\text{CO}_2$. *T. reniformis* corals maintained host metabolic demands when enriched with NO_3^- (Dobson et al., 2021). My findings show that NO_3^- additions do not have adverse effects on anemone-algal symbiosis, in ambient or heated conditions, rather NO_3^- and NH_4^+ additions have similar effects on the symbiosis. Despite nitrogen additions leading to decreased cell density in heated

treatments compared to anemones grown in ASW, heated NO_3^- and NH_4^+ enriched anemones were able to increase percent of photosynthate translocated and perform similarly to animals grown in heated ASW.

Unlike prior research on scleractinian corals, I observed no detrimental or clear beneficial response of one nitrogen form over another in the sea anemone, *E. diaphana*. The different responses I observed in anemones could be due to differences in experimental design and nutrient availability. $5 \mu\text{M}$ NO_3^- or NH_4^+ were continually pumped to respective treatment conditions, however, within two hours, I found singular animals rapidly drawing down nutrients, despite increasing flow rates to attempt to maintain these nutrient levels. Since water changes only occurred weekly, animals were only exposed to full $5 \mu\text{M}$ nutrient enrichments once a week. Fernandes de Barros Marangoni et al. (2020) and Ezzat et al. (2015) had similar findings within their experiments, with NO_3^- having deleterious effects and NH_4^+ benefiting *S. pistillata*; however, they delivered nutrients to experimental tanks $\sim 3\times$ faster than I did. Negative effects of NO_3^- enrichments were also noted in *Acropora* and *Pocillopora* when fertilizer was added to the water (Burkepile et al., 2020). Meanwhile, when bowls were spiked with fresh $5 \mu\text{M}$ additions every day, NO_3^- had positive effects on corals in the absence of heat stress (Tanaka et al., 2007). Variations in experimental design could explain some of the observed differences; however, this is not to say one method is better than another. My animals were still exposed to nutrient enrichments, despite reducing nutrient levels rapidly and showing no long lasting effects of nutrients.

Additionally, using a sea anemone as a cnidarian host could explain why my findings do not align with previous work. Other studies examined the effects of

nitrogen enrichments on corals, with calcium carbonate skeletons, rather than sea anemones. Different carbon demands depending on cnidarian host could also contribute to the different findings. To understand if carbon demands of the host are driving this response, I suggest repeating this experiment with the same methods used by Fernandes de Barros Marangoni et al. (2020) as they also fed animals throughout the experiment and added nutrients in balanced N:P ratios.

Interestingly, although I did not see a differential response in nitrogen form, I saw that nutrients and heat could have had an effect of carbon dynamics. Upon heating, symbiont density significantly declined for NO_3^- and NH_4^+ treatments, yet percent photosynthate translocated to the host increased. As symbiont density declined, carbon availability could have increased, thus leading to increased translocation. Davy and Cook (2001) noted symbionts of *E. diaphana* were able to maintain percent carbon translocation and increase photosynthetic rates despite decreased symbiont density under starvation stress and linked this response to increased carbon availability. I also observed a decline in symbiont density by increasing water temperature. Additionally, higher temperatures could affect Rubisco activity, thus allowing heated symbionts to maintain or increase host carbon demand.

2.5.2 Heterotrophy

During thermal stress, increased trophic plasticity could offer some relief and allow cnidarians to meet their metabolic demands. However, despite decreased symbiont densities in all heated treatments, only heated NO_3^- treatments consumed more *Artemia* than respective ambient treatments. Although symbiont density declined, other thermal stress response cues were not evident. Symbionts still were able to meet or exceed metabolic demands of cnidarian host, and thus these animals

might have not needed to rely heterotrophic feeding to maintain symbiosis.

Alternatively, the nutrients supplied from heterotrophic feeding could have masked the effects of NO_3^- or NH_4^+ additions, as zooplankton are a source of NH_3 . Additionally, heterotrophic food sources can serve as a different carbon source than photosynthetically fixed carbon. Photosynthetically fixed carbon is transported to the host primarily in the form of glycerol (Muscatine, 1967; Trench 1971 a,b).

Conversely, heterotrophy could provide a different carbon source to the host that might be more beneficial and readily used. Ezzat et al. (2019) found that heterotrophy increased in stressed corals but these increased nutrients were not able to be assimilated by the holobiont. Future work would need to be done to assess if there was a difference in the amount of auto- or heterotrophically derived nutrients impacting the holobiont.

2.5.3 Conclusion

Despite previous findings, showing nutrient enrichment impacts thermal susceptibility of cnidarians, I found that nutrient additions had no significant impact on the holobiont, using a sea anemone host and a thermally sensitive alga. In addition, heating did cause a bleaching response as evident by the loss of algal symbionts, but symbionts were able to maintain metabolic demands of the host. Results from my study shed new light onto how cnidarian-symbioses might be affected by shifting nutrient regimes, specifically localized nutrient enrichments and warming waters.

FIGURES

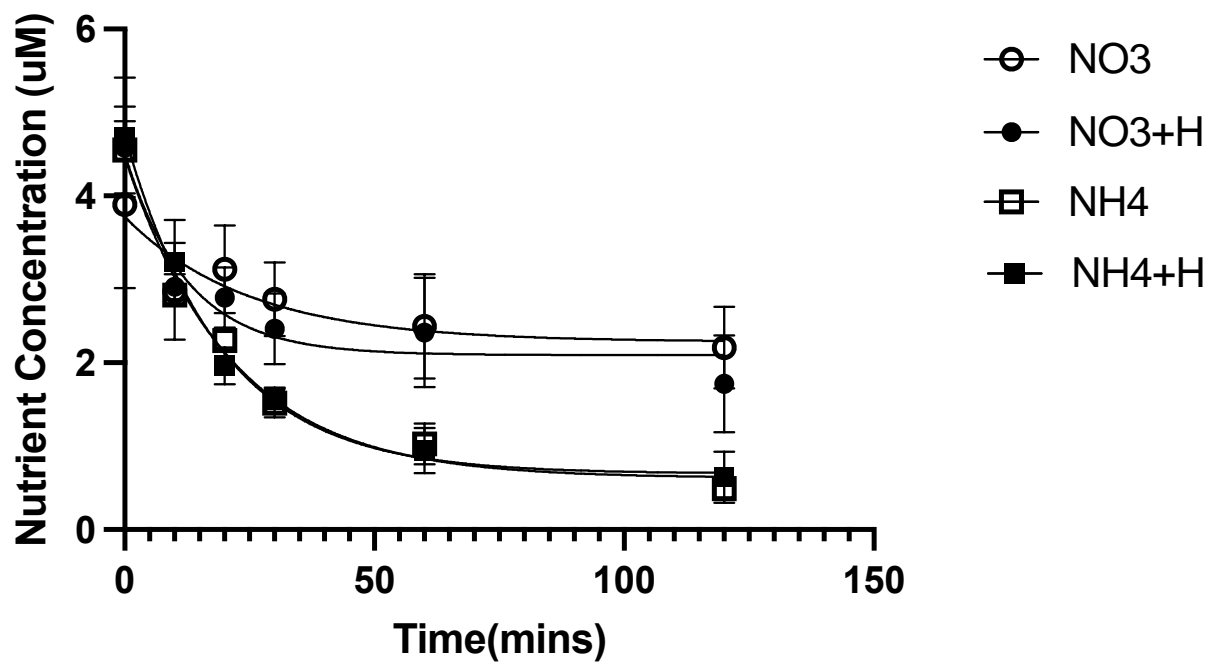


Figure 2.1 Nutrient drawdown of individual anemones in beakers over two hours ($n = 4$). Mean \pm SD are shown for ambient and heated NO_3^- and NH_4^+ treatments. Open symbols are ambient and closed are treatments heated to 33°C .

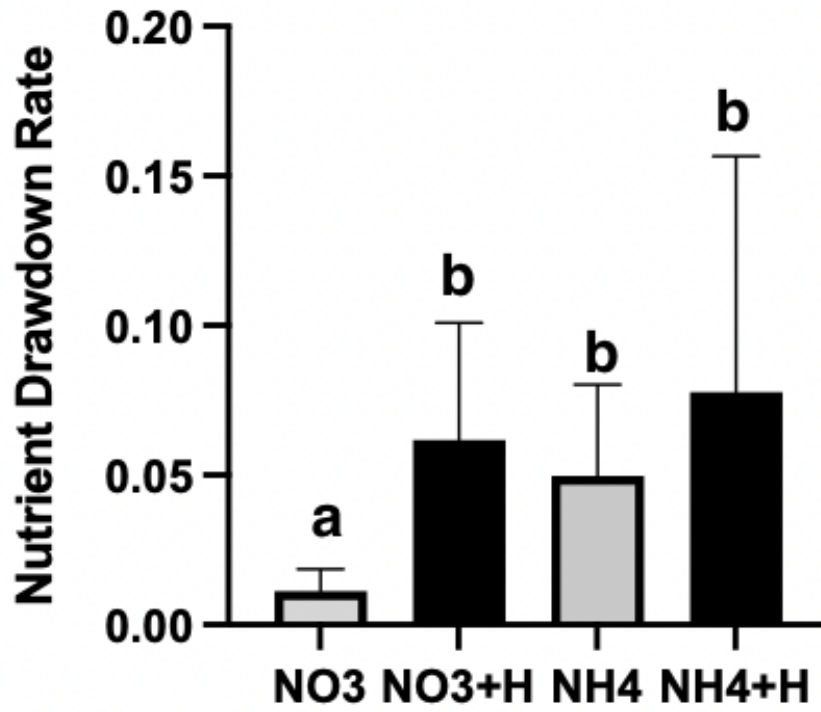


Figure 2.2 Nutrient drawdown rate constant of individual anemones in beakers (n = 4). Mean \pm SD are shown for ambient and heated NO_3^- and NH_4^+ treatments.

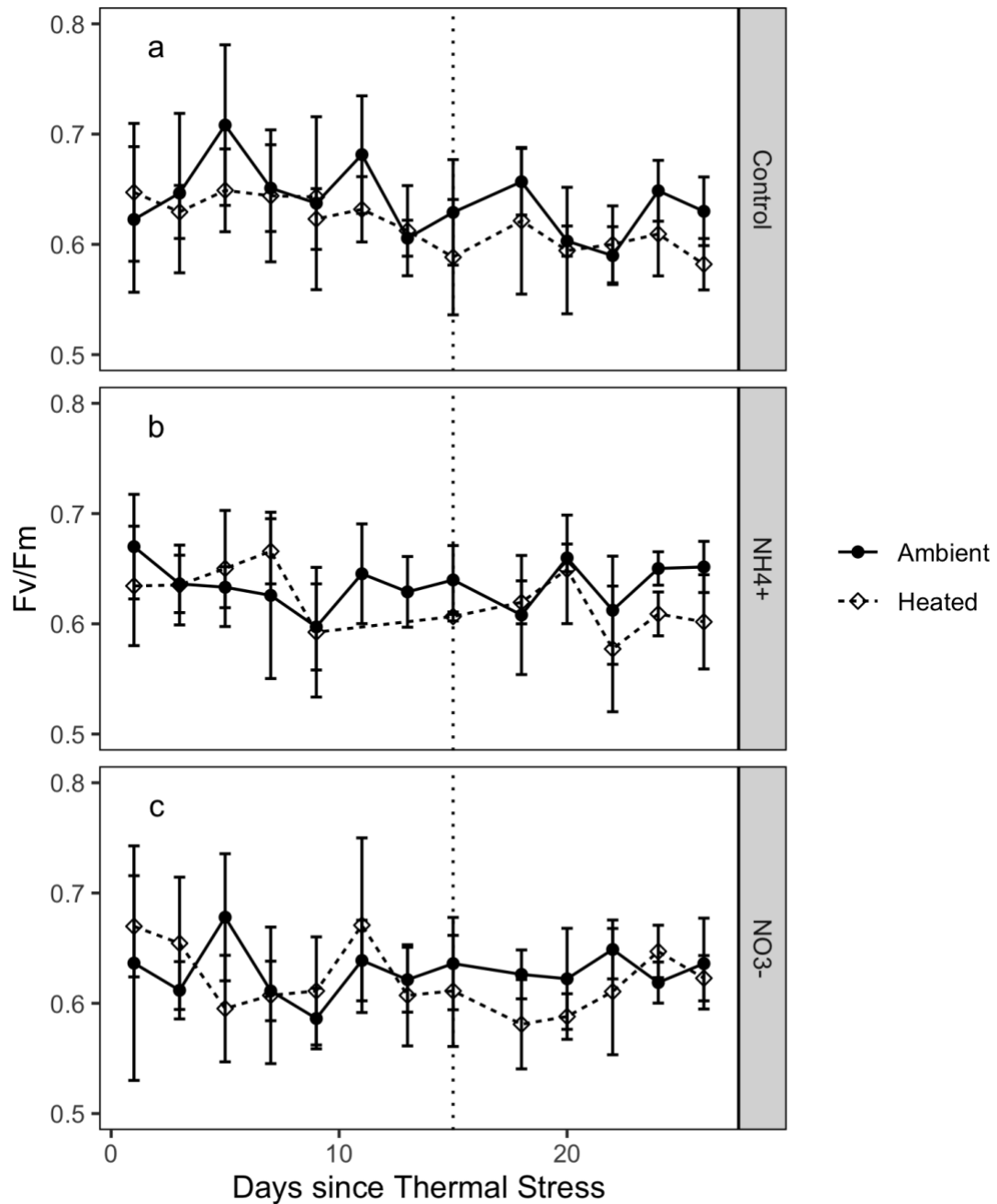


Figure 2.3. Maximum quantum yield of PSII (F_v/F_m^{MT}) under three different nutrient regimes ($n=4$). Mean \pm SD are shown for ambient and heated treatments (ambient = closed circles, heated = open circles). The dashed line represents the day when heated treatments were shifted from 32° C to 33° C.

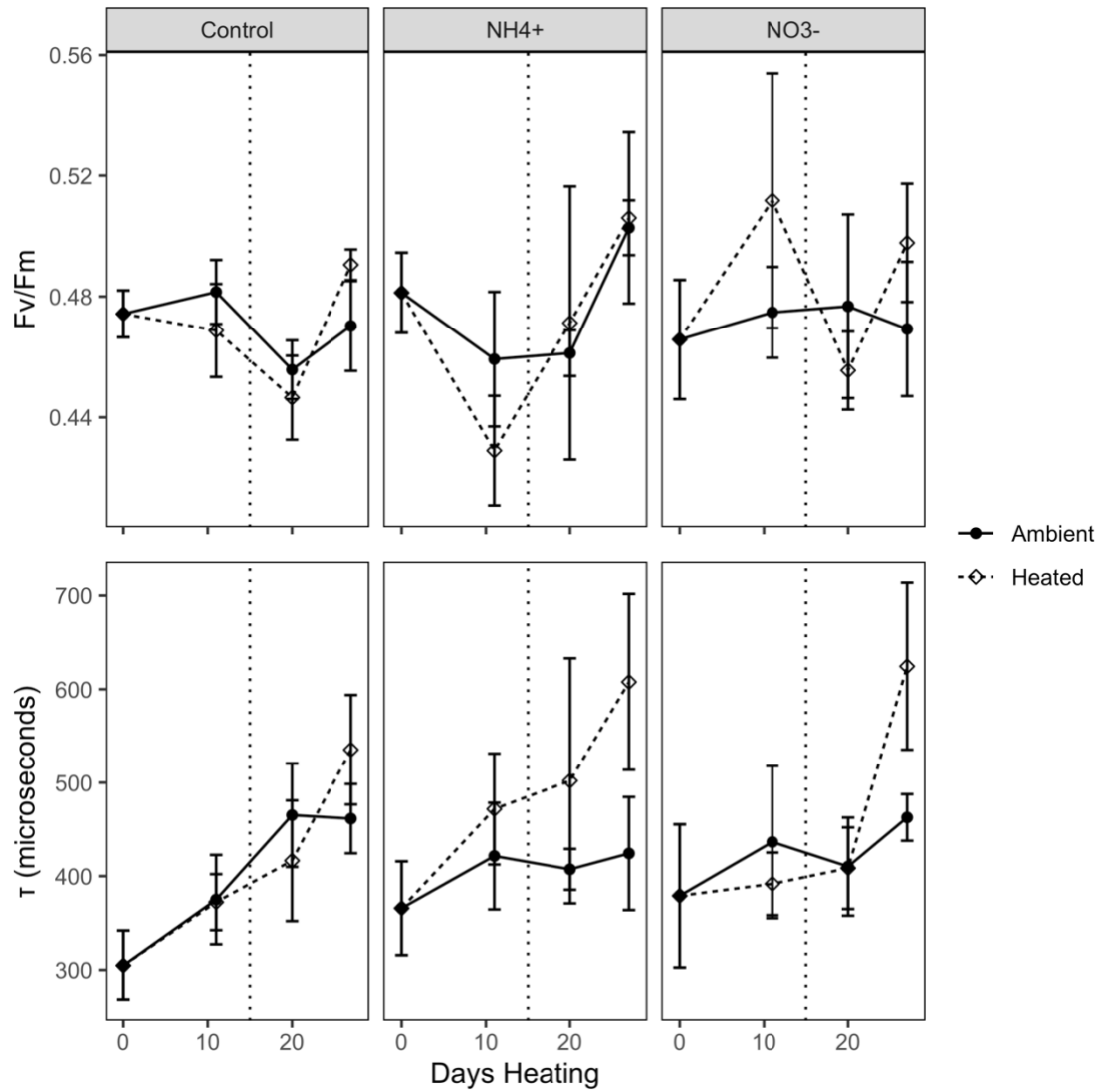


Figure 2.4. Maximum quantum yield of PSII (Fv/Fm) under three different nutrient regimes and ambient and heated conditions (a-c) Dark acclimated reoxidation rate of PSII (d-f) under three different nutrient regimes for ambient and heated conditions. Mean \pm SD are shown for ambient and heated treatments (ambient = closed circles, heated = open circles, n = 4). The dashed line represents the day when heated treatments were shifted from 32° C to 33° C.

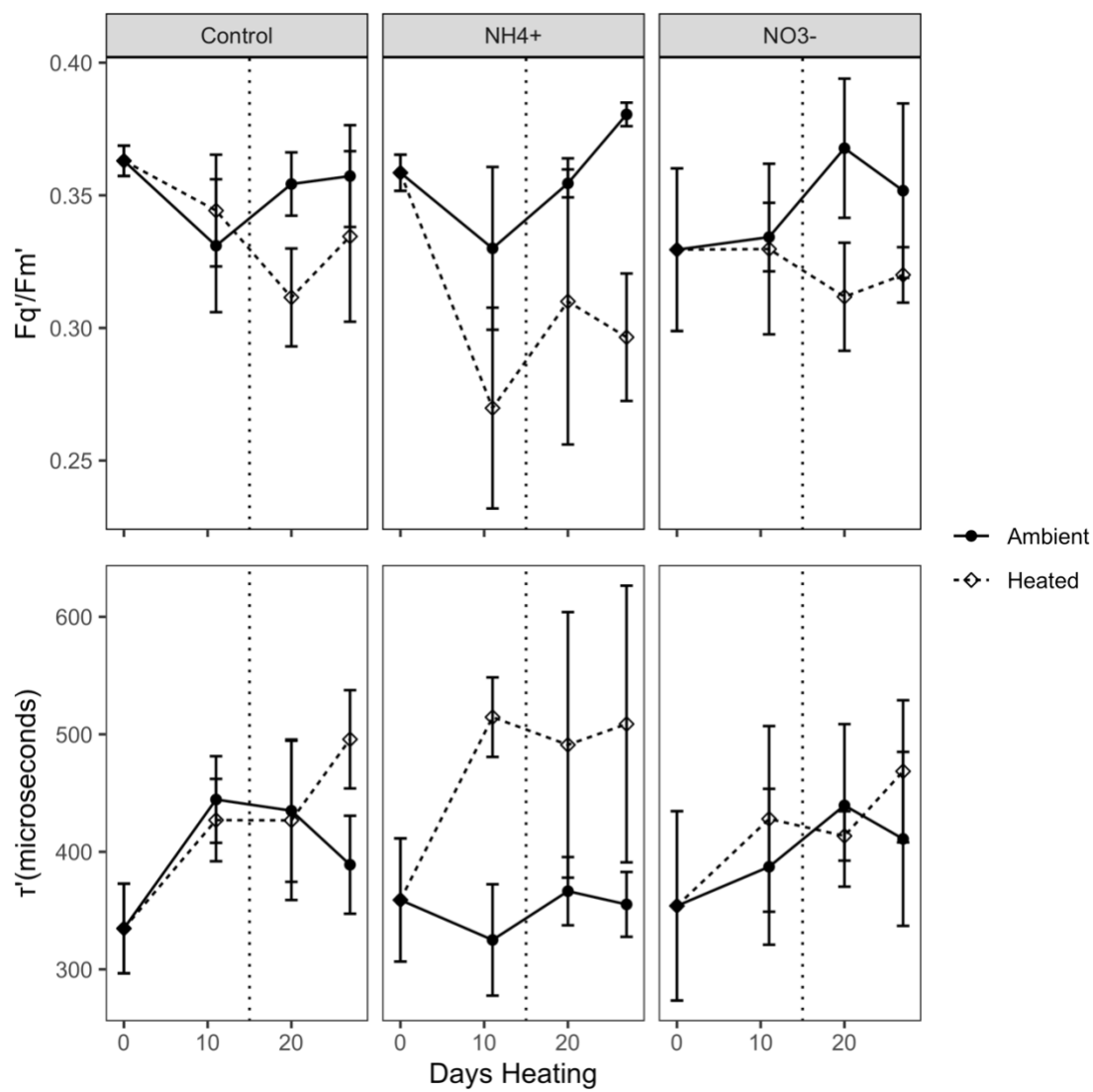


Figure 2.5. Effective quantum yield of PSII (Fq'/Fm') under three different nutrient regimes for ambient and heated conditions (a-c) Light acclimated reoxidation rate of PSII (d-f) for three different nutrient regimes under ambient and heated conditions. Mean \pm SD are shown for ambient and heated treatments (ambient = closed circles, heated = open circles, $n = 4$). The dashed line represents the day when heated treatments were shifted from 32° C to 33° C.

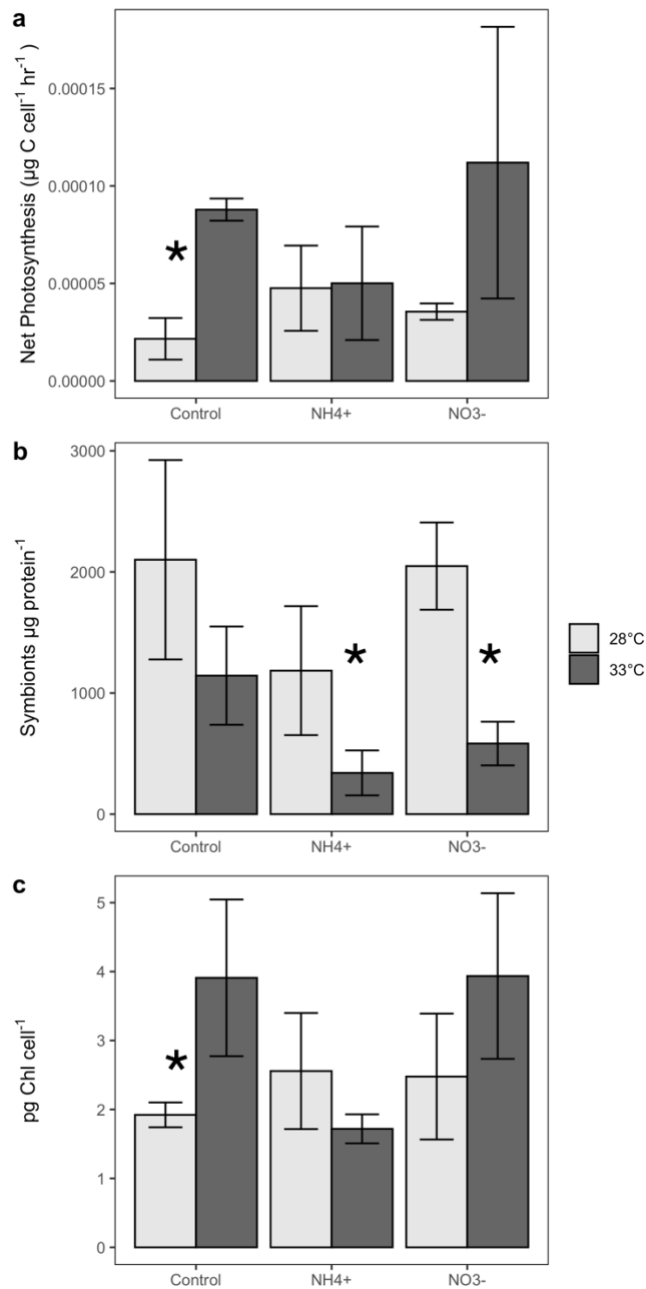


Figure 2.6. Net photosynthesis (a), symbiont cell density (b), and Chl a cell⁻¹ (c) of ambient and heated anemones after 28 days of heat exposure and under three different nutrient regimes. Mean \pm SD are shown for each treatment (n = 4). Asterisks denote significant differences between ambient and heated treatments.

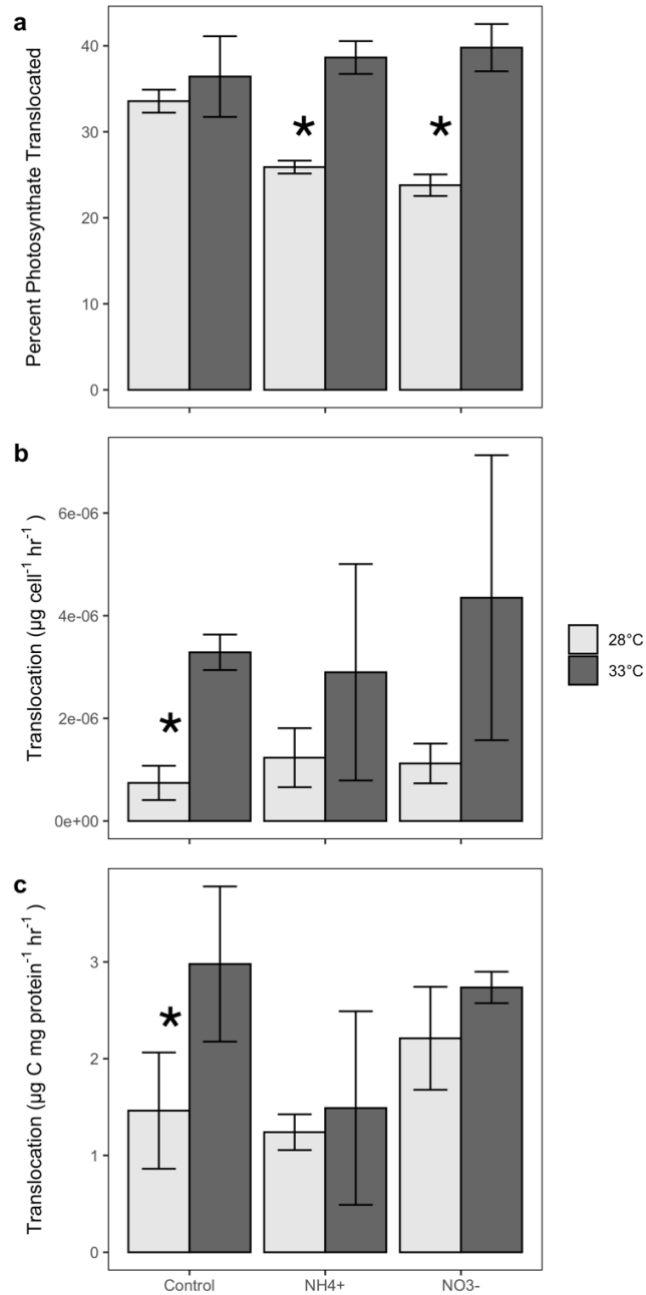


Figure 2.7. Percent of photosynthate translocated to the host (a), carbon translocation rate normalized to algal cell number (b), and carbon translocation rate normalized to host protein (c) of ambient and heated anemones after 28 days of heat exposure and under three different nutrient regimes. Mean \pm SD are shown for each treatment (n=4). Asterisks denote significant differences between ambient and heated treatments.

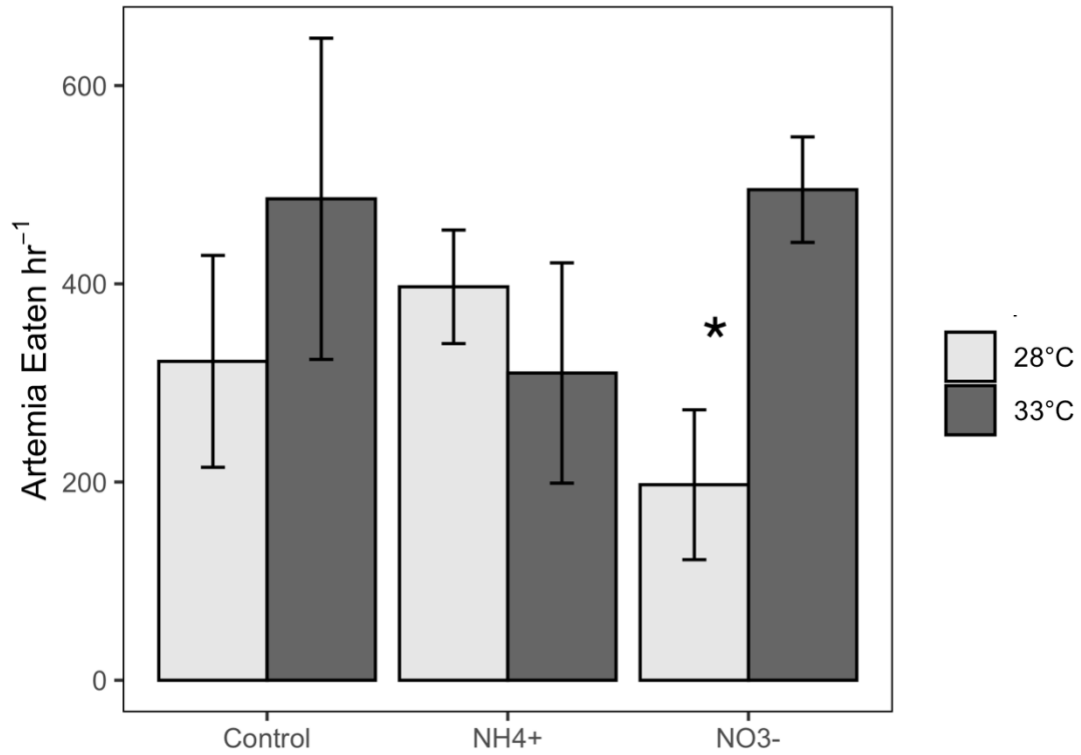


Figure 2.8. Heterotrophy of ambient and heated anemones after 28 days of heating and under three nutrient regimes. Mean \pm SD are shown for each treatment (n=5). Note the asterisks denotes a significant increase in feeding for heated NO₃⁻ anemones compared to ambient NO₃⁻ anemones (p < 0.01 nutrient x heat).

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