2018

NITROGEN LOADING, CLIMATE CHANGE, PATHOGENS: IMPACTS OF MULTIPLE STRESSORS ON RHODE ISLAND SHELLFISH

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BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

BIOLOGICAL AND ENVIRONMENTAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2018

MASTER OF SCIENCE IN BIOLOGICAL AND ENVIRONMENTAL SCIENCES

OF

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2018

ABSTRACT

Filter feeding bivalves, such as the eastern oyster, *Crassostrea virginica* and the blue mussel, *Mytilus edulis* are valued for their role in the marine nitrogen cycle; specifically, their ability to facilitate the process of denitrification (reduction of nitrate to an inert N_2 gas), and thereby the removal of reactive nitrogen from the system. Historically, these organisms have been victim of overharvesting along much of the east coast, however Rhode Island has undergone a vast expansion of oyster production through the development of a prosperous aquaculture industry within the state, potentially contributing to the restoration of this valuable ecosystem service. However, the success of all of Rhode Island oyster populations (wild, resorted, cultured) are threatened by anthropogenic stressors, such as warming waters and increased nitrogen loads into coastal habitats, which many interrupt and/or alter the rates at which *C. virginica* and *M. edulis* are able to perform the process of nitrogen removal. Of particular concern is incomplete processes of denitrification that may lead to an accumulation of nitrous oxide (N_2O), a potent greenhouse gas with global warming potential nearly 300 times more powerful than that of carbon dioxide.

Warming waters are also known to favor common oyster pathogens such as *Haplosporidium nelsoni, H. costale* and *Perkinsus marinus*. The combination of high temperature and nitrogen loads is likely to cause physiological stress to these organisms, leading to increased susceptibility of the organisms to pathogens and, therefore, further impacting the environmental benefits provided by bivalves.

The goal of this study was to investigate how the combination of current and projected temperatures and nitrogen loads may impact the health status, rates of nitrogen removal (N_2 production), and rates of N_2O production of *C. virginica* and *M. edulis*. This was accomplished through separate studies conducted on *C. virginica* and *M. edulis*. Two types of experiments were performed with *C. virginica*: Experiment 1, which was a controlled, laboratory-based study, in which organisms were maintained in a gradient of ammonium nitrate levels (20µM, 40µM, 70µM, 100µM), crossed by contrasting temperatures (18°C, 24°C) (i.e. 8 combinations total). Organisms were maintained in these conditions for 3 months, and rates of denitrification and N_2O production were measured at 3 time points over the incubation period in order to determine how these gas productions may change with time of exposure to experimental conditions. Upon completion, prevalence of three common oyster pathogens listed above was determined in a subsample of oysters from each treatment. A similar mesocosms experiment was performed with blue mussels with slightly different experimental conditions (5µM, 10µM, 1.5µM, 25µM and 18°C, 21°C), selected based on biological and ecological differences between oysters and mussels.

With the realization that *C. virginica* tolerates much more variation in environmental conditions within their habitat than just temperature and nitrogen, Experiment 2 was performed, and consisted of two (2016 & 2017), 3-month field manipulations in which oysters where maintained in contrasting ends of the estuarine gradient of Point Judith Pond, in Narragansett, RI were performed. At each location, organisms were deployed in experimental setups and left at either ambient or enriched (20µM) conditions. Oyster growth and mortality and water quality measurements were

made at selected time points over the 3 month period and, upon completion, experimental organisms were brought back to the laboratory for a single incubation at ambient conditions (18°C, unenriched site water) in Year 1 (2016), and at contrasting temperatures and high nutrient levels (18°C, 24°C, and 100µM) in Year 2 (2017). The goal was to reveal how previous exposure of oysters in the field to different environmental factors (salinity, pH, chlorophyll-a, oxygen, temperature, and nutrient loading) may have impacted the rates of gas production (N_2 and N_2O) under high temperature and nitrogen loading. In 2017, a random subset of experimental organisms were sacrificed and analyzed for the prevalence of common oyster pathogens.

The major hypotheses for this study included: (1) Temperature will initially increase rates of denitrification of both *C. virginica* and *M. edulis*. (2) Increased nitrogen loads will increase rates of denitrification and nitrous oxide production of both *C. virginica* and *M. edulis*; (3) The combination of warming and high nitrogen levels over long terms will compromise the health of the organism, causing physiological stress for both *C. virginica* and *M. edulis*, and higher nitrous oxide accumulation (possibly via incomplete denitrification).

Several major conclusions emerged from this study. Based on both mesocosms and field experiments, temperature appeared to be an initial driver of denitrification for *C. virginica*, however long-term exposure may act as a stressor, possibly inhibiting the process, indicated by the greater level of mortality within warmer laboratory treatments, over time ($F_{48,71}$=4.80, p=0.001), and general lack of enhanced rates in association with temperature for both experiments. Additionally, increased temperature may lead to increased N_2O production, indicated by the field study (F=-2.76, p=0.014). The

combination of nitrogen loading and warming appears to promote N_2 consumption, as opposed to denitrification (N_2 production), ($F_{16,23}$=5.21, p=0.011; F=-2.92, p=0.010; lab and field study respectively) as well as increased N_2O production of *C. virginica* over time ($F_{16,23}$= 4.10, p=0.024; lab study).

M. edulis generally supported net denitrification (nitrogen removal) in all tested scenarios of nitrogen availability and temperature (average rate across treatments: 28.01 (+/- 23.93) mmol m^{-2} day^{-1}), which indicates a greater role of N removal than previously reported, as most past studies have focused solely on sedimentary denitrification. Nitrous oxide production was greatest in the cooler treatments ($F_{48,71}$=5.17, p=0.027) throughout the *M.* edulis examination. However, with only ¼ of the nitrogen availability, *M. edulis* produced N_2O similar in range of *C. virginica.* Finally, in most conditions, both species produce N_2 at several orders of magnitude greater than N_2O, indicating that environmental benefits of filtering feeding bivalves, at this time, greatly outweigh the negative effects caused by the tested anthropogenic stressors.

ACKNOWLEDGMENTS

I have had the exceptional fortune to be able to work with a talented and diverse group of mentors during the completion of my graduate work. I would first like to thank my advisor, Dr. Serena Moseman – Valtierra, for seeing potential within me that was worthy of this position. When I decided to continue my education with graduate school, I found Serena's lab website and was so excited by her "big picture" approach and the multidisciplinary methods she used for her research on changing coastal environments. I feel very proud to have been a part of a lab where so much exceptional work has developed, and which thus far, has been comprised of a group of strong women scientists. I would also like to wish the sincerest thank you to my co-advisor, Dr. Marta Gómez – Chiarri. I am in awe of your ability to balance everything you do with such a positive and enthusiastic attitude. Most of all, the level of commitment you have for your students is inspiring, and I can never adequately thank you for the amount of time and consideration you have dedicated to me. Finally, many thanks to Dr. Austin Humphries for joining my committee. It has been a privilege to learn from you and receive your input on this project and manuscript.

Additionally, the support of all the members of the "MV Lab" has made all the difference. A special thank you to Dr. Elizabeth Brannon for her patience in guiding me through methods, protocols, and graduate life in general. And to Katelynn Szura, thank you for your encouragement and friendship, I cannot imagine navigating through this crazy journey without you! I have also had the wonderful opportunity to work with many undergraduate research assistants during my time at URI – thank you to Joseph

Barnes, Jennie Warrell, Lauren Richards, Tania Santos, Talia Santos, Dylan Barrett and Nicholas Letarte – without your assistance in both the field and lab, this work could not have been accomplished. I will forever appreciate your dedication and eagerness to learn. I have much appreciation to all the members of the Gómez – Chiarri lab as well, thank you allowing me to share resources, lab space, and welcoming me as a member of your team.

Additional mentors and colleagues I would like to thank include: Dr. Craig Tobias and his entire laboratory at the University of Connecticut, Avery Point. Not only did you offer me my first "real" science job after undergrad, you sparked my interest in nitrogen cycling and aquaculture, which has completely guided my path ever since. If that was not enough, your lab also came to my rescue, when allowing me to use your MIMS, without which I literally could not have presented the majority of data within this manuscript. Thank you to Susan Smith, also of the University of Connecticut, for your friendship and our many after work chats about science and life. I have extreme gratitude towards Dr. Rodrigue Spinette of the Graduate School of Oceanography; there have been countless times you have gone out of your way to help me, as well as offer much needed advice and encouragement, and it has been appreciated beyond words. Thank you to Ed Baker and everyone at the Ann Gall Durbin Marine Research Facility, your expertise and guidance in all things aquarium set up and maintenance has made all the difference. I gratefully thank Dr. Roxanna Smolowitz, and the Aquatic Diagnostic Laboratory at Roger Williams University, for disease diagnostic and histology data for this manuscript.

I would also like to acknowledge Harvey Catalado of Bluff Hill Cove Oysters for allowing me access to my southern field site, as well as all the advice and many laughs that has come with working on his farm over the last two years. Thank you to Cindy and John West of Cedar Island Oyster Company as well, for access to my northern field site, and for the kindness and encouragement you offered as I pursued my degree.

Finally, I would like to thank my family. First my grandparents, Michael and Lynda Ieronimo, who instilled the importance of an education and the joy of learning within me from a very young age. Thank you to my parents, Karen Griffin and Jason Hogan for always teaching me "the sky's the limit." I realize there must have been many times when you questioned my career choices, and I cannot thank you enough for letting me feel nothing but love and support as I found my way. Last, but never least, to my best friend and loving husband, Jason Hamilton. Thank you for handling the stress that comes along with deadlines, field work, and data analysis since high school. Without your encouragement, sacrifice, and the occasional long day assisting in the field, I would not be *where* I am, or *who* I am today.

DEDICATION

To my grandmothers – Lynda Ieronimo and Judy Hogan. Thank you both for my love of nature, which has led to the overwhelming desire to understand and preserve its beauty.

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INTRODUCTION

1.1 Ecosystem services provided by bivalves

The eastern oyster, *Crassostrea virginica,* and the blue mussel, *Mytilus edulis*, are both ecologically, commercially, and culturally relevant species within the waters of New England. These marine resources have been an integral part of the area's coastal landscape since Native Americans first began harvesting these bivalves over 3000 years ago (Rick et al. 2014), denoting their long standing cultural value. Additionally, these reef-forming organisms provide a number of ecosystem services, including the provision of habitat and food for other significant marine species (Coen et al. 2007); the stabilization of sediments, which mitigates against erosion and storm surge events (Meyer et al. 1997); and the improvement of water quality through their filtration capabilities (Newell 2004).

Historically, shellfish populations have endured significant overharvesting in New England waters, but the success of the aquaculture industry has resulted in an expansion of oyster production within the area in more recent times (Beutel 2018). Oyster aquaculture endeavors within Rhode Island specifically, have increased nearly 300% in the last decade (Alves 2005; Beutel 2018). This translates into over 8 million oysters being sold for consumption, with net profits of nearly $5.5 million, and almost 200 farming jobs in 2015 (Beutel 2018). Blue mussel cultivation is also a growing industry within the state, although to a lesser extent than oysters (Beutel 2018).

1.2 The increasing threat of pathogens to shellfish populations

This substantial increase in cultured shellfish populations has become challenged by outbreaks of several diseases, including the protistan pathogens *Haplosporidium nelsoni, H. costale* (Taylor et al. 2016), and *Perkinsus marinus* (Smolowitz 2013); as well as the bacterial pathogen *Roseovarius crassostreae* (Boettcher et al. 2005; now named *Aliiroseovarius crassostreae*). Specifically, *P. marinus*, the causative agent of Dermo disease (Mackin & Owen 1950), and *H. nelsoni*, which causes MSX disease (Haskin et al. 1966) have resulted in high levels of oyster mortality along the entire United States east coast (Burge et al. 2014). *H. costale* is the causative agent of SSO disease (Stokes and Burreson 2001) and was first identified along the U.S. east coast in the 1960s (Andrews et al. 1962), yet the known range of the parasite is not as wide as that of *H. nelsoni* (Wang et al. 2010).

Dermo disease began causing large mortalities within the Gulf of Mexico in the 1940s and quickly spread up the coast, into the Chesapeake and Delaware Bays. Disease continued to intensify and spread northward over the next 40 years, eventually finding its way as far north as New Hampshire and Maine (Burge et al. 2014). The causative agent of MSX disease (*H. nelsoni*) is an introduced parasite (Burreson et al. 2000), first identified in Delaware Bay in the late 1950s (Haskin et al. 1966). Similar to Dermo, MSX spread up the coast and intensified in the decades to follow (Burge et al. 2014). Temperature is a known driver of both diseases (Ford & Tripp 1996), and it is thought that increases in the average winter water temperatures during the 1990s contributed to the northward expansion and increased prevalence and intensity of these parasites (Cook et al. 1998; Hofmann et al. 2001, Ford & Smolowitz 2007).

The interactions between the host and pathogen with the environment strongly dictate disease dynamics and outbreaks (Harvell et al. 2004, Mydlarz et al. 2006). Environmental stressors such as ocean warming and hypoxia are thought to compromise invertebrate resistance to disease, yet understanding the direct and indirect effects of this stress upon immunity remains complex and elusive (Mydlarz et al. 2006). Because diseases threaten the success of the aquaculture industry and ecosystem function, there has been notable investments towards the development of selectively bred disease resistant lines of oysters, in addition to much research on the mechanisms of disease resistance (Gómez-Chiarri et al. 2015). The development of selective breeding programs began in the 1990s on the Atlantic coast, with the goal of breeding oysters resistant to both Dermo and MSX disease (Calvo et al. 2003). Results thus far have been encouraging, but environmental uncertainties remain the major challenge for disease pressure and host susceptibility (Degremont et al. 2015).

Parasites such as trematodes, parasitic copepods, and shell-boring polychaetes are the major threats to blue mussel health (Buck et al. 2005). However, despite the growing commercial market of the blue mussel, the effects of its parasites and pathogens are less well studied within Rhode Island. This provides the opportunity in research to not only more thoroughly understand the challenges that oysters are currently experiencing, but also decrease the gap in knowledge of the host-pathogen relationship of the blue mussel within Rhode Island waters.

1.3 Human impacts on the marine environment

Global surface ocean temperatures are predicted to rise 1.3 – 2.6°C by 2100 due to rising greenhouse gas concentrations in the atmosphere (Mora et al. 2013). While the recent range expansion of several shellfish diseases within New England may be in response to pathogens favoring warmer waters (Harvell et al. 1999; Harvell et al. 2002; Burge et al. 2014) there are many other environmental parameters that also affect bivalve disease susceptibility, such as decreased dissolved oxygen (Keppel et al. 2015), decreased pH (Keppel et al. 2015), and inputs of anthropogenic pollutants, such as heavy metals (Parry & Pipe 2004). Additionally, increased temperature may affect the host itself, as warming waters are known to compromise the growth of young bivalves, promote mortality, and increase respiration rates (Dove & Sammut 2007; Dickinson et al. 2012; Matto et al. 2013; Mackenzie et al. 2014).

Furthermore, much of New England's coastal waters are threatened by anthropogenic nitrogen inputs via wastewater and septic system discharge and runoff (Craig 1994, Malham et al. 2009; Carmichael et al. 2012). Excess nitrogen is known to cause stress in benthic organisms, such as oysters and mussels, directly, through ammonia toxicity (Hand & Poxton 1993; Gray et al. 2002) and indirectly, through the promotion of hypoxia (Galloway et al. 2008; Diaz & Rosenberg 2008). Moreover, the current warming of coastal waters can potentially exacerbate the effects of nitrogen loading through increased water column stratification and therefore restriction of physical flushing, which inhibits the mixing of nutrients and replenishment of oxygen to benthic organisms (Diaz & Rosenberg 2008).

1.4 Nitrogen cycling and nitrous oxide production by shellfish

Bivalves, including *C. virginica* and *M. edulis,* are extremely efficient filter feeders (Newell 2004), which make them active participants in the marine nitrogen cycle, and particularly have a significant role in nitrogen removal via denitrification and nutrient retention (Kellogg et al. 2014). Denitrification is the anaerobic reduction of nitrate (NO_3^-) to an inert, dinitrogen gas (N_2), which exists as the major component of the atmosphere (Knowles 1982) (**Figure 1**).

Bivalve-mediated nitrogen removal in the form of denitrification is performed by microbes within the gut and/or exterior of the shellfish (Newell et al. 2002). The anaerobic environment of the oyster gut allows for microbial-mediated reduction of reactive nitrogen (NO^-_3) pollution, to N_2 gas, and thereby removal from the marine ecosystem (**Figure 1**). Although there is a growing body of work highlighting that oysters are a quintessential source of denitrification in marine and estuarine environments (Smyth et al. 2013; Kellogg et al. 2013; Humphries et al. 2015; Caffrey et al. 2016), the rates and controls of this process are not well constrained at this time (Kellogg et al. 2014). Dynamic environmental conditions, such as dissolved oxygen, chlorophyll–a, and temperature, are likely to play a part in determining these rates (Carmichael et al. 2012, Symth et al. 2013, Humphries et al. 2016). The amount and form of nitrogen (N) available to induce denitrification is also a factor, and it is possible that the combination of environmental factors and excess nitrogen within the marine environment, these rates can further be altered.

Nitrous oxide (N_2O), a potent greenhouse gas with a global warming potential 265 times more powerful than that of carbon dioxide (IPCC 2014), is a byproduct in the first step of nitrification (Goreau et al. 1980, Bange et al. 2010) (**Figure 1**). Further,

incomplete denitrification processes may result in an excess accumulation of N_2O (Zumft 1997). Denitrification may terminate prematurely for a number of reasons, including non-ideal environmental factors for the process, such as oxygen concentrations greater than 3µmol L^{-1}, and lack of organic carbon availability (Babbin et al. 2015), or the absence of the required enzymes in members of the microbial communities, such as nitrous oxide reductase (*nosZ*), which reduces N_2O to N_2 (Jenkins et al. 2008). The few studies that have attempted to quantify N_2O emissions of marine invertebrates found that filter-feeding organisms are potentially large emitters (Heisterkamp et al. 2010 & 2013). This is due to the ability to harbor a large amount of N_2O producing bacteria in their guts (Karsten & Drake 1997, Stief et al. 2009 & 2010, Heisterkamp et al. 2010 & 2013) combined with the ideal *in situ* conditions of the organisms' gut, including anoxia, high concentrations of labile carbon, and presence of NO_3^- and nitrite (NO_2^-) (Drake et al. 2006). Therefore, shellfish populations may be a significantly overlooked source of N_2O in certain environmental conditions. Furthermore, the role of shellfish in N_2O production becomes increasingly more complex due to environmental stressors and the threat and presence of the before mentioned pathogens.

Shellfish pathogens are hypothesized to influence nitrogen cycling by compromising the health of their hosts. Oysters' innate immune system responds to both pathogen presence and environmental stressors (Guo et al. 2015). Thermal stress is known to down regulate functional genes of *C. virginica*, such as those associated with growth (Guo et al. 2015), presumably to allow the organism to allocate more energy to respond to that stressor. Furthermore, exposure to a combination of stressors, for

example, temperature and pollution (Lanning et al. 2006), and temperature and pH (Keppel et al. 2015), have shown to cause high levels of mortalities and/or chronic effects of energy metabolism, when compared to a single stressor. Therefore, we hypothesize, that if *C. virginica* is exposed to a combination of anthropogenic stressors (high T + high N), their efficiency to respond to pathogens is likely to be compromised, as well as other functional responses such as the ability to facilitate N removal through filtration. Therefore, attempting to more clearly establish the connection between an organisms' health status and biogeochemical function, as well as, discerning the rates at which this gas may be produced under current and future environments is essential in order continue to benefit from the ecosystem system services which bivalves provide.

PROJECT OBJECTIVES

The goal of this study was therefore, to test how warming waters and increased nitrogen loads affect the eastern oyster, *Crassostrea virginica* and the blue mussel, *Mytilus* edulis, with regard to health status, rates of nitrogen removal, and nitrous oxide production and/or consumption. The study utilized both laboratory experiments and a field study to advance understanding of how multiple aspects of environmental change interact while impacting shellfish health and biogeochemical functions. The major hypotheses for this study included: (1) Increased temperature (T) will initially increase rates of denitrification (N_2 production) of both *C. virginica* and *M. edulis*. This is expected due to higher temperatures promoting higher filtration rates. (2) Increased nitrogen (N) loads will increase rates of denitrification and N_2O production of both *C. virginica* and *M. edulis*. (3) The combination of high T and high N over long terms will compromise the health of the organism, causing physiological stress for both *C. virginica* and *M. edulis*, and higher N_2O production (possibly via incomplete denitrification).

CHAPTER 1

Nitrogen loading and warming: Understanding how a combination of stressors may impact *Crassostrea virginica's* nitrogen cycling capabilities and health status

MATERIALS AND METHODS

Experiment 1: (Laboratory study)

2.1: Overview

Controlled laboratory experiments examined the effects of nitrogen and temperature on *C. virginica's* health status and rates of denitrification and N_2O production using a gradient of nitrogen levels and two contrasting temperatures for a period of 3 months. The nitrogen ($NH_4^+NO_3^-$) gradient used for *C. virginica* incubations was 20µM, 40µM, 70µM, and 100µM (enrichment above ambient seawater), and the contrasting temperatures were 18°C and 24°C. These target nitrogen concentrations are based on the observed range of total nitrogen in Narragansett Bay (Oviatt et al. 2002), and temperature levels represent current and projected summer bottom temperatures within Narragansett Bay (Mora et al. 2013). Ammonium nitrate ($NH_4^+NO_3^-$) was chosen, due to both nitrogen species being common anthropogenic inputs to coastal environments via wastewater and septic systems (Galloway et al. 2003; Galloway et al. 2008). This experiment therefore included 8 different combinations of N and T, each analyzed in triplicate, for a total of 24 experimental tanks (**Figure 2**). Each tank initially

housed 10 organisms. Juvenile oysters (1.5 inch, ~3.8cm) were purchased from Bluff Hill Cove Oyster Farm, located in Narragansett Rhode Island in June 2017 for this laboratory experiment. Organisms were maintained within these tanks, under experimental conditions for approximately three months (June – September 2017). The following parameters were measured during the experiment: growth (length, width), wet weight, temperature, dissolved oxygen, pH, salinity, NH_4^+ concentration, and on select days denitrification (N_2) and N_2O production rates.

2.2: Controlled laboratory experimental setup

Experimental organisms were typically maintained in 7L, glass aquaria, in a controlled environmental chamber (Holman Engineering) set to 18°C at the Marine Sciences Research Facility (MSRF) at the Graduate School of Oceanography, Narragansett, Rhode Island. All experimental aquaria sat within water baths, in order to ensure a more consistent water temperature among each experimental unit (**Figure 2**). Filtered seawater pumped directly from Narragansett Bay was stored in large 100L reservoir containers, enriched with ammonium nitrate ($NH^+_4NO^-_3$) to target levels, and pumped to each experimental aquarium utilizing a MasterFlex© multi-channel peristaltic pump, with the target flow rate of 7mL min^{-1}.

Temperature in the aquaria for the 24°C treatments was controlled by two submersible heaters (EHEIM Jager Aquarium Thermostat Heater, 75 watts) within the water bath. Submersible pumps (Hydro Empire ©) were utilized to ensure even distribution of the heated water around each experimental bivalve aquarium. To avoid isolative segregation, there were three water baths per temperature treatment, and

aquaria of the various nitrogen levels were randomly placed within these water baths (Hurlbert 2009; Cornwall et al. 2016). There were two representative nutrient reservoirs per nitrogen treatment, which allowed for the random interspersion of nitrogen inputs among the aquaria (**Figure 2**). Based on the target flow rate of 7mL min^{-1}, the target turnover time for each experimental aquarium was approximately 24 hours, and the large nutrient reservoirs were refilled roughly twice per week.

2.3: Bivalve growth, mortality and health

Shellfish were randomly assigned within each experimental aquarium. Growth (length and width) and biomass (weight) measurements were made monthly, as well as immediately prior to each incubation event. Organisms were fed 70 µl of Reed Mariculture Shellfish Diet © daily (Monday – Friday) and the tanks checked for mortalities. Deceased individuals were removed from the experimental aquaria as necessary and when possible, these tissues were preserved for disease analysis (detailed in Section 2.9). Upon completion of Experiment 1, a subset of remaining organisms from each treatment (n=5) were analyzed for pathogen prevalence, as detailed below in Section 2.8.

Water quality parameters such as dissolved oxygen, (DO), pH, salinity, temperature, and nitrogen (ammonium) were measured weekly as specified in Section 2.4. Water changes of the tanks were performed as necessary to maintain target experimental parameters, which on average resulted in every two to three weeks.

2.4: Incubation procedure for measurement of gas production rates

Approximately 12 hours prior to an incubation event (Day 5, 53, 89) all experimental organisms were transferred from the 7L glass aquaria to 2L plastic tanks (Freund Containers) and allowed to acclimate overnight, with continued flow from the peristaltic pump (**Figure 3**). Three (2L, plastic – Freund Containers) filtered seawater tanks (no organisms, no enrichment) were also added to the 18°C water baths and served as controls.

Water quality parameters were measured from each tank and nutrient reservoir on the morning prior to, and following, each 6-8 hour incubation period. Water quality parameters included dissolved oxygen (DO), pH, temperature, salinity and dissolved inorganic nitrogen (ammonium: NH_4^+). DO and pH were measured with an Orion © RDO / pH handheld monitoring probe, calibrated prior to use. Salinity was measured with a handheld refractometer (Fisher Scientific) and temperature with a simple handheld thermometer. Dissolved inorganic nitrogen samples were taken by collecting 60mL of seawater from each tank via a syringe, and filtering the sample through a 0.22µm EMD Milipore Millex™ filter, intro a pre-labeled sampling vial and immediately frozen in -20°C freezer until analysis.

Once all the initial water quality measurements were recorded, the pump was stopped and a gas-tight, foam lined, screw top lid was secured to each 2L incubation tank, thereby eliminating gas exchange with the outside atmosphere. Lids were equipped with a 12 cm inflow line, which attached to a peristaltic pump (MasterFlex © L/S variable speed drive) via approximately 2 meters of Tygon MasterFlex © tubing, (1/8 inch inner diameter, Cole Parmer); and an outflow line secured with a three-way, stopcock male valve, which was able to connect to the 60mL syringes used for gas and

nutrient sampling (**Figure 3**). The inflow and sampling lines in the gas tight caps were maintained in the upper and lower portion of the tank, respectively, to promote mixing during the sampling time points (**Figure 3**).

Immediately after the lids were secured, a small amount of water (<5mL) was manually pulled via a 60mL syringe and discarded to rid the outflow line of air and/or water from any previous sample. Next, 60mL of water was pulled from each of the 27 tanks (3 controls, 24 experimental). Once a sample was collected from each incubation tank and the three-way valves were back in the "off" position, the peristaltic pump, set to 10 mL min^{-1}, was allowed to run for 6 minutes, thereby replacing the sampled water, with fresh seawater from the appropriate nutrient reservoir. After 6 minutes, the pump was once again halted until the next sampling time point, approximately 1.5 hours later; this procedure continued for a total of 4 time points.

Each of the 60mL samples were aliquoted into two 12mL Labco© Exetainers, by removing the exetainer cap, and taking precaution to slowly and steadily transfer the sample from the syringe to the exetainer to discourage mixing and avoid bubble formation. Water within each exetainer was then immediately fixed with 50% (weight/volume) zinc chloride (Sigma Aldrich) to terminate any biological activity that could potentially alter gas concentrations within the sample. These samples were then stored upside-down, underwater, at 17°C until they were analyzed on a Membrane Inlet Mass Spectrometer (MIMS) for N_2 concentration (detailed in Section 2.5). The remaining water within each sampling syringe was discarded until the 20mL mark, at which point, the syringe was secured with a three-way valve in the "off" position. These syringes were then stored overnight in a cooler on ice [average temperature 8.2 (+/-

0.05) °C] until they were analyzed for nitrous oxide (N_2O) concentration using gas chromatography (Section 2.6).

2.5: Sampling processing – Denitrification rates

Water samples were analyzed for dinitrogen (N_2) gas concentrations using a Membrane Inlet Mass Spectrometer (MIMS) located at the University of Connecticut (Groton, CT) and the N_2/Ar method (Kana et al. 1994). N_2 production rates were determined using linear regressions of concentration versus time of the incubation based on 4 (duplicate) data points. If the regression line had a $R^2 \geq 0.65$ then a production rate was considered significant (Prairie 1996). The changes in N_2 over the course of the incubation were then normalized to the gram wet weight of oyster biomass that existed in each tank during the experiment. Positive rates are indicative of net denitrification within the tank during the incubation period (Kana et al. 1994), while negative rates indicate N_2 consumption exceeds production (i.e. N_2 fixation) (Purvaja et al. 2008).

2.6: Sampling processing – Nitrous Oxide Production

Within 12 hours of an incubation event, the 20mL water samples in the syringes (previously stored on ice) were equilibrated with 15mL of ultra-high purity helium (AirGas) following methods detailed in Moseman-Valtierra et al. (2015). After the helium addition into the syringe, the sample was vigorously shaken for 60 seconds, to equilibrate the dissolved gases within the water sample equilibrating into the headspace. The 20mL of water was then discharged, and the temperature and salinity of each sample was measured and recorded. The 15mL headspace left in the syringe was then

stored in a refrigerator until analysis on a Gas Chromatograph (Shimadzu GC-2014), for a maximum of 4 days after an incubation event.

Nitrous oxide concentration values of each sample were corrected for the dilution of the gas phase and the equilibrate distribution of N_2O between gas and water phases according to the following equation presented by Walter et al (2010):

$$C_w = [k_0 x' P V_{wp} + (\frac{x' P V_{hs}}{RT}) / V_{wp}]$$

Where C_w is the dissolved concentration of nitrous oxide (nmol L^{-1}); $k_0$is the solubility coefficient for N_2O (mol L^{-1} atm^{-1}); x' is the dry gas mole fraction of N_2O in the sample headspace (ppb); P is the atmospheric pressure (1atm); V_{wp} is the volume of water phase (Ml); V_{hs} is the volume of the headspace (Ml); R is the gas constant (L atm K^{-1} mol^{-1}); and T is the temperature upon equilibration (K). (Weiss and Price 1980; Walter et al. 2010; Garate 2016).

Nitrous oxide production rates were then determined using linear regressions of concentration versus time of the incubation based on 4 data points. Similar to N_2, if the regression line had an $R^2 \geq 0.65$ then a production rate was considered significant (Prairie 1996). The changes in N_2O over the course of the incubation were then normalized to the gram wet weight of oyster biomass that existed in each tank during the incubation period.

2.7: Sampling processing – Dissolved inorganic nitrogen

Pre- and post- incubation water samples were kept frozen until the morning of analysis. Standard colorimetric techniques were utilized to determine ammonium concentrations (Solorzano 1969). An Orion Aquamate 7000 VIS Spectrophotometer©

was used for dissolved ammonium concentrations; and nitrite and nitrate samples were analyzed by the Marine Science Research Facility (Narragansett RI) with a QuickChem QC8500 automated ion analyzer (Lachat ©).

2.8: Pathogen prevalence and intensity

Upon completion of the laboratory study, all remaining experimental organisms were shucked, and small samples of gill, mantle, and rectum were collected, stored in 95% ethanol, and stored in a -80°C freezer until DNA extraction and analyses. Pathogen (*P. marinus, H. nelsoni, H. costale*) prevalence and intensity were determined for a subset of 30 randomly selected individuals per treatment. This was completed using a modification of a quantitative polymerase chain reaction protocol (qPCR) (Proestou et al. 2016), carried out by the Aquatic Diagnostic Laboratory at Roger Williams University, Bristol, RI.

2.9: Statistical Analysis

The goal of this laboratory experiment was to test whether environmental drivers (nitrogen, temperature) can considerably and predictability affect oyster growth, mortality, pathogen prevalence, and rates of denitrification and nitrous oxide production. Critical thresholds of nitrogen and/or temperature were defined as those which significantly decreased *C. virginica* performance as defined by a decrease in N_2 and an increase in N_2O production, or increased mortality. Preliminary analyses were done via Three-Way ANOVAs, testing the effect of temperature, N-level, and/or time; therefore, Two-Way ANOVAs were used to analyze N level and time at each

temperature separately; and One-Way ANOVAs investigated both temperature and N-level (independently), at each time point. When statistically different significant differences arose, a Tukey's HSD test followed. A Principal Component Analysis (PCA) was used to test potential correlations between water quality parameters and mortality as well as the rates of denitrification and nitrous oxide production.

Denitrification rates from incubation 3 (day 89) were excluded from statistical analysis due to the belief that the experimental setup was compromised. During this portion of the study, one section of one roller of the MasterFlex © peristaltic pump became loose and therefore 1) wore down tubing at an accelerated and unpredictable pace, and 2) allowed a large amount of air to be pumped into certain aquaria. Statistical analysis, as described above, was still performed for N_2O samples, due to the belief that air bubble accumulation did not skewed this data as severely as the N_2/Ar method (Kana et al. 1994; Eyre et al. 2002; Humphries et al. 2016).

Experiment 2 (Field Manipulation)

2.10 Overview: Response to N addition across a dynamic estuarine gradient

Since multiple environmental variables affect oyster health and function, a field component was also carried out in order to examine how oysters respond to increased nitrogen inputs under dynamic estuarine conditions. Point Judith Pond (Narragansett, RI) was chosen as a field site due to active oyster aquaculture practices (Beutel 2018) and because it experiences a wide range of tidal influence. Tidal mixing is strongest at the southern end of the pond (closer proximity to open ocean) and weakens northward (**Figure 4**). This field site was used to test how *C. virginica* responds to experimental

nitrogen loading with regard to pathogen resistance and denitrification efficiency under complex field conditions. This field investigation was carried out in both Summer 2016 and 2017 for approximately four months each, targeting peak periods for oyster growth.

2.11: Field location

Point Judith Pond (41°N 24'N; 71°W 31'W) is an 8 km estuary which connects on its seaward end to Block Island Sound (**Figure 4**). This system experiences semi-diurnal tides, with flood tides approximately 2-3 m in height. This tidal range allows for the introduction of cold, oligotrophic water, which decreases the temperature of the water by 2-6°C and chlorophyll concentrations as much as 80% (Rheault and Rice 1996). There are significant variations in salinity, oxygen, temperature, and chlorophyll over spatial and temporal tidal cycles (CRMC 1999). Billington Cove Marina served as the northern field site (hereafter referred to as "northern site") and Bluff Hill Cove Oyster Farm served as the southern site ("southern site").

2.12: Field deployment and nitrogen additions

Oysters were deployed in experimental containers (buckets) at contrasting ends of the estuarine gradient of Point Judith Pond and were either enriched with organic nitrogen or left at ambient conditions. Experimental oyster containers were made from 5-gallon buckets, sturdy plastic netting with 13mm openings to allow for flow, and weights attached to the bottom to secure the experimental unit in place (**Figure 5**). Nitrogen amendments in the enriched treatment were in the form of Milorganite slow release pellet fertilizer (Worm et al. 2000), with the overall enrichment goal of 20µM,

based on the assumption the Milorganite fertilizer was composed of 10% inorganic nitrogen (~2% phosphorus). Six buckets were deployed at each location (3 per treatment, ambient or control and nitrogen enriched), and each housed 30 randomly assigned juvenile oysters, purchased from Bluff Hill Cove Oyster Farm, (approximately 3.5cm length) to start both years' experiments. Experimental buckets and oysters were deployed on June 24, 2016 and June 8, 2017 for each of the respective field manipulations. Buckets were grouped by treatment (ambient vs. enriched) to limit the introduction of N addition into the ambient setups. Replicates of the same treatment were grouped approximately 1 meter apart, and the treatment groups were spaced approximately 10 meters from one another at both locations.

2.13: Oyster growth, survival, and health

Observations of mortality of each experimental bucket were made 1-3 times per week for the entire duration of both field deployments. Deceased organisms were removed as necessary, and the remaining tissues preserved for pathogen presence when possible. Monthly measurements of growth (cm) using calipers, and wet weight (g), using a portable 600g balance (Fisher Scientific), were made on each oyster. Growth (length, weight) measurements were made biweekly in 2016, and monthly in 2017.

2.14: Water quality parameters

Water ($\leq$60mL) was collected from discrete samples, by using tubing fitted to a syringe (~3 meters in length), which allowed collection from each experimental bucket. In both years' experiments, samples were analyzed for dissolved inorganic nitrogen

concentrations and compared between locations, treatments, and tidal heights. In 2016, a YSI © 6 Series Multiparameter Water Quality Sonde (Model 6920VS) was used to monitor water quality parameters. Due to limited instrument availability, the sonde was deployed for alternating 2 week periods at each of the two field locations, from August to October. The instrument was position approximately 5m from the ambient bucket setups. Measurements of dissolved oxygen (DO), salinity, chlorophyll, temperature and pH were recorded every 15 minutes, and calibration occurred as recommended by the manufacturer (every 4-6 weeks). For a more detailed temporal analysis, a 12-hour sampling day of each experimental unit at each location allowed for the comparison of environmental factors between location, enrichment treatment, and tidal height. This was achieved by calibrating the instrument to record a measurement every minute, and at each sampling point the sonde was positioned within the experiment unit for 5 minutes. The data from the middle recording (minute 3) was used for analysis for all timepoints. Timepoints were chosen based on approximate high and low tide levels (US Harbors). Additionally, a discrete water sample was collected from each bucket during each 5 minute sonde recording. This 12-hour profile occurred on September 23, 2016. The field portion of the experiment was concluded on and incubations for measurement of gas rates were performed on October 15, 2016.

In 2017, an additional water quality sonde (YSI © EXO2) was obtained and deployed exclusively at the northern field site while the 6 series sonde (Model 6920VS) was stationed at the southern site. Both probes were deployed from June to August and set to take measurements of DO, salinity, chlorophyll, temperature and pH every 15 minutes. Due to calibration issues with the YSI sonde located at the southern field site,

that instrument was reading pH values too high; therefore, *differences* in value are able to be compared between treatments at the site, however true pH measurements are invalid. Instruments were calibrated approximately every 5 weeks. Additionally, water samples collected for DIN (NO_2^-, NO_3^-, NH_4^+) analysis were collected from each experimental bucket, each month, at high and low tide. The field portion of the experiment was concluded and incubations for measurements of gas rates were performed on September 5, 2017.

2.15: Denitrification potential and nitrous oxide production of oysters from contrasting estuarine sites

Upon completion of both years' field seasons, three randomly selected oysters from each experimental bucket (both locations) were collected for a laboratory incubation to measure potential denitrification and N_2O production rates as described in Section 2.4. These procedures were conducted at the Marine Life Science Facility in the Graduate School of Oceanography. In both years, oysters were incubated in tanks which reflected their original field locations (northern site vs. southern site), as well as their field treatment (ambient vs. enriched). While these factors remained the same, modifications in environmental controls were applied to the 2016 and 2017 incubation setups as described below.

The goal of the 2016 field oyster incubation experiment was to discern how short-term field conditions (location and organic fertilization treatment) impacted an oyster's physiological ability to denitrify in unaltered, Point Judith Pond waters (i.e. collection of site water from both locations). The site water from both locations (~100L)

was collected via multiple 25L carboys, from the surface and in proximity to the ambient treatment field buckets. Alongside the collected site water, oysters from each bucket (both locations and treatments; n = 3) were randomly selected to participate in the incubation event to follow. Once in the laboratory, oysters were placed into 2L incubation tanks (three per treatment), as described in Section 2.4 and unenriched site water was allowed to flow into each experimental tank, as well as the control tanks, which contained no organisms (**Figure 6**). Organisms were allowed to acclimate overnight, and approximately 12 hours later, the incubation began. Oysters were kept at a control temperature of 18°C for the entirety of the incubation. The procedure for incubation and measurement of denitrification and nitrous oxide rates of production was as described in Sections 2.4 – 2.7.

The 2017 field oyster incubation took into consideration more complex environmental stressors. The goal of this year's field oyster incubation was to understand how short-term field conditions (location and organic fertilization treatment) impacted an oyster's physiological ability to potentially remove nitrogen when exposed to a "pulse" of intense nitrogen, as well as observing how current and projected oceanic temperatures (18°C and 24°C, respectively) may influence these potential rates of denitrification and/or nitrous oxide production.

Randomly selected oysters (n = 6) from each field bucket were divided in two groups of 3 and placed in incubation tanks labeled according to field location (northern and southern) and treatment (ambient and enriched) and incubation temperature (18°C and 24°C). Tanks were filled with 100µM $NH_4^+NO_3^-$ (enrichment above seawater), filtered seawater (**Figure 7**). Therefore, each laboratory temperature treatment

contained 12 experimental tanks (treatments: northern field enriched, northern field ambient, southern field enriched, southern field ambient; 3 replicates per treatment). Additionally, 3 control tanks (no organisms) were incubated at 18°C. All 27 incubation tanks (24 experimental, 3 control) received a continued flow of 100µM $NH_4^+NO_3^-$ pulse of nitrogen for an acclimation period of approximately 48 hours. The procedure for incubation and measurement of denitrification and nitrous oxide rates of production was as outlined in Sections 2.4 – 2.7.

2.16: Pathogen prevalence and intensity under dynamic environmental conditions

Remaining field oysters from 2017 were processed for prevalence and intensity of common pathogens (*P. marinus, H. nelsoni, H. costale*) as described in Section 2.8.

2.17: Statistical analysis of data from field experiments

Three-way ANOVAs were utilized to test the effect of location, N level (field enrichment), and time of exposure to field conditions on oyster performance parameters and environmental parameters. In 2016, two-way ANOVAs initially examined the effect of location and N level on nitrogen cycling rates, with one-way ANOVAs following in order to further analyze the interaction terms. Due to an altered experimental set up in 2017, nitrogen cycling rates were analyzed with three-way ANOVAs, due to the two-temperature treatment setup for the incubation. Therefore, field location, field enrichment, and incubation temperature were analyzed within these results, with two-way ANOVAs following for further analysis of location and N level for each incubation temperature.

Principal Component Analyses (PCA) were used to investigate the relationship of organisms' long or short environmental conditions with rates of denitrification and nitrous oxide production for both years' data.

RESULTS

Experiment 1: Impact of N & T on oyster DNF potential, N_2O production, and health

3.1: Growth, mortality and pathogen prevalence

The N addition treatments (100 and 40 µM) experienced significantly higher mortality compared to the control (20µM, $F_{96,143}$= 4.85, p=0.003) (**Figure 8**). Moreover, the 24°C / 100µM (most extreme conditions) treatment had the highest percentage of mortality, and the 18°C / 20µM (least extreme conditions), the lowest ($F_{96,143}$= 3.00, p=0.034). Growth (length) and biomass was not significantly different between any of the 8 treatments. Overall, mortality was low, with only 8% of the total organisms perishing throughout the entirety of the experiment.

When mortality was analyzed at each temperature independently, N-level (2-way ANOVA, $F_{48,71}$= 9.14, p=<0.0001) and time of exposure ($F_{48,71}$= 6.33, p=0.0001) significantly impacted mortality within the 24°C experimental tanks, with the 100µM treatments having significantly higher mortality compared to the 20µM for the majority of the experiment (**Table 1, Figure 8, Appendix 1 & 2)**. Most mortalities in the 24°C treatments were observed during the final days (between days 59 and 90 and between days 90 and 99) of the 3-month experiment (**Figure 8**). These time intervals showed the highest levels of mortality compared to the first time period ($F_{96,143}$= 3.45, p=0.006) (**Appendix 1 & 2**). Within the cooler (18°C) treatments, the 40µM tanks experienced the highest level of mortality and the 20µM the lowest (**Figure 8, Appendix 1 & 2**).

The water quality parameters within the tanks of the highest mortality (24°C, 100µM) showed a noticeable rise in temperature, and decrease in both DO and pH when

the rise in mortality occurred (~Day 55-60). When considering the lowest mortality treatment in the warmer tanks (24°C, 20µM) this trend did was not as pronounced (**Figure 9**). When making the same comparison within the cooler treatments, the highest mortality tanks (40µM) had consistently lower pH and DO levels for the large portion of the experiment, when compared against the 20µM (lowest mortality) tanks (**Figure 9**). The PCA results revealed that DO and pH values are often tightly correlated with one another (**Figure 12**), and by Day 53 (Incubation 2), the change of DO and pH (during the incubation) is also tightly correlated with the percentage of deceased organisms at that time period (**Figure 12**). This also aligns within a reasonable timeframe with the drop in the average DO and pH within the 24°C tanks (~Day 55), as well as the spike in mortality in the 24°C tanks (~Day 51) (**Figure 9**).

Results from qPCR analysis displayed no detectable levels of *P. marinus, H. nelsoni,* or *H. costale* in the subsample of oysters collected upon completion of Experiment 1 (data not presented).

3.2: Water quality parameters in laboratory experiment (Experiment 1)

Average water quality conditions in experimental aquaria (overall)

Average water temperatures across the eight treatments were consistently in range of the target (18°C or 24°C) with no more than 1.1°C variation over the course of the three-month lab experiment (**Table 2**). DO, pH, and salinity values were all in range typical of estuarine parameters throughout the entirety of the experiment (**Table 2**). All parameters with the exception of salinity, significantly changed with time (**Appendix 3, Table 3**); specifically, tanks became warmer, less oxygenated, more acidic, and more

enriched (higher NH_4^+ concentrations) over the course of Experiment 1, presumably due to exposure to the controlled conditions (**Appendix 4**). Additionally, pH was significantly higher in aquaria with the warmest treatment ($F_{48,71}$= 48.89, p<0.0001) while the highest N treatment (100µM) had significantly lower pH ($F_{48,71}$= 9.81, p<0.0001). Finally, there was a significant interaction between target temperature and N-level such that the cooler tanks with highest N levels (18°C, 70 & 100µM) were the most acidic ($F_{48,71}$= 13.56, p= <0.0001). DO also displayed a significant interaction between temperature and nitrogen treatments (**Appendix 3 & 4**), specifically 18°C, 70µM treatment experienced the lowest oxygen levels overall, and the 18°C 40 & 20µM and 24°C, 70µM treatments exhibiting significantly highest DO levels than all other treatments ($F_{48,71}$= 5.71, p= 0.001) (**Appendix 3).**

Change in water quality over the course of N_2 and N_2O incubations

Due to the metabolic activity of the oysters and microbes within gas-tight tanks, dissolved oxygen declined over the course of the incubations for measurement of gas rates. During the first measurement, when oysters had been exposed to the incubation conditions for nearly 4 hours (day 5) starting values of DO, averaged for all treatments, started at 5.25 (0.28) mg/L, and ended at 3.40 (0.24) mg/L. Two of the treatments (24°C, 40 & 100µM) experienced temporary hypoxic (≤3.00 mg/L) conditions (**Table 4**); this did not occur for oysters that had been exposed to experimental conditions for a longer period of time (53 or 89 days). Warmer temperature treatments (24°C) experienced the largest drop in DO during the first measurement (Day 5) ($F_{16,23}$= 9.10, p=0.008) (**Table 5, Appendix 5**). By Day 89, oxygen values remained more similar (average starting:

5.46(0.22) mg/L; ending: 4.51(0.20) mg/L), and hypoxia was not observed in any of the treatments (**Table 4**). Water in the incubation tanks with oysters exposed to the lowest nutrient levels (20µM) showed a significantly higher decrease in pH during N_2 and N_2O incubations than water in the 100µM treatment (**Table 4**). The change in DO and pH within the control tanks (18°C, no N additions, no oysters) was minor, in comparison, with the average change in DO being 0.15(+/-0.20) mg/L, and average change in pH -0.02(0.02) overall.

3.3: Denitrification (N_2) rates

Rates of oyster denitrification were generally variable with both positive (net N_2 production) and negative (net N_2 consumption) values (**Figure 10**). Rates were significantly but non-linearly impacted by N treatment (3way ANOVA, $F_{32,47}$= 3.47, p=0.027) (**Appendix 6 & 7**), with the 40µM tanks displaying significantly higher rates overall than the 70µM treatment. Rates were also significantly impacted by the interaction of temperature, N level, and time ($F_{32,47}$= 3.31, p=0.032) (**Appendix 6 & 7**). Thus, highest denitrification rates were not found in the highest N levels, contrary to our hypotheses. Largest denitrification rates (positive and negative) were found in the warmest temperature (24ºC) treatment (Figure 10).

When examining each time point independently, denitrification rates were initially similar across all treatments (Day 5, **Table 6**). By 53 days, denitrification rates in oysters exposed to nitrogen enrichment at 24ºC were impacted by N level ($F_{16,23}$=6.68, p=0.004) (**Table 6, Appendix 8**), with tanks with oysters exposed to the lowest nutrient levels (20 and 40µM) exhibiting significantly higher denitrification rates

than the 70 µM treatment (**Figure 10**). The interaction of temperature and N level was also significant on Day 53 ($F_{16,23}$=5.21, p=0.011) (**Table 6, Appendix 8**), with the highest rates of denitrification displayed within the high temperature, low nutrient tanks (24°C, 20 & 40 µM); and the lowest rates within a high temperature, moderately high nutrient tank (24°C, 70µM) (**Figure 10, Appendix 7**).

Data from the third incubation (Day 89) was not included in any analysis, due to an unreliable MasterFlex pump allowing air flow into a number of tanks, which is known to greatly skewed N_2/Ar analysis on a membrane inlet mass spectrometer (Kana et al. 1994; Eyre et al. 2002; Humphries et al. 2016).

3.4: N_2O production rates

N_2O production rates displayed variability, with rates ranging from N_2O consumption to production (**Figure 11**). There were significant interactions between temperature and time ($F_{48,71}$=4.07, p=0.023) as well as between N level and time ($F_{48,71}$=3.05, p=0.013) (**Appendix 6 & 7**). Overall, warmer and/or higher N-enriched tanks resulted in higher N_2O production with longer-term exposure to conditions (**Figure 11, Appendix 4**).

N_2O production patterns differed between the two temperature treatments (**Figure 11**). At 24ºC, N level significantly and consistently increased N_2O production ($F_{24,35}$=3.90, p=0.021), with 100µM being significantly higher than the 70µM treatment (2way ANOVA, Tukey's HSD) (**Figure 11, Appendix 7**). Longer exposure to conditions (time) also led to significantly higher N_2O production rates within the 24°C treatments (**Figure 11, Appendix 7**), such that the moderate to high nutrient tanks (40,

70, 100 µM) switched from N_2O consumption at Day 5 (Incubation 1) to N_2O production Days 53 and 89 (Incubation 2 & 3) (**Figure 11**). There was more variability and less predictive patterns observed in the 18°C treatment tanks (**Figure 11**), however the 70µM treatment on Day 89 was significantly higher than it was on Day 5 (**Appendix 7**), indicating that exposure to N level may factor into N_2O production.

When each of the three incubation time points were analyzed independently from one another to examine the effect of N level and temperature, initially (Day 5), lower temperature treatments resulted in higher rates of N_2O ($F_{16,23}$=7.22, p=0.016), (**Table 7, Figure 11, Appendix 8**). By Day 53 (Incubation 2), N level became the significant factor within the results ($F_{16,23}$=4.10, p=0.025) (**Table 7, Figure 11, Appendix 8**), with the 100µM treatments producing significantly more of the greenhouse gas compared to the 20 or 40µM treatments (Tukey's HSD: µM: 100A, 70AB, 20B, 40B). Day 89 (Incubation 3), overall displayed higher production rates when compared to the start of the experiment (**Figure 11**), but values were not significantly related to any of the experimental factors (**Table 7**).

3.5: Relationship between N_2 and N_2O production rates and environmental conditions

A PCA was used to test correlations between the average, and pre- and post-incubation water characteristics on rates of *C. virginica* nitrogen gas production. For oysters that had been exposed to experimental conditions for 5 days, 58% of the variation able to be described (**Figure 12**). Rates of denitrification were most closely related to temperature (average, and incubation) while N_2O production was closely

related to the decrease in DO over the course of the incubation for measurement of gas rates (**Figure 12**). Other factors appear to be correlated with N_2O rates include the change of NH_4^+ and final pH values over the course of the incubation (**Figure 12**). Denitrification and N_2O production were found to be inversely related (**Figure 12**).

For Day 53 (Incubation 2), the PCA was able to describe 46.9% of the variation within the results (**Figure 12**). Similar to Day 5 (Incubation 1), rates of denitrification and nitrous oxide production were once again inversely related within the Incubation 2 results (**Figure 12**). Contrary to Incubation 1, rates of denitrification were most closely related to incubation factors, such as the ending DO and pH values. Denitrification was also very closely related to the average flow rate leading up to the Day 53 experiment (**Figure 12**). Also, the rates of N_2O production measured for Day 53 were more closely correlated to average factors (within the 7L aquaria), such as temperature and pH. N_2O was also related to the observed temperature during the incubation (**Figure 12**).

Experiment 2: Response to N along a dynamic estuarine gradient

3.6: Growth, mortality, and oyster pathogen prevalence

Location significantly affected oyster growth rates in terms of size ($F_{8,11}$= -3.38, p=0.009) and biomass gains ($F_{8,11}$=-3.12, p=0.014) in 2016 (**Table 8**), with the oysters in the southern site growing faster in regard to both metrics, by 1.2 mm $month^{-1}$ and 2.5 g $month^{-1}$, respectively (**Figure 13 & 14**). Neither N-level, nor the interaction between N-level and location significantly affected growth rates (**Figure 13 & 14**). Oyster mortality in the field was not affected by location ($F_{8,11}$= -0.09, p=0.934), N-level ($F_{8,11}$=-0.26, p=0.800), nor the interaction of these two factors ($F_{8,11}$= 1.30, p=0.231).

Contrary to 2016, the north site supported significantly higher *C. virginica* growth rates ($F_{8,11}$ =2.74, p=0.002) and biomass ($F_{8,11}$ = 3.07, p=0.001) (**Table 8**, **Figure 13 & 14**) by 1.5 mm month^{-1} and 1.9 g month^{-1}, respectively, when compared to the southern site in 2017 (**Appendix 9**). Across both years, organic nitrogen enrichment resulted in greater biomass by 1.2 g month^{-1} on average (F=5.35, p=0.034) (**Appendix 9, Figure 14**).

Mortality remained low in the 2017 field season (~12% overall), and did not differ by location ($F_{8,11}$=-0.70, p=0.503), N-level ($F_{8,11}$=-2.16, p=0.062), or the interaction between the two ($F_{8,11}$=0.44, p=0.669). There were no detectable levels of *P. marinus* or *H. nelsoni* were observed in the tested oysters. The parasite *H. costale* was detected in 7.5% of the oysters (indicating low levels of intensity); however there was no effect of location ($F_{8,11}$= -0.26, p=0.803) or N-level ($F_{8,11}$= -0.26, p=0.803) on the prevalence of the disease.

3.7: The dynamic environment of Point Judith Pond

On average, the northern location was warmer, less saline, had higher Chl-a, and lower DO levels than the southern site in 2016 (**Table 9**). Additionally, the northern location experienced a larger range in pH, chl-a, and DO (**Table 9**).

The 2016 12-hour profile of environmental conditions allowed for the analysis of the effect of location, N-level, and tidal height on the environment experienced by the oysters within a single day (**Table 10**, **Figure 15, Appendix 10 & 11**). During this sampling day, the general difference observed between sites during 2016 (**Table 9)** held true, with the northern location being warmer (3 Way ANOVA, $F_{40,47}$=10.71,

p<0.0001), less saline (3 Way ANOVA, $F_{40,47}$= -20.96, p=<0.0001), experiencing higher Chl-a levels (3 Way ANOVA, $F_{40,47}$= 5.61, p=<0.0001), and lower NH_4^+ (3 Way ANOVA, $F_{40,47}$= -2.51, p=0.016) (**Appendix 10 & 11**). Additionally, temperature ($F_{40,47}$= -2.85, p=0.006), DO (3 Way ANOVA, $F_{40,47}$= -2.37, p=0.0226), and pH (3 Way ANOVA, $F_{40,47}$= -2.42, p=0.020) were significantly higher at high tide than at low tide (**Figure 15, Appendix 10 & 11**).

Similar environmental trends were observed in 2017 when both sites were monitored simultaneously. The northern field site on average was once again warmer, less saline, experienced higher chlorophyll levels, and also generally had less dissolved oxygen (**Table 11, Figure 16**). All four environmental parameters showed significant differences between location, month and tidal heights, confirming Point Judith Pond as a dynamic system, both spatially and temporally (**Appendix 12 & 13**). On average, the northern field site also experienced higher concentrations of dissolved NH_4^+, NO_3^-, and NO_2^-, with NH_4^+ being the major DIN species at both locations in 2017 (**Table 12**). Trends in field parameters largely stayed the same between the 2016 and 2017 field seasons, with the exception of NH_4^+, which was higher in the southern site in 2016 and in the northern site in 2017 (**Table 13**).

3.8: Denitrification (N_2) rates

Location ($F_{8,11}$= -5.05, p=0.001) and the interaction between location and N-level ($F_{8,11}$= 4.69, p=0.001) significantly affected denitrification rates of oysters from the 2016 field experiment (**Figure 17**). Specifically, field-enriched oysters from the southern site exhibited higher rates of denitrification than oysters from any other

treatments (**Figure 17**). The opposite trend appears for the northern location's oysters ($F_{4,5}$= 6.43, p=0.006), with organisms kept at ambient conditions denitrifying at higher rates on average than those which received N additions in the field (**Figure 17**).

In 2017, when potential denitrification rates were measured from oysters via exposure to a pulse of high N levels (100µM), the denitrification rates were generally an order of magnitude higher than those from 2016. However, there was a significant interaction between incubation temperature and field N-level at the Southern Location (F=-2.87, p=0.021) (**Table 14, Appendix 14**). Oysters in the 18°C southern site, enriched treatment and the 24°C northern site ambient treatment displayed the highest rates (**Figure 17 & 18**). The 24°C incubated oysters which had received organic N enrichment in the field, from both the northern and southern site, switched from a net source of N_2 (i.e. net denitrification) at 18°C, to a net sink of N_2 at 24°C (**Figure 18**). This was not true for the oysters who were maintained at either field location under ambient (low N) conditions (**Figure 18**).

3.9: N_2O production rates

Nitrous oxide was not detected for oysters from the 2016 field trial (data not shown).

Oysters from the 2017 field experiment that were exposed to a pulse of 100µM $NH_4^+NO_3^-$, showed production of N_2O. Overall, production rates were low (≤0.30 nmol N_2O g wet weight^{-1} hr^{-1}) (**Figure 19**). However, N_2O production was higher at warmer temperatures, from the oysters which were maintained at the northern location (F=-2.81, p=0.021) (**Table 14, Figure 19, Appendix 14 & 15**).

3.10: Relationship between N_2 and N_2O production rates and field environmental conditions

A PCA was utilized with both the 2016 and 2017 field data (separately), in order to observe potential correlations between environmental factors and N_2 and N_2O gas production. In 2016, 76.88% of the variation was able to be described (**Figure 20)**. However, very few factors appeared tightly correlated to denitrification rates. The two factors that were somewhat related included the percentage of oyster mortality observed in the field, and evening low tide NH_4^+ values (**Figure 20**). Factors that the PCA found inversely related to rates of denitrification included evening low tide pH and DO, evening high tide Chl-a, and morning low tide chl-a (**Figure 20**). In 2016, the only DIN species that was able to be analyzed was NH_4^+, and the only factors that were used for the PCA were the long term field conditions (no incubation conditions).

In 2017, 62.46% of the variation was able to be described with field data and pre– and post– incubation characteristics (**Figure 20**). Rates of denitrification were most closely related to field DIN concentrations, including July high tide NH_4^+, June high tide NO_3^-, and August low tide NO_3^-. Denitrification was also related to one incubation factor, which was post – incubation DO (**Figure 20**). N_2O production was most closely correlated to DIN incubation factors, which were pre-, post -, and overall change in NH_4^+ concentrations during the incubation experiment. Field factors that showed correlation to N_2O production were June high and low tide NO_2^- concentrations (**Figure 19**). Finally, the PCA revealed that rates of denitrification and rates of nitrous oxide production appear to be inversely related (**Figure 20**).

DISCUSSION

4.1: Overview of major findings

As hypothesized, temperature initially resulted in increased rates of denitrification, as Experiment displayed higher rates in the warmer (24°C) tanks on Day 5 (Incubation 1). Experiment 2, 2017 results also indicate that temperature is a partial driver of N_2O production as well. Compromised health from long term exposure to increased temperature may be associated with these findings, as organisms maintained longer term in warmer treatments succumbed more frequently with time (**Figure 8**).

It was initially thought that increased N loads would increase rates of both denitrification and nitrous oxide production. Results indicate that nitrogen loads appear to influence N_2O production in a more linear response compared to denitrification rates, which switch between sinks and sources of N_2 (**Figure 10**). In Experiment 1, the 24°C treatments show a clear trend of higher N_2O rates with higher N levels in the later incubations (Day 53, 89), also indicating that exposure (time) influences production rates. However, this trend did not hold true for Experiment 2, where field enrichment had no additional effect on potential N_2O production (**Figure 19**), most likely due to the pulse of 100µM $NH_4^+NO_3^-$ dominating the response.

Finally, the interaction of high T + high N resulted in inhibited rates of denitrification. In Experiment 1 and 2, long term N enrichment (70 & 100 µM and field fertilization, respectively), combined with warming (24°C), resulted in a switch of net N_2 production (denitrification) to consumption (nitrogen fixation). In regard to N_2O production, Experiment 1 showed increased greenhouse gas production at high N levels in the warmer treatments, and Experiment 2, the oysters which experimented higher N

loads in the field produced more N_2O on average when incubated at a higher temperature.

4.2: Prolonged exposure to warmer temperatures may inhibit denitrification and favor N_2O production of *C. virginica*

In regard to the impact of temperature to the health of *C. virginica's* health status, it was thought that prolonged exposure to higher temperatures would induce stress, and eventually compromise the organisms' health, leading to higher rates of mortality and/or increased susceptibility to pathogens. Experiment 1 indicated that there is an association with high T (24°C) and mortality, as organisms maintained in the warmer aquaria succumbed more frequently with time (i.e. exposure to stressor), and N level (100µM) (**Table 1, Figure 8, Appendix 1 & 2**). Further investigation of the change of water quality parameters revealed that an increase in temperature and drop in DO and pH occurred within the same time frame in which the 24°C / 100µM experienced a spike in mortality (**Figure 9**), indicating that the experimental factors (high T + high N) induced several expected changes of water parameters (DO, pH), possibly resulting in physiological stress of the organisms and their eventual demise. This was not the case for highest mortality treatment (40µM) within the cooler tanks, which did not experience a noticeable difference of DO or pH drop from the treatment with the lowest morality (20µM) (**Figure 9**). This was further verified with the PCA analysis For Day 53, which indicated the relationship between mortality and the change of both DO and pH (**Figure 12**). There was no clear association of pathogen presence

with organisms maintained within higher temperature aquaria and/or location, suggesting that physiological stress from poor water quality alone was enough to induce oyster mortality.

It was hypothesized that warmer conditions may favor N_2O production (possibly via incomplete denitrification) due to compromised health of the organism. In regard to N_2O production, exposure to increased temperature and nitrogen were significant factors (**Appendix 6**), as observed most clearly under warmer conditions (in 24°C tanks) where our hypothesis of higher N_2O emissions with higher N loads and warmer conditions was generally supported (**Figure 11, Appendix 7**). Further, supporting our hypothesis, N_2O production was significantly higher ($p=0.014$) during the Experiment 2, 2017 incubation experiment within the 24°C tanks compared to the cooler, 18°C treatments (**Figure 19, Appendix 15**). The N levels used for Experiment 2 (2017) were intentionally high in attempt to test potential thresholds of N loading, and therefore represent quite extreme scenarios. Results from both Experiment 1 & 2 therefore indicates that *C. virginica* N_2O production is dependent on N availability, as well as the duration in which they are exposed to increased warming.

The hypothesis of higher temperatures resulting in higher rates of denitrification were not supported for *C. virginica*. In the short-term during the laboratory experiment (Day 5, Incubation 1), the experimental warming (24°C treatments) resulted in significantly higher N_2 production rates (**Figure 10**). However, this association of higher denitrification rates with higher temperatures was not exhibited in any prolonged exposure to temperature. Oysters maintained in either high temperature tanks (24°C) in later incubations (**Figure 10**) did not exhibit higher denitrification rates.

Oysters maintained in the field (Experiment 2) withstood a wide range of environmental factors, including temperature, in both years' studies. The northern site was on average warmer (~23 – 25°C), yet the southern location experienced a wider range of T on average (~17 – 23°C) (**Table 9, Figure 15 & 16, Appendix 11 & 13**). This further justifies that these organisms are physiologically capable of withstanding temporary temperature changes within their natural habitats (Shumway 1996), and places Experiment 1's chosen temperature range (18°C, 24°C) into a realistic context. Similar to Experiment 1, the warmer field locations in Experiment 2 (northern field site in the 2016 and 2017 field incubation) (**Figures 16, 18**) did not exhibit higher denitrification rates. These results suggest that temperature is only one of several drivers of nitrogen fluxes associated with shellfish. The lack of a prolonged effect of temperature on denitrification may be explained in part by higher temperatures promoting higher shellfish filtration rates (Ehrich and Harris 2015) but long-term exposure to higher temperatures may become a stressor to the organism, as highlighted in the mortality data previously.

The different response of these two N transformations to warming, specifically, the inhibition of denitrification versus promotion of N_2O with prolonged exposure to warming, warrant further consideration. N_2O has multiple potential sources in addition to what is produced as a byproduct of denitrification. Such processes include the oxidation of NH_4^+ to NO_3^- during the first step of nitrification (Goreau et al. 1980), dissimilatory reduction of nitrate to ammonium (Smith and Zimmerman 1981), and as a byproduct during nitrifier – denitrification (Wrage et al. 2001). It is also likely that coupled nitrification – denitrification is a factor, due to favorable conditions for both

processes (Wrage et al. 2001), and the well supported idea that shell biofilm is a major contributor to bivalve – associated N_2O production (Svenningsen et al. 2012; Heisterkamp et al. 2013). Additionally, N_2 production rates calculated for this study, reflected both N_2 fixation and denitrification. Further analysis of the different pathways may reconcile the lack of predictable relationship between N_2 and N_2O production within the data.

The PCAs revealed an inverse relationship of denitrification and N_2O, production within the Incubation 1, Incubation 2, and 2017 Field Incubation datasets (**Figure 12 & 20**), suggesting that N_2 and N_2O may be viewed as alternative products of one pathway in regard to *C. virginica* nitrogen cycling. These data suggest that *C. virginica* will either facilitate the full process of denitrification, acting as a sink of N_2O (Chapuis-Lardy et al. 2007), or the N-removal process will terminate early, leading to an accumulation of the greenhouse gas. Furthermore, in Experiment 1, temperature was initially related to N_2 production and inversely related to N_2O production (Day 5 / Incubation 1), but overtime (Day 53 / Incubation 2), that relationship switched (**Figure 12**). Therefore, the results presented here likely indicate that the longer *C. virginica* experiences extreme and/or rapid warming scenarios: (1) denitrification rates largely depends on N availability and time scale of high T exposure; results indicated that rates may not be significantly enhanced and may even possibly experience a decrease and (2) N_2O production is likely to continue to rise under conditions of high nitrogen availability.

4.3: Nitrogen cycling of *C. virginica* likely depends on duration of N exposure

We hypothesized that increasing N loads would enhance both denitrification and N_2O production rates of *C. virginica* due to potentially a more active microbial gut community and greater N availability. Our results, however, results only partly support this notion, as denitrification did not appear to be enhanced by increasing N loads. The effects of N level are more pronounced within the N_2O production rates. When considering the Experiment 1, the 24°C treatments begin to show a clear, linear trend of higher N with higher N_2O rates, starting at Day 53 (Incubation 2), and becoming significant by Day 89 (Incubation 3) (**Figure 11**). Additionally, N level (p=0.021), time (0.001), and N level X time (p=0.006) were all significant factors within the warmer incubation tanks in Experiment 1, indicating that long term exposure to high N loads results in higher rates of N_2O production by *C. virginica*, likely due to increased nutrient availability for the nitrogen cycling microbes (Kroeze and Seitzinger 1998) within the tank and/or oyster gut.

When considering denitrification rates, Experiment 1 resulted in very few N-related trends. In some cases, N level did increase rates; for example, in Incubation 2, the 24°/ 20µM and 24°/ 40µM showed higher N levels resulting in higher denitrification rates, on average (**Figure 10**). Observing this trend in the warmer tanks is likely caused by a more active microbial community (Kroeze and Seitzinger 1998; Lindermann et al. 2016). However, seeing the trend at lower N levels (20 & 40µM) opposed to the higher N levels (70 & 100 µM), where there is no difference and/or reversal of N_2 production, is unexpected (**Figure 10**). This finding can potentially be explained by a limited microbial population size within Experiment 1, as filtered seawater (~1 micron) was

used throughout the 3 month experiment, therefore reducing the introduction of new microbes within the aquaria. Specifically, the microbial enzymes found in marine sediments, which mediate the process of denitrification may be lacking: nitrate reductase (*Nar*), nitrite reductase (*Nir*); nitric oxide reductase (*Nor*), and nitrous oxide reductase (*Nos*) (Zumft 1997).

Laboratory nitrogen enrichments were in reasonable range of the target, as ambient seawater, on average, had a NH_4^+ concentration of 40.82 (+/-4.01) µM. Subtracting that value from the averaged nutrient reservoir concentrations, presented in **Table 2**, results in average NH_4^+ concentrations of 24.09, 53.32, 72.99, and 96.68 µM for the 20, 40, 70, and 100 µM reservoirs, respectively. NO_3^- was also a nitrogen species that was added as part of this enrichment, but those concentration values were not tested. We acknowledge that these enrichment levels are high in referenced to N inputs that Narragansett Bay currently receives (Oviatt 2017), and higher than typical Point Judith Pond values (Moran et al 2014), yet they are not unrealistic values that these systems have received historically (Oviatt et al. 2002; Moran et al. 2014).

Water samples collected from each experimental bucket during Experiment 2 (2016 & 2017) largely did not fully capture the increased enrichment level within those which received fertilizer additions (both locations). This is likely due to the dynamic nature of the system and the difficulty which arose when attempting to take discrete samples within a strong, tidally driven environment. Had there been a monitoring probe or a long term sonde that had the ability to measure DIN concentrations, there may have been more accurate readings for Experiment 2, as we did see some significant findings associated with N additions; for example, biomass when considering both year's data

(F=5.35, p=0.034; **Appendix 9, Figure 14**), and denitrification from 2017's incubation (F=-2.92, p=0.010; **Appendix 14, Figure 18**), suggesting the N additions were at least marginally successful.

The field experiment (Experiment 2), partially supported the original hypothesis of denitrification increasing with N loads. The 2016 field results showed significantly higher denitrification rates from oysters who received enrichment in the southern field site when compared to their ambient counterpart, however this this trend was not found at the northern site (**Figure 16**). The 2016 southern field oysters were larger by both metrics (**Figure 13 & 14**) than those from the northern site, potentially linking the larger sized organisms with more abundant denitrifiers (i.e. more microbial biomass). This positive correlation of invertebrate N_2O production and biomass has been found in a number of past studies (Stief & Eller 2006; Stief et al. 2009; Stief & Schramm 2010), and is driven by the organism's increased gut size being able to hold more N_2O producing microbes.

In the 2017 field experiment, there is a trend of higher denitrification rates for both the northern and southern oysters, who received field organic N enrichment, within the 18°C treatments (**Figure 18**); however, this trend does not hold true for the 24°C treatments. Finally, comparing the rates from 2016, which were not given incubation N enrichments, to the rates in 2017, which were given a 100µM $NH_4^+NO_3^-$ incubation enrichment, rates of denitrification were an order of magnitude higher in 2017, showing what may happen when oysters receive a high spike in N loads (100µM) compared to a more realistic and slow dilute of N inputs, like in the 2016 data, suggesting that a response to N may only be evident at high enrichment levels.

While Experiment 2, 2017's enrichment level is less than half of the typical total DIN (~35 µM) within Point Judith Pond, the northern most section of the system has experienced groundwater N concentrations as high as 1470 µM (total DIN; 99.8% NH_4^+) (Moran et al 2014). These high concentrations are found further north than the northern site used for Experiment 2, and are within an area closed to shellfishing (RI DEM 2017). Overall, the findings suggest that denitrification is related to N availability, as expected, but the rates associated with the process appear to be controlled by many addition complex environmental factors, most prominently temporal DIN availability, as highlighted by the PCA analysis (**Figure 20**).

4.4: The combination of stressors (high T + high N) may inhibit denitrification and favor N_2O production of *C. virginica*

A surprising interaction was found between the interactions of stressors (high T + high N) on denitrification rates, were observed in both laboratory and field experiments. Experiment 1 and Experiment 2 (2017) both showed that with a combination of long-term N enrichment (70 & 100µM; field fertilization, respectively) and warming (24°C), oyster denitrification switched from N_2 production (i.e. denitrification) to consumption (**Figure 10 & 18**). N_2 consumption indicates net nitrogen fixation (at rates exceeding denitrification rates) and results in the production of NH_3 (ammonia, i.e. reactive N) (Purvaja et al. 2008), therefore recycling reactive nutrients back into the system, as opposed to removal through denitrification. This switch from N removal (net N_2 production) to N production (net N_2 consumption) did not occur within the lower nutrient treatments of Experiment 1, nor the oysters left at

ambient conditions (of either field location) within the 2017 Experiment 2 field manipulation (**Figure 10 & 18**).

The 2016 field denitrification rates (Experiment 2) show a more subtle but similar trend to Experiment 1 and 2017 Experiment 2 (**Figure 17**). The southern field site experienced an average temperature of 19.2°C over the course the entire 2016 field deployment, and oysters which received N loading denitrified at significantly higher rates than those left at ambient (low N) conditions within the southern location. However, the northern location, which experienced an average temperature of 24.4° during the field deployment displayed the opposite trend, with oysters which received enrichment in the field denitrifying at a lower rate, on average, than those left at ambient locations (**Figure 17**).

While it is counter intuitive that an increased concentration of reactive N would lead to decreased rates of denitrification, a number of factors may be responsible for this switch from N_2 production to N_2 consumption. In Experiment 1, we saw tanks with the highest mortality levels within the high T + high N treatments (**Figure 8**), and also a noticeable diverge of DO and pH (decrease, both factors) within these tanks (**Figure 9**). N loading into coastal habitats often results in decrease pH and DO values within the system (Diaz and Rosenberg 2008). It is not unreasonable to assume that the field manipulations (N additions) within the enriched buckets created differences in localized water quality within the experimental units, very much similar to what was observed in Experiment 1 (**Figure 9**). Additionally, DO and pH are both known drivers of N cycling processes, including denitrification. This fact, combined with the findings that tanks with high enrichment experienced high mortality (Experiment 1), suggest that localized

effects of N loading appear to cause physiological stress, as a result of altered water parameters, and this stress may also tie into *C. virginica's* ability to facilitate the denitrification process, as seen in both Experiment 1 and 2. It also appears that exposure time to these factors is an important aspect of these findings.

In regard to N_2O production, Experiment 1 showed that temperature and N level were both significant factors, but at different times within the experiments (**Table 7**). Furthermore, in Experiment 2 (2017), oysters which experienced higher N loads within the field (**Table 12**), produced more N_2O, *on average,* when incubated at a high temperature (**Figure 19**). Similar to the denitrification data, these results suggest that the threshold for N, on N_2O production, is dependent upon temperature, but also the duration of N exposure (**Figure 11 & 19, Appendix 6**). Specifically, increased exposure to T and N leads to increased N_2O production and decreased N removal (denitrification) rates.

The environmental data collected in Point Judith Pond for the 2016 and 2017 field experiment indicate that the system experiences temperatures of 24°C or above for approximately 5.3 days at the southern site, and 38.6 days at the northern site. The typical range of DIN (~35 µM, Moran et al. 2017) within most of Point Judith Pond is well under the concentrations where we observed these negative effects (particularly N_2 consumption) of the combined stressors of high T + high N, however it does raise concern for oysters in the northern portion of the system, as the northern most portion of Point Judith has measured groundwater N loads as high as 1470 µM (Moran et al. 2017). It could also be assumed that temperatures in this location are similar to what was measured in the northern field site.

Currently, aquaculture and shellfishing activities are prohibited in this area of extremely high N (and presumable high T). While this restriction is based on human safety in regard to shellfish consumption, the results presented here may suggest that the closure may also be advantageous for the environment. By limiting the amount of oysters within this area (through aquaculture restrictions), it may also limit shellfish N_2O production, as well as increased nitrogen fixation rates.

Based on the findings of this study, oyster populations (wild, restored reefs, aquaculture practices) within Point Judith Pond appear to be a net benefit to the system, as typical DIN values are low, and the system does not experience temperatures above 24°C for long durations of the year, therefore limiting the combination of severe stressors on the organisms. If shellfish operations were expanded in the northern most portion of the system (further north than the northern study site), there would first need to be a vast reduction of N inputs, in order to avoid an increase of N_2O production of the bivalves.

4.5: Potential implications

Historically, Narragansett Bay has received N loads as high as 100µM in the northern end of the watershed (Oviatt et al. 2002), but the inputs have been reduced to approximately 40µM in more recent times (Oviatt et al. 2017). If we average all 100µM N_2O production rates (18°C and 24°C) measured in Experiment 1, convert the values to a unit of area (m^2), upscale the values to yearly production rates and finally, multiply the values by approximately 1/4 the area of Narragansett Bay (estimated area effected by high N), the final value is an astonishing 7.391E+11 µM N_2O yr^{-1}. The same

conversions with the 40µM data, result in a final value of 2.13E+11 µM N_2O yr^{-1}; and while this value is still high, it nearly a 30% reduction of N_2O emissions, highlighting the large-scale implications and importance of reducing anthropogenic N loads into coastal estuaries.

In order to understand the role of bivalves on the N cycle, it is important to understand the conditions affecting these rates. Oysters are very much valued for their water purification capabilities (Kennedy and Newell 1996; Newell 2004; Newell and Koch 2004; Grizzel et al. 2008; Dame 2012), and in scenarios which potentially decrease and/or eliminate this ecosystem service warrant further consideration. These conclusions suggest that there is a threshold (>18°C + >40µM) to which oysters may not facilitate denitrification to their full potential, possibly due to physiological status of the organism in response to a combination of stressors, a change in their microbial community, or in response to the effects that the interaction of high T + high N has within the environment. While the average temperature within Narragansett Bay is slowly rising (Narragansett Bay Estuary Program 2017), the system as a whole has undergone a vast reduction of N inputs in recent times (Narragansett Bay Estuary Program 2017), thereby reducing the number of anthropogenic stressors to our coastal habitats. This is reassuring, as oyster production is continuing to rise within Rhode Island, and therefore can still benefit both the economy and the environment.

Overall, the findings presented in this study have the potential to aid in decision making regarding how to maximize the greatest economic and ecosystem benefits from aquaculture and restored reefs. For example, shellfish managers can identify coastal areas which will promote growth of *C. virginica*, and therefore benefit Rhode Island's

aquaculture market, as well as target eutrophic areas for restored reef projects, with the goal of N reduction either through oyster denitrification or nutrient retention. It also highlights the importance of monitoring (and reducing when necessary) the N inputs into coastal habitats, as we may experience the inhibition of important ecosystem services and/or an increase in greenhouse gas emissions from valued coastal resources, in scenarios of excess N.

CHAPTER 2

Impact of nitrogen addition (N) and warming (T) on *Mytilus edulis* N cycling capabilities

MATERIAL AND METHODS

5.1: Overview

Controlled laboratory experiments were used to investigate the effects of the increased nitrogen loads and rising temperatures on *M. edulis*' nitrogen cycling ability, and therefore potential contribution to global climate change due to nitrous oxide (N_2O) production. This goal was achieved by establishing a gradient of ammonium nitrate ($NH_4^+NO_3^-$) inputs at two temperatures. The N gradient used for *M. edulis* incubation experiments was 5µM, 10µM, 17.5µM, and 25µM (enrichment above seawater), and the temperatures were 18°C and 21°C. Ammonium (NH_4^+) and nitrate (NO_3^-) are both common anthropogenic nitrogen species, and most often enter coastal environments through wastewater and septic systems (Galloway et al. 2003; Galloway et al. 2008). With the realization that *M. edulis* is bivalve species sensitive to environmental change and/or stress (Carrington et al. 2009; Jones et al. 2009 & 2010; Dijkstra et al. 2011; Sorte et al. 2017), the goal was to test whether small alterations to their ecosystems can potentially translate to large environmental problems. Therefore, the nitrogen gradient chosen for the experiments is only a fraction of the actual observed range of N concentrations into Narragansett Bay (Oviatt et al. 2002); and contrasting temperatures represent current average bottom temperatures of Narragansett Bay (Nixon et al. 2009)

and the lower end of projected global surface temperatures by 2100 (Mora et al. 2013). This experiment therefore included 8 different combinations of N and T, and each was analyzed in triplicate and maintained for 3 months (**Figure 2**).

To start this laboratory investigation, each experimental mussel tank contained 11 organisms. Mussels (~2.5 cm in size) were purchased from American Mussel Harvesters in Jamestown Rhode Island during December 2016. After being randomly placed within the tanks, organisms were marinated under the set conditions (described above) from January – March. Incubations of the organisms for measurements of N_2 and N_2O production rates occurred on day 0, 51, and 103.

5.2: Controlled laboratory experimental setup

The experimental 7L glass aquaria were maintained within a controlled environmental chamber (Holman Engineering) at the Marine Science Research Facility (MSRF), located at the Graduate School of Oceanography, Narragansett, Rhode Island. The environmental chamber was set to a controlled temperature of 18°C. The experimental aquaria, which contained the organisms, sat within large water baths to maintain consistent temperatures among each experimental unit (**Figure 2**). 21°C treatment tanks also contained a submersible heater (EHEIM Jager Aquarium, Thermostat Heater, 75 watts) within the water bath, as well as submersible pumps (Hydro Empire ©) to distribute the heated water evenly around the higher temperature tanks (**Figure 2**). Temperature was monitored every 2 – 4 days and the heaters were adjusted as necessary to maintain the target warming conditions.

Filtered seawater from Narragansett Bay was stored in large 100L reservoir containers, and enriched with pre-prepared aliquots of ammonium nitrate ($NH^{+}_{4}NO^{-}_{3}$). Water from these nutrient reservoirs was pumped into the appropriate mussel tanks using a MasterFlex © multi-channel peristaltic pump. The flow rate was adjusted as necessary to achieve a target turnover rate of 24 hours for each experimental tank. Based on this turnover rate, nutrient reservoirs needed to be refill roughly twice per week.

To avoid pseudorepliation, there were three water baths per temperature treatment, and tanks of four target nitrogen levels were randomly placed within each water bath. Additionally, there were two nutrient reservoirs per nitrogen level in order to allow for the interspersion of nitrogen inputs among the various treatments (**Figure 2**) (Hurlbert 2009; Cornwall et al. 2016).

5.3: Blue mussel growth, morality and health

After being randomly assigned among each experimental tank, initial mussel size (length and width) and biomass (wet weight) measurements were made on each organism. These measurements were also made monthly, as well as prior to each of the three incubation events (Day 5, 53, 105). Growth rates were later averaged across the treatments. Organisms were fed 70 µl of Reed Mariculture Shellfish Diet © Monday – Friday and the tanks were also checked for mortalities. Deceased individuals were removed from the aquaria as necessary.

Water quality measurements (dissolved oxygen (DO), pH, salinity, temperature, and dissolved inorganic nitrogen (NH_4^+)) were measured weekly for each tank and nutrient reservoir. Complete water changes for the aquaria were performed as necessary

in order to maintain target parameters, which on average resulted in every three to four weeks.

5.4: Incubation procedure for measurement of gas production rates

Approximately 12-16 hours prior to an Incubation event (Day 5, 53, 105) all experimental organisms were transferred from the 7L glass aquaria to the 2L, plastic tanks (Freund Containers) where they were allowed to acclimate overnight. Flow from the peristaltic pump continued during this period. During the time of transfer, three filtered seawater tanks (no organisms, no enrichment) were added to the 18°C water baths to act as controls. The morning of each incubation event, initial water quality parameters were recorded for each tank and nutrient reservoir, including: dissolved oxygen and pH using a handheld monitoring probe (Orion © RDO / pH), salinity with a handheld refractometer (Fisher Scientific), and temperature (handheld thermometer). Due to an issue with the monitoring probe, pH was not able to be recorded for the third incubation (Day 105). Additional samples included dissolved NH_4^+ concentration, obtained from filtering 60mL of water from each tank through a 0.22µm EMD Milipore Millex™ filter, which were stored on ice until frozen in -20°C conditions. The same procedure was repeated immediately after the incubation, in order to observe the overall changes.

Once all initial sampling concluded, the pump was stopped, and each incubation tank was fitted with a gas-tight, screw top lid which thereby eliminated gas exchange with the outside atmosphere. These gas tight lids contained an 12 cm inflow line which connected to their respective nutrient reservoirs via the peristaltic pump via

approximately 2 meters of Tygon MasterFLex © tubing (1/8 inner diameter, Cole Parmer), and a 30 cm sampling line that was secured with a three-way stopcock, male valve, allowing for the connection of a 60mL sampling syringe (**Figure 3**). The inflow line was maintained in the upper portion of each 2L tank, while the sampling line existed in the lower portion in the hopes of promoting mixing during each sampling time point (**Figure 3**).

Immediately following the securing of the lid; as well as prior to each sampling time point, a small amount of water (<5mL) was pulled from each tank and discarded. This was a precaution, to rid the sampling line of any air and/or water from the previous sample. Next, 60 mL of water was sampled from each of the 27 tanks (24 experimental, 3 controls), after which the three-way valves were positioned back in "off" direction. Finally, the peristaltic pump, which was set to a rate of 10 mL min^{-1}, was turned back on and allowed to flow for 6 minutes, this resulted in replacing the sampled water promptly after each time point. The pump was once again paused until this entire procedure was repeated again, approximately 1.5 hours later for the next sampling point. A total of 4 time points were collected from each experimental tank during the incubation events.

All but 20mL of the sampled water from each time point was then carefully aliquoted to two 12mL Labco© Exetainers. It was of high priority to avoid any bubble formation into the Exetainer during this transfer, as this would greatly skewed dissolve gas concentrations within the sample, which is especially a concern for N_2 (g). Each exetainer was immediately fixed with a 50% (weight/volume) zinc chloride (Sigma Aldrich) solution in order to terminate any potential gas concentrations alterations via

biological activity. These samples were then stored upside-down, underwater, at 18°C until analyzed on a Membrane Inlet Mass Spectrometer (MIMS) for N_2 concentrations (detailed below in Section 5.5). The remaining 20mL of sampled water was stored overnight in a cooler and under ice (average temperature: 8.5 (+/- 0.3) °C) until they were prepped and analyzed for nitrous oxide (N_2O) concentration (detailed in Section 5.5).

5.5: Sample processing – Dissolved nitrogen gas (N_2O and N_2)

The morning after each incubation, the 20mL of water stored in the syringes (under ice) were prepped in order to be analyzed for N_2O concentrations on a 2014 Greenhouse Gas Chromatograph (Shimadzu). 15mL of ultra-high purity helium (AirGas) was added to each syringe, (following methods presented by Moseman-Valtierra et al. 2015) and then vigorously shaken for 60 seconds in order to equilibrate the dissolved gases within the water sample into the inert, helium headspace. The water portion of the sample was then discharged, and the temperature and salinity of each was measured and recorded for later calculations. The remaining 15mL helium headspace samples were stored in the refrigerator until analysis. All samples were analyzed on the Gas Chromatograph within four days of collection. Concentrations of each samples were corrected for the dilution of the gas phase and the equilibrate distribution of N_2O between gas and water phases as outlined in Chapter 1 (Weiss and Price 1980; Walter et al. 2010, Garate 2016).

The Exetainer samples were analyzed for dinitrogen (N_2) gas concentrations using a Membrane Inlet Mass Spectrometer (MIMS) located in the Marine Science

Research Facility (MSRF) located at the Graduate School of Oceanography (Narragansett RI). The N_2/Ar method was followed (Kana et al. 1994). Analysis typically occurred within 2 weeks of collection. This timeframe excludes the third time point; due to complications with the instrument these samples were analyzed approximately 2 months after collection and analyzed using a MIMS located at the University of Connecticut (Groton Ct).

For both N_2O and N_2 concentrations, fluxes were determined using linear regressions of concentration versus time. If the regression line had a $R^2 \geq 0.65$ then a flux was calculated (Prairie 1996). Positive fluxes of N_2 were indicative of net denitrification (Kana et al. 1994), and positive fluxes of N_2O indicated production from the organisms during the incubation. These values were then normalized to the gram wet weight of mussel biomass within each tank during time of sampling.

5.6: Sample processing – Dissolved inorganic nitrogen

Water samples collected from each tank prior to and following the incubation experiments were kept frozen at -20°C until the morning of analysis. Dissolved ammonium (NH_4^+) was analyzed with an Orion Aquamate 7000 VIS Spectrophotometer ©, using standard colorimetric techniques (Solorzano 1969).

5.7: Histology

Upon completion of this long-term lab study, a subsample of remaining organisms (n=5) were sacrificed and fixed for histology, using standard procedures

(Howard et al. 2004). Cross sections of the organisms were prepared and analyzed by the Aquatic Diagnostic Laboratory at Roger Williams University, Bristol, RI.

5.8: Statistical Analysis

Three-way ANOVAs were used to examine the impact of temperature, N level, and time (exposure to experimental conditions) on mortality rates, water quality parameters, and nitrogen gas production (N_2 and N_2O). To further clarify the interaction terms, in regard to denitrification and nitrous oxide production rates, two-way ANOVAs analyzed the effect of N-level and time at each temperature; and one-way ANOVAs were used to investigate both temperature and N level (independently) at each incubation time point. Tukey HSD tests followed when significant differences were revealed. A Principal Component Analysis (PCA) was performed for each incubation time point, in order to reveal possible correlations between environmental conditions and nitrogen gas production rates. The overall goal was to determine whether there are critical thresholds which result in a decrease in *M. edulis'* performance – which is defined as a significant decrease in N_2 (denitrification) and/or an increase in N_2O (greenhouse gas production).

RESULTS

6.1: Growth, mortality, and health

Changes in biomass of *M. edulis* were significantly dependent upon N level within the 18°C treatments ($F_{8,11}$=5.12, p=0.028), with the organisms in the 17.5 µM tanks (moderately high N) displaying the fastest growth among the four N levels. N level had no impact within the 21°C treatments ($F_{8,11}$=0.76, p=0.546). Temperature ($F_{16,23}$=0.81, p=0.380) nor N level ($F_{16,23}$=1.41, p=0.276) had an impact on the biomass gain.

On average, *M. edulis* grew in the 0.2 – 0.6 mm/month range throughout the laboratory experiment. There was no significant difference in shell growth rates between different N levels within either the 18°C or 21°C treatments ($F_{8,11}$=0.54, p =0.665; $F_{8,11}$=1.19, p -0.372, respectively). Furthermore, neither temperature ($F_{16,23}$= 0.009, p=0.925) nor the interaction of temperature and N level ($F_{16,23}$=0.38, p=0.770) had an effect of the shell growth rates.

Overall, the percentage of deceased organisms was low, with less than 7% of the total organisms to start the experiment perishing, and no single treatment experiencing greater than 30% mortality on average (**Figure 21**). Nitrogen level (3 way ANOVA, $F_{80,119}$=8.86, p=<0.0001) and time of exposure (3 way ANOVA, $F_{80,119}$=2.79, p=0.031) both significantly impacted the percentage of moribund mussels (**Appendix 16 & 17**). When the data was analyzed at each temperature, independently, mortality was significantly dependent upon N level within both the 18°C (2 way ANOVA, $F_{40,59}$=4.06,

p=0.001) and 21°C ($F_{40,59}$=6.77, p=0.0008) treatments (**Table 15**), with the highest N levels having the highest mortality, expect at the warmer temperature when there was only significantly higher mortality within the 17.5 µM treatments (**Figure 21, Appendix 17**). Temperature had no significant impact for any mortality observation (**Figure 21**), although it is noteworthy that there was no mortality for mussels within the most extreme conditions (21°C / 25µM tanks). When the lowest and highest mortality levels for each temperature treatment (5µM, 17.5µM, respectively for 18°C; and 25µM, 17.5µM, respectively for 21°C) were compared against the water parameters of the tanks (DO, pH, NH_4^+), there were very few divergences of the water quality between the nutrient levels (**Figure 22**). Within the cooler, 18°C tanks, one difference between the 15µM (lowest mortality) and the 17.5µM (highest mortality) treatments, was that the latter experienced a temporary, yet large drop in DO to 5.18 (+/- 0.85) around day 16. In the following weeks, (~day 30), is when the spike in mortality levels occurred within those tanks as well. Otherwise, pH and NH_4^+ in the 18°C treatments, and all parameters of the warmer (21°C) tanks were very similar between the high and low mortality levels of each temperature (**Figure 22**).

6.2: Water quality parameters

Weekly water quality conditions in experimental aquaria

Average water temperatures were within range of the target by 0.6°C across the eight treatments (**Table 16**). On average, dissolved oxygen ranged from approximately 7.0 – 7.8 mg/L, pH was generally 7.50 or greater, and salinity was 34.4ppt, on average, over the 3 month experiment (**Table 16**), indicating a well oxygenated, slightly basic,

and high saline environment for experimental organisms. On average, NH_4^+ was in the 50 – 65 µM range (**Table 16**). Oxygen was differed significantly between N level treatments (3 way ANOVA, $F_{176, 263} = 10.44$, p=<0.0001) (**Appendix 18**), such that the lower nutrient tanks (5 and 10 µM) exhibiting the highest DO levels, higher nutrient tanks (17.5 and 25µM) exhibiting the lowest (**Table 17**, **Appendix 19**) (Tukey's HSD (µM: 5A, 10A, 25AB, 17.5.B).

Change in water quality over the course of N_2 and N_2O incubations

Over the course of the three incubations, no experimental treatment experienced hypoxic conditions (<3.00 mg/L) (**Table 18**). The largest decreases in oxygen occurred during Incubation 1 (Day 2) due to the gas-tight closure of the tanks for approximately 4 hours. Average (overall) starting DO levels for Incubation 1 were 10.56(0.03) mg/L and ending values were 6.02(0.14) mg/L. By Incubation 2 (Day 103), although the average starting values for DO was much lower (4.95(0.31) mg/L), the overall drop in oxygen over the course of the incubation was much smaller (**Table 18),** with final values averaging 4.24(0.23) mg/L. The decrease in DO during Incubation 1 ($F_{16,23}$=26.51, p=<0.0001), differed significantly according to temperature treatments, with the higher temperature treatment experiencing larger decrease in oxygen. Neither temperature nor N level had any effect on the change of DO within Incubation 2 or 3 (**Table 19, Appendix 20**).

Following the same trend as DO, the decrease in pH was not as severe in Incubation 2 compared to Incubation 1, however no data for pH was able to be collected for Incubation 3 (**Table 18**). The change in pH in both Incubation 1 ($F_{16,23}$= 29.24,

p=<0.0001) and Incubation 2 ($F_{16,23}$=10.58, p=0.005) varied between temperature treatments, with the higher temperature treatment resulting in a larger drop in pH for both instances (**Table 19, Appendix 20**).

Changes in NH_4^+ concentrations were highly variable, ranging from production to uptake (**Table 18**), with both temperature and N level having no impact on the production or consumption of NH_4^+ in any of the three incubations (**Table 19**).

6.3: Denitrification & N_2O Production Rates

M. edulis' rates of denitrification over the course of the three incubations (103 days) were highly variable (**Figure 23**). Rates did not significantly differ with regard to N level, temperature or time (**Table 20, Figure 23, Appendix 21& 22**).

Both temperature ($F_{48,71}$= 5.17, p=0.027) and N level ($F_{48,71}$=3.22, p=0.030) significantly influenced rates of N_2O production (**Appendix 22 & 23**), such that the lower temperature (18°C) and higher N levels (Tukey's HSD: μM: 25A, 17.5AB, 10AB, 5B) resulted in increased N_2O production rates. Over the course of the three incubations, *M. edulis* consistently produced N_2O. There was never an instance of N_2O consumption (**Figure 24**). Highest rates (average: 0.66 +/- 0.21 nmol g wet weight $^{-1}$ hr $^{-}$) were seen during Incubation 2 (Day 48) under the 18°C temperature and highest N level (25 uM) (**Figure 24**). On average, N_2O production within the 21°C treatments showed little variation, and were generally in the 0.1 – 0.2 nmol g wet weight $^{-1}$ hr $^{-1}$ range (**Table 21, Figure 24).**

6.5: Relationship between N_2 and N_2O production rates and environmental conditions

PCAs were utilized in order to discern correlations between environmental characteristics, over both long (average tank conditions) and short-term (incubation conditions), and *M. edulis* nitrogen gas production. During Incubation 1 (Day 2), 55.3% of the variation within the data was able to be explained. Rates of denitrification and N_2O production were very tightly correlated with one another and with the average tank pH prior to the first incubation (**Figure 25**). Denitrification and N_2O rates were inversely related to many incubation factors, including NH_4^+ concentrations as well as pre-incubation DO (**Figure 25**).

During Incubation 2 (Day 53) 44.5% of the variation within the data was able to be explained through a PCA (**Figure 25**). Denitrification rates were most tightly correlated to the average tank temperature prior to Incubation 2 and the change in DO during the incubation (**Figure 25**). N_2O rates were almost completely independent of any factor, according to the PCA results, but most closely align with final NH_4^+ concentrations during Incubation 2; production rates were found to be inversely related to DO and pH values (**Figure 25**). Denitrification and N_2O rates showed no correlations to one another (**Figure 25**).

Finally, for Incubation 3 (Day 105), 39.5% of the variation within the data was able to be explained utilizing a PCA. Rates of denitrification were most closely correlated to the percentage of mortality leading to Incubation 3 and the average DO leading into the incubation (**Figure 25**). N_2O production rates were correlated to the change in concentration of NH_4^+ during Incubation 3 (**Figure 25**). Finally, similar to

Incubation 2, denitrification and N_2O were not correlated in the Incubation 3 results (**Figure 25**).

DISCUSSION

7.1: Overview of major findings

The original hypothesis was that both temperature and N level would increase rates of nitrogen gas production. The results indicate that *M. edulis* nearly always supports net N_2 production (i.e. denitrification, and therefore N removal) at low ($\leq$ 25µM) nutrient conditions. However, the response to warming and N additions is inconsistent, as N_2 production rates were similar across all treatments and time. Additionally, it was found that nitrogen is a very strong driver of N_2O production of *M. edulis*, as a relatively small increase of N inputs (+20µM $NH_4^+NO_3^-$) led to a significant increase in production rates. Finally, higher *M. edulis* N_2O production rates were exhibited at the cooler (18°C) temperature treatment, which many be a reassuring finding when considering the anticipated ocean warming trends.

7.2: *M. edulis* exhibits high potential for denitrification

Initial hypotheses of this study included that warming would initially increase denitrification rates, as would higher N loads, and the combination of the two anthropogenic stressors, with time, lead to incomplete denitrification processes and higher N_2O production. While *M. edulis* showed a wide range of denitrification potential, with average rates ranging from N_2 consumption (i.e. net nitrogen fixation), of -83.6 nmol g wet weight^{-1} hr^{-1}, to high rates of N_2 production (i.e. net denitrification)

of 295.8 nmol g wet weight^{-1} hr^{-1}; the hypothesized relationships to N and T were not observed within this experiment. Therefore, it appears that though *M. edulis* does nearly always show net N_2 production, we have yet to see a strong, consistent response to warming or N additions. However, this study does generally support N removal via denitrification by *M. edulis* at low nutrient ($\leq$ 25µM) conditions (**Figure 23**).

It is important to note that this experiment took place from January– March 2017, with experimental organisms collected on December 29, 2016. A study which examined the microbial communities of *C. virginica* found that season (winter) negatively impacted the structure and richness within the organism, with temperature being the driving factor (Pierce et al. 2016). Based on these findings, the gut microbiome of experimental *M. edulis* potentially could have been low during the time of year which the experiment took place. *M. edulis* facilitates N cycling processes, but the nitrogen transforming microbes, ingested by the organism, are what actually carry out the conversion (Wahl et al. 2012; Steif et al. 2013; Mouton et al. 2016). Therefore, it is possible that the rates measured in this study many not fully represent denitrification potential of *M. edulis* due to a less abundant, less active and/or less sensitive microbial gut community.

Despite the lack of strong response of M. edulis-associated denitrification to N and T, this study provided a step to fill in the gap in knowledge by quantifying denitrification rates associated with *M. edulis* in and of themselves. Most, if not all, previous studies which quantify N removal rates of *M. edulis* focus either on: the assimilation of nutrients within the organism and thereby removal once harvested (Edebo et al. 2000; Lindahal et al. 2005), or denitrification within the sediment

underlying mussel beds (Kaspar et al. 1985; Gilbert et al. 1997, Christenson et al. 2003; Stadmark and Conely 2011). Kaspar et al. 1985 measured *in situ* denitrification rates of sediments underlying a mussel farm (via an acetylene block technique) and found that mussel presence can potentially enhance sedimentary denitrification rates as much as 5.22 mmol m^{-2} day^{-1}, when compared to a reference site (Kaspar et al. 1985). When the rates measured within this study are converted to similar units and averaged across all treatments and incubation times, the final value is 28.03 +/- 23.93 mmol m^{-2} day-^{1}. While this value shows high variability, it does indicate that *M. edulis* may have a larger, direct role in N removal than previously thought. Nonetheless, *M. edulis* plays a strong ecological engineering role in many coastal habitats (Jones et al. 1994), and therefore increasing substrate area for denitrification to occur, as well their ability to filtrate and retain nutrients from the water column may be the primary means by which they affect N removal.

7.3: Sensitivity of *M. edulis* N_2O production to N addition and surprising response to warming

The effect of N level followed the initial hypothesis, with higher N concentrations (µM) producing higher N_2O emissions (Tukey's HSD: 25A, 17.5AB, 10AB, 5B) (**Figure 24**). The gradient of N used for this experiment is small relative to actual DIN levels within Point Judith Pond (Moran et al. 2014), and Narragansett Bay as a whole (Oviatt et al. 2002; 2017), indicating that production rates within a natural system may be much larger in magnitude. While denitrification (N removal) potential

of *M. edulis* is orders of magnitude higher than N_2O production across all experimental treatments (**Figure 23 & 24**), this negative effect of N level on greenhouse gas production should be considered as Rhode Island expands its efforts of blue mussel cultivation.

Overall, temperature and N level both had a significant impact on *M. edulis'* N_2O production rates (p=0.027; 0.030, respectively) (**Appendix 22**), but they did not display the predicted synergism. Temperature had the opposite effect than predicted, with cooler 18°C treatments producing more of the greenhouse gas than the higher, 21°C treatments (**Figure 24**). The negative effect of increased temperature (21°C) on N_2O production may be reassuring given anticipated warming trends (Mora et al. 2013). It is plausible that the increased temperature (21°C treatments) induced stress on the organism to a point where functional responses, such as filtering were compromised (Guo et al. 2015). This may explain the unanticipated findings of N_2O production in the cooler treatments.

A number of water quality parameters, besides temperature, may be influencing these findings. For example, during Incubation 2, the 18°C / 25µM treatment tanks exhibited the highest average N_2O production rate and was significantly higher than the 21°C / 25µM treatment (**Figure 24**), with the final pH values of Incubation 2 being consistently more acidic within the cooler (18°C) tanks (**Table 18**). The opposite relationship of ending pH values and temperature treatments existed for Incubation 1 (**Table 18**) where N2O production rates were not significantly different. Decreased pH is a well-known driver of increased N_2O production within soils (Tate 1995; Cuhel et al. 2010), and potentially can explain why the lower temperature treatments, particularly

the 25µM enrichment, resulted in higher N_2O production when compared to its warmer counterpart (**Figure 24**). Additionally, low DO concentrations can result in higher N_2O via a disruption in the denitrification process (Robertson et al. 1995; Baumann et al. 1996) and during Incubation 2, the 18°C / 25µM treatment was considerably less oxygenated than the 21°C / 25µM treatment (**Table 18**). It is counter intuitive that the cooler treatment would display lower pH and DO values, as increased temperature should theoretically increase respiration rates and therefore decrease both DO and pH. However, if the warmer temperature did compromise the health status of the organisms to a point where filtering abilities were reduced, the water quality within the cooler treatments would make sense, which presumably contained healthier and fully functional organisms.

With *M. edulis* aquaculture practices being a relatively new endeavor within the state of Rhode Island (Beutel 2018), the data provided in this document, as well as past studies, are important considerations in future spatial planning of farmed mussel beds. Future studies that would like to expand on this work, should attempt to quantify a larger array of N-cycling processes, such as nutrient assimilation and coupled nitrification-denitrification within the organism, rates of organic matter deposition from organism to sediment, and denitrification and dissimilatory nitrate reduction to ammonium within the underlying sediments across a gradient of environmental conditions. Overall, despite an unexpected trend with temperature, this data adds to the growing body of work which highlights filtering feed bivalves as potentially overlooked emitters of N_2O (Stief et al. 2009 & 2010; Heisterkamp et al. 2010 & 2013; Svennningsen et al. 2012; Garate 2016),

and that there are many complex factors that impact *M. edulis'* N cycling rates and capabilities.

CONCLUSION

Bivalves increase benthic pelagic coupling processes and therefore are active participants in the marine nitrogen cycle (Newell 2004). There are many pathways in which organisms such as *C. virginica* and *M. edulis* many remove, recycle, or regenerate reactive nutrients within their habitat (Kellogg et al. 2014). However, variables based on location, health status and environmental stress have made it difficult to determine rates associated with these processes. This study attempted to address these unknowns, including the impact of a combination of environmental stressors (N and T) on filter feeding bivalves, and how those factors would interact to facilitate N removal (denitrification) and greenhouse gas production (N_2O). Additionally, we attempted to survey the health status of organisms under a gradient of N levels and contrasting temperatures in attempt to reveal whether a connection existed with bivalve biogeochemical function.

To place the nitrogen transformation rates (denitrification and N_2O production) measured within this study into context, the rates were compared to a number of coastal habitats and invertebrates. Much variation existed between the studies from which denitrification rates were compared (Kellogg et al. 2014; Humphries et al. 2016; Caffrey et al. 2016) but were generally were in the same order of magnitude for both *C. virginica* and *M. edulis* (**Table 22**). In the majority of experimental conditions both organisms typically support net denitrification, with *M. edulis* appearing to be an efficient net N_2 producer at low nutrient conditions. However, denitrification rates did not follow

simple, linear patterns that were expected with increasing N level and/or increased temperature, further highlighting that the rates associated with process are dependent on a suite of interacting, environmental factors in addition to common stressors (N & T) within coastal environments.

Nitrous oxide production of both *C. virginica* and *M. edulis* fell with a reasonable range with many marine invertebrates studied by Heisterkamp et al. 2010 (Heisterkamp et al. 2010) (**Table 23**) but are drastically minor when related to coastal sediments in different environments (Murray et al. 2015, and references therein). To compare to global estuarine environments, the highest measured N_2O production rates from each species (1527.76 and 1.08 nmol N_2O m^{-2} hr^{-1} for oysters (Experiment 2,Year 2) and mussels, respectively) were upscaled to the total area of current (oyster) aquacultured waters within Rhode Island (~275 acres, Beutel 2018) and revealed that N_2O emissions from bivalves within the state is minuscule in comparison (6.56E-7 and 4.66E-10 TG $N2O^{-1}$ $yr^{-1,}$ oysters, mussels respectively). Based on the rates across different N and T treatments examined in this study, this showed that Rhode Island aquacultured bivalves are not likely to produce feedback on climate via N_2O production, except under high N loads. The magnitude of this will also depend on species, as well as exposure to temperature.

It is noteworthy that N_2O production rates were similar between *C. virginica* and *M. edulis* within this study, despite *M. edulis* experiencing a lower gradient of N and a less severe warming scenario (**Figure 11, 19, 24**). Overall, N level is the stronger driver of N_2O production for both *C. virginica* (**Figure 11**), and *M. edulis* (**Figure 24**). Temperature is not consistently important for *C. virginica* N_2O production, based on the

positive relationship exhibited in Experiment 2 (**Figure 19**), yet largely lacking correlation within Experiment 1 (**Figure 11**). *M. edulis* N_2O production exhibited an opposite relationship with temperature than expected (**Figure 24**), which may be tied to compromised health in the warming scenarios.

In this manuscript, several prominent conclusions emerge: 1) rates of N removal largely surpass rates of greenhouse gas production for both bivalve species (**Figures 10, 11, 23, 24**), indicating that the benefits of the recent increase in oyster and mussel production within Rhode Island outweigh the costs associated with it under the range of conditions we tested; 2) *M. edulis* appears to be a less efficient facilitator of denitrification, when compared to *C. virginica*, as a fraction of N inputs produce nearly identical N_2O emissions (**Figures 11, 24**), suggesting the process of denitrification is more likely to be carried to completion by microbes associated with *C. virginica*; 3) the combination of high N and high T, lowers (**Figure 17**) or induces a switch from net N_2 production to N_2 consumption (**Figures 10, 18**) for *C. virginica*. This was not observed for *M. edulis* (**Figure 23**), possibly due to the reduced stressors in the *M. edulis* experiments. However further investigation of this finding is warranted in scenarios of increased levels of nitrogen and temperature, as results are based upon a fraction of the N and T which *C. virginica* experienced within this study; and finally 4) this study focused on the macro-organisms which facilitate N cycling processes, it would be greatly beneficial for future studies to include analysis of the microbes who actually perform the process (Wahl et al. 2012; Stief et al. 2013; Mouton et al. 2016) to determine how environmental stress impacts the abundance and assemblage of the organisms' gut microbiome, therefore having a more complete picture of these complex processes.

TABLES & FIGURES

Table 1: Results from a Two-Way ANOVA testing the effect of N-level and time of exposure on the percentage of mortality of *C. virginica* at each temperature (Experiment 1)

	% Mortality *C. virginica* (Experiment 1)			
	Testing the effect of N level and time			
	18°C		24°C	
	$F_{48,71}$-value	p- value	$F_{48,71}$ -value	p- value
N Level	2.01	0.125	9.14	**<0.0001**
Time	0.75	0.589	6.33	**0.0001**
N Level X Time	0.14	0.999	1.04	0.434

Table 2: Average water parameters (based on weekly sampling) over the course of the 3 month *C. virginica* laboratory experiment (Experiment 1); levels not connected by the same letter are significantly different, based on Tukey's HSD post-hoc tests.

Treatments:			Average (+/- SE) water quality parameters				
Temp (C)	N Level (uM)		**Temperature (C)**	**DO (mg/L)**	**pH**	**Salinity**	**NH_4^+ (µMl)**
18	20		**17.9** (0.08) B	**7.62** (0.25) A	**7.66** (0.02) A	**31** (0.00)	**76.02** (3.80) A
18	40		**17.9** (0.09) B	**7.64** (0.33) A	**7.68** (0.03) A	**31** (0.00)	**102.45** (3.32) A
18	70		**17.9** (0.08) B	**6.91** (0.12) B	**7.55** (0.01) B	**31** (0.00)	**103.39** (7.36) A
18	100		**17.9** (0.05) B	**7.08** (0.11) AB	**7.54** (0.02) B	**31** (0.00)	**105.37** (13.21) A
24	20		**22.9** (0.09) A	**7.41** (0.10) AB	**7.69** (0.02) A	**31** (0.00)	**82.38** (1.46) A
24	40		**23.0** (0.12) A	**7.28** (0.11) AB	**7.67** (0.02) A	**31** (0.00)	**93.33** (12.10) A
24	70		**22.9** (0.05) A	**7.61** (0.10) A	**7.74** (0.02) A	**31** (0.00)	**107.56** (4.87) A
24	100		**22.9** (0.04) A	**7.35** (0.18) AB	**7.65** (0.02) A	**31** (0.00)	**109.85** (12.43) A
Target and Observed: Average NH_4^+ (+/-SE)				**20 µM**	**40 µM**	**70 µM**	**100 µM**
				64.91 (7.12)	**93.14** (7.99)	**113.81** (8.53)	**137.50** (10.70)
Tukey's HSD (nutrient reservoirs)				C	BC	AB	A

Table 3: Two-way ANOVA results, testing the effects of, N-Level and time on weekly water quality parameters at each temperature treatment of *C. virginica* experimental tanks over the course of 3-month experiment (Experiment 1)

Experiment 1, Weekly Water Quality Measurements												
	18°C						24°C					
	DO		**pH**		**NH_4^+**		**DO**		**pH**		**NH_4^+**	
	$F_{40, 59}$	p-value	$F_{48, 71}$	p-value	$F_{24, 35}$	p-value	$F_{40, 59}$	p-value	$F_{48, 71}$	p-value	$F_{24, 35}$	p-value
N Level	5.07	**0.004**	15.91	**<0.001**	2.296	0.103	1.48	0.234	5.591	**0.002**	1.992	0.142
Time (Days)	2.32	0.073	13.75	**<0.001**	15.689	**<0.001**	1.46	0.232	12.190	**<0.001**	19.26	**<0.001**
N Level X Time	0.379	0.963	1.01	0.464	1.57	0.199	0.679	0.760	0.966	0.503	0.435	0.848

Table 4: Average (+/- SE) pre-, post-, and overall change in water quality parameters over the course of each *C. virginica* incubation experiment

Water quality parameters over course of incubation (Average +/- SE)										
Treatments:		Incubation 1 (Day 5)								
		Dissolved oxygen (mg/L)			**pH**			**NH_4^+ (µM)**		
Temp (C)	N Level (uM)	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change
18	20	**6.50** (0.21)	**5.17** (0.30)	**-1.33** (0.11)	**7.42** (0.05)	**7.19** (0.05)	**-0.22** (0.02)	**52.32** (8.28)	**53.69** (6.11)	**1.37** (8.01)
18	40	**5.89** (0.51)	**4.03** (0.74)	**-1.86** (0.28)	**7.42** (0.04)	**7.18** (0.02)	**-0.24** (0.04)	**95.48** (10.68)	**88.95** (6.59)	**-6.53** (4.19)
18	70	**5.20** (0.09)	**3.47** (0.29)	**-1.73** (0.20)	**7.08** (0.03)	**7.13** (0.01)	**-0.19** (0.02)	**120.96** (2.19)	**140.33** (16.94)	**19.37** (15.17)
18	100	**4.90** (0.10)	**3.73** (0.34)	**-1.17** (0.33)	**7.29** (0.03)	**7.11** (0.01)	**-0.18** (0.03)	**147.70** (10.20)	**142.65** (4.81)	**-5.05** (13.48)
24	20	**5.62** (0.10)	**3.64** (0.21)	**-1.98** (0.36	**7.37** (0.01)	**7.07** (0.04)	**-0.30** (0.02)	**73.80** (3.19)	**65.37** (2.19)	-8.42 (2.23)
24	40	**4.58** (0.04)	**2.09** (0.27)	**-2.49** (0.30)	**7.30** (0.01)	**7.04** (0.01)	**-0.26** (0.02)	**114.43** (10.68)	**106.75** (13.80)	**-7.68** (3.20)
24	70	**5.09** (0.33)	**3.09** (0.29)	**-1.99** (0.12)	**7.35** (0.03)	**7.09** (0.02)	**-0.25** (0.02)	**137.59** (10.59)	**109.48** (8.43)	**-28.11** (2.38)
24	100	**4.21** (0.50)	**1.96** (0.43)	**-2.25** (0.16)	**7.24** (0.04)	**7.06** (0.01)	**0.18** (0.04)	**154.12** (9.80)	**163.59** (10.37)	**9.47** (16.19)
		Incubation 2 (Day 53)								

Treatments:		Dissolved oxygen (mg/L)			pH			NH_4^+ (µM)		
Temp (C)	N Level (uM)	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change
18	20	**5.87** (0.31)	**4.75** (0.10)	**-1.11** (0.38)	**7.60** (0.03)	**7.44** (0.05)	**-0.16** (0.02)	**45.07** (3.23)	**45.07** (0.33)	**0.00** (3.20)
18	40	**5.88** (0.29)	**5.11** (0.34)	**-0.76** (0.15)	**7.59** (0.02)	**7.50** (0.04)	**-0.09** (0.02)	**59.35** (5.26)	**46.96** (5.23)	**-12.39** (6.26)
18	70	**6.07** (0.43)	**5.33** (0.43)	**-0.74** (0.04)	**7.08** (0.01)	**7.49** (0.02)	**-0.06** (0.02)	**62.54** (1.21)	**70.58** (6.47)	**8.04** (6.45)
18	100	**5.63** (0.31)	**5.47** (0.43)	**-0.17** (0.17)	**7.52** (0.02)	**7.47** (0.04)	**-0.05** (0.02)	**98.48** (9.80)	**93.41** (2.29)	**-5.07** (12.03)
24	20	**5.84** (0.6)	**5.18** (0.23)	**-0.66** (0.50)	**7.57** (0.05)	**7.47** (0.01)	**-0.10** (0.04)	**36.52** (5.04)	**45.72** (7.01)	**9.20** (4.15)
24	40	**4.73** (0.09)	**4.02** (0.12)	**-0.71** (0.21)	**7.50** (0.01)	**7.42** (0.01)	**-0.08** (0.01)	**45.07** (2.48)	**56.09** (2.59)	**11.01** (4.41)
24	70	**5.23** (0.14)	**4.42** (0.09)	**-0.81** (0.16)	**7.49** (0.01)	**7.41** (0.02)	**-0.08** (0.01)	**69.57** (9.44)	**54.93** (4.46)	**-14.64** (6.74)
24	100	**4.54** (0.65)	**3.88** (0.14)	**-0.66** (0.51)	**7.47** (0.02)	**7.39** (0.01)	**-0.08** (0.03)	**106.45** (10.03)	**102.68** (14.12)	**-3.77** (7.02)

Treatments:		Incubation 3 (Day 89)								
		Dissolved oxygen (mg/L)			pH			NH_4^+ (µM)		
Temp (C)	N Level (uM)	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change

18	20		**6.95** (0.37)	**5.23** (0.35)	**-1.72** (0.28)		**7.80** (0.01)	**7.49** (0.07)	**-0.30** (0.07)		**54.57** (6.42)	**50.58** (2.56)	**-3.99** (4.49)
18	40		**6.11** (0.64)	**5.34** (0.65)	**-0.76** (0.22)		**7.70** (0.02)	**7.54** (0.06)	**-0.15** (0.04)		**59.28** (5.75)	**47.32** (4.61)	**-11.96** (5.71
18	70		**4.85** (0.13)	**4.31** (0.24)	**-0.54** (0.11)		**7.08** (0.01)	**7.51** (0.04)	**-0.09** (0.03)		**41.59** (4.27)	**50.00** (1.88)	**8.41** (4.74)
18	100		**3.87** (0.34)	**3.41** (0.39)	**-0.46** (0.10)		**7.49** (0.02)	**7.45** (0.02)	**-0.04** (0.03)		**54.28** (4.88)	**52.46** (6.57)	**-1.81** (2.41)
24	20		**6.00** (0.29)	**5.15** (0.40)	**-0.85** (0.13)		**7.60** (0.02)	**7.49** (0.04)	**-0.12** (0.02)		**32.03** (2.96)	**45.36** (1.54)	**13.33** (3.28)
24	40		**5.31** (0.22)	**4.50** (0.13)	**-0.80** (0.10)		**7.57** (0.01)	**7.47** (0.01)	**-0.10** (0.03)		**45.94** (3.92)	**42.10** (1.54)	**-3.84** (5.64)
24	70		**5.67** (0.31)	**4.57** (0.47)	**-1.11** (0.16)		**7.56** (0.04)	**7.47** (0.04)	**-0.09** (0.01)		**66.30** (5.12)	**52.46** (2.05)	**-13.84** (7.08)
24	100		**4.95** (0.38)	**3.53** (0.18)	**-1.43** (0.24)		**7.50** (0.02)	**7.39** (0.01)	**-0.11** (0.02)		**64.78** (3.79)	**71.30** (6.12)	**6.52** (3.39)

Table 5: Results from Two-Way ANOVAS, testing the effect of temperature and N-level on the *change* of water quality parameters over the course of each *C. virginica* incubation (Experiment 1)

Change in water parameters over the course of each *C. virginica* incubation						
Testing the effect of Temperature and N-Level						
	Incubation 1 (Day 5)					
	Change in DO (mg/L)		Change in pH		Change in NH_4^+ (µM)	
	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values
Temp	9.10	**0.008**	2.70	0.120	1.65	0.217
N-Level	1.15	0.360	2.31	0.115	0.21	0.888
Temp * N-Level	0.58	0.632	0.58	0.637	2.38	0.108
	Incubation 2 (Day 53)					
	Change in DO (mg/L)		Change in pH		Change in NH_4^+ (µM)	
	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values
Temp	0.003	0.954	4.93	**0.041**	0.23	0.638
N-Level	0.56	0.649	0.35	0.788	0.47	0.708
Temp * N-Level	0.53	0.671	1.25	0.326	2.70	0.080
	Incubation 3 (Day 89)					
	Change in DO (mg/L)		Change in pH		Change in NH_4^+ (µM)	
	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values
Temp	1.27	0.277	2.05	0.172	0.48	0.499
N-Level	2.17	0.132	4.00	**0.0272**	1.81	0.186
Temp * N-Level	6.65	**0.004**	3.22	0.051	4.32	**0.021**

Table 6: Results from a Two-Way ANOVA, analyzing the effect of temperature and N level on rates of denitrification for *C. virginica* incubation experiments (Day 5, 53). Day 89 was excluded from analysis due to a technical issue.

C. virginica denitrification rates (Experiment 1)								
Testing the effect of Temperature and N level								
		Incubation 1 (Day 5)			**Incubation 2 (Day 53)**		**Incubation 3 (Day 89)**	
		$F_{16,23}$-*value*	*p-value*		$F_{16,23}$-*value*	*p-value*	$F_{16,23}$-*value*	*p-value*
Temp		4.46	0.051		0.0003	0.988		
N Level		0.22	0.883		6.68	**0.004**	**X**	**X**
Temp * N Level		1.01	0.413		5.21	**0.011**		

Table 7: Results from a Two-Way ANOVA, analyzing the effect of temperature and nitrogen treatment on rates of nitrous oxide production for each *C. virginica* incubation experiment (Day 5, 53, 89).

C. virginica N_2O production rates (Experiment 1)								
Testing the effect of Temperature and N level								
		Incubation 1 (Day 5)			**Incubation 2 (Day 53)**		**Incubation 3 (Day 89)**	
		$F_{16,23}$-*value*	*p-value*		$F_{16,23}$-*value*	*p-value*	$F_{16,23}$-*value*	*p-value*
Temp		7.22	**0.016**		1.70	0.210	0.64	0.437
N Level		1.14	0.365		4.10	**0.025**	2.44	0.102
Temp * N Level		1.52	0.249		0.34	0.799	2.36	0.110

Table 8: Results from a Two-Way ANOVA, analyzing the effect of location and N level on *C. virginica* growth rates, biomass gains, and % mortality for Experiment 2, analyzed each year (2016 & 2017)

Experiment 2, Growth and Mortality												
	2016						2017					
	Growth Rates		**Biomass Gains**		**% Morality**		**Growth Rates**		**Biomass Gains**		**% Morality**	
	$F_{8,11}$	p-value	$F_{8,11}$	p-value	$F_{8,11}$	p-value	$F_{8,11}$	p-value	$F_{8,11}$	p-value	$F_{8,11}$	p-value
Location	-3.38	**0.009**	-3.12	**0.014**	-0.09	0.934	2.74	**0.025**	3.07	**0.015**	-0.70	0.503
N Level	0.54	0.603	-1.84	0.1026	-0.26	0.800	-0.92	0.385	-1.40	0.199	-2.16	0.062
Location X N Level	0.60	0.565	-0.42	0.683	1.30	0.233	-0.03	0.979	0.52	0.616	0.44	0.669

Table 9: Average water quality parameters of 2016 field season, as well as range in conditions (Experiment 2, 2016)

Northern Location					
August 22 – September 1, 2017					
	Temp (°C)	Salinity (ppt)	pH	Chl-a (µg/L)	DO (mg/L)
Average (+/- SE)	25.79 (0.03)	29.71 (0.05)	7.88 (0.005)	12.33 (0.18)	6.69 (0.05)
Min	23.65	22.67	6.63	2.00	3.19
Max	27.77	30.38	8.21	32.65	10.78
Southern Location					
September 2 – 12, 2017					
	Temp (°C)	Salinity (ppt)	pH	Chl-a (µg/L)	DO (mg/L)
Average (+/- SE)	22.18 (0.05)	30.54 (0.02)	8.00 (0.003)	3.85 (0.05)	7.25 (0.04)
Min	19.17	23.53	7.65	0.70	4.7
Max	25.18	31.02	8.26	16.83	10.81
Northern Location					
September 12 – 22, 2017					
	Temp (°C)	Salinity (ppt)	pH	Chl-a (µg/L)	DO (mg/L)
Average (+/- SE)	23.03 (0.03)	29.65 (0.02)	8.20 (0.009)	39.95 (1.27)	6.71 (0.07)
Min	20.92	25.57	7.57	5.31	1.49
Max	25.14	30.33	8.77	478.72	11.14
Southern Location					
September 27 – October 13, 2017					
	Temp (°C)	Salinity (ppt)	pH	Chl-a (µg/L)	DO (mg/L)
Average (+/- SE)	17.35 (0.03)	30.60 (0.02)	8.03 (0.002)	11.10 (1.46)	8.12 (0.02)
Min	13.34	24.46	7.82	0.24	5.64

Max	20.27	31.27	8.25	54.28	10.96

Table 10: Results from a Two-Way ANOVA which analyzed the effect of N level and tidal height on the environmental measurements at each location, from a "one day profile" sampling event in the 2016 field season (Experiment 2, Year 1)

Experiment 2 (2016) All Day Profile												
	Northern Location											
	Temp (°C)		**DO (mg/L)**		**Salinity**		**pH**		**Chl-a (ug/L)**		**NH_4^+**	
	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value
N Level	0.05	0.964	-0.06	0.951	1.45	0.162	-0.08	0.933	-1.44	0.164	-0.40	0.693
Tidal Height	-0.59	0.561	-2.87	**0.009**	0.12	0.907	-1.34	0.194	-0.24	0.811	-1.63	0.119
N Level X Tidal Height	-0.36	0.724	0.61	0.546	0.63	0.537	-0.42	0.679	0.94	0.356	-0.34	0.739
	Southern Location											
	Temp (°C)		**DO (mg/L)**		**Salinity**		**pH**		**Chl-a (ug/L)**		**NH_4^+**	
	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value	$F_{20,23}$	p-value
N Level	0.55	0.585	0.32	0.750	0.80	0.435	1.00	0.328	1.27	0.220	-1.12	0.277
Tidal Height	-3.07	**0.006**	-2.12	**0.046**	-1.90	0.071	-2.36	**0.028**	-0.80	0.434	1.32	0.201
N Level X Tidal Height	0.43	0.669	-0.13	0.899	0.77	0.448	0.41	0.685	1.03	0.316	0.33	0.741

Table 11: Results from 2017's field season (Experiment 2), highlighting the significant differences between location, month, and tidal height on environmental parameters

Experiment 2 (2017) Environmental Parameters										
	Northern Location									
	Temp (°C)		**DO (mg/L)**		**Salinity**		**pH**		**Chl-a (ug/L)**	
	$F_{824,829}$	p-value	$F_{824,829}$	p-value	$F_{824,829}$	p-value	$F_{824,829}$	p-value	$F_{824,829}$	p-value
Month	71.308	**<0.0001**	62.042	**<0.0001**	815.328	**<0.0001**	25.653	**<0.0001**	237.540	**<0.0001**
Tidal Height	2.649	0.104	4.417	**0.035**	15.231	**0.0001**	0.445	0.504	7.476	**0.006**
Month X Tidal Height	0.670	0.511	8.150	**0.0003**	15.501	**0.002**	0.303	0.738	12.201	**<0.0001**
	Southern Location									
	Temp (°C)		**DO (mg/L)**		**Salinity**		**pH**		**Chl-a (ug/L)**	
	$F_{824,829}$	p-value	$F_{824,829}$	p-value	$F_{824,829}$	p-value	$F_{824,829}$	p-value	$F_{824,829}$	p-value
Month	53.155	**<0.0001**	13.598	**<0.0001**	821.121	**<0.0001**	231.698	**<0.0001**	3.875	**0.021**
Tidal Height	0.094	0.759	0.060	0.806	0.049	0.823	1.308	0.253	0.488	0.485
Month X Tidal Height	14.187	**<0.0001**	0.737	0.478	4.256	**0.014**	3.024	**0.049**	0.025	0.974

Table 12: 2017 Field DIN averaged values for location, treatment, month, and tidal height; Average range of each location overall listed along bottom

	Experiment 2: 2017 Field DIN (Average +/- SE)								
	June			July			August		
Concentration (µM):	**NH_4^+**	**NO_3^-**	**NO_2^-**	**NH_4^+**	**NO_3^-**	**NO_2^-**	**NH_4^+**	**NO_3^-**	**NO_2^-**
Northern Ambient Low	**22.58** (1.81)	**8.49** (3.52)	**0.04** (0.04)	**21.02** (2.51)	**0.24** (0.18)	**0.08** (0.04)	**23.06** (3.57)	**0.38** (0.15)	**0.10** (0.01)
Northern Ambient High	**22.89** (2.88)	**4.81** (1.97)	**0.03** (0.01)	**28.13** (2.18)	**0.20** (0.07)	**0.09** (0.04)	**51.60** (5.33)	**0.94** (0.17)	**0.08** (0.04)
Northern Enriched Low	**23.24** (1.23)	**44.4** (18.13)	**0.15** (0.08)	**27.96** (1.25)	**0.09** (0.02)	**0.07** (0.02)	**24.00** (1.51)	**0.77** (0.57)	**0.09** (0.04)
Northern Enriched High	**25.07** (3.11)	**19.33** (12.13)	**0.05** (0.01)	**23.02** (2.98)	**2.63** (1.16)	**0.10** (0.03)	**38.44** (0.49)	**1.10** (0.46)	**0.02** (0.02)
Southern Ambient Low	**31.51** (8.00)	**10.00** (4.14)	**0.09** (0.01)	**25.33** (4.87)	**0.48** (0.08)	**0.08** (0.04)	**25.29** (3.51)	**0.31** (0.07)	0.10 (0.04)
Southern Ambient High	**20.58** (1.630	**11.52** (9.33)	**0.04** (0.04)	**23.42** (2.83)	**0.56** (0.05)	**0.09** (0.04)	**19.64** (3.42)	**0.33** (0.08)	**0.09** (0.01)
Southern Enriched Low	**22.67** (2.57)	**9.06** (3.35)	**0.09** (0.02)	**19.33** (1.86)	**0.26** (0.16)	**0.06** (0.04)	**27.64** (3.42)	**0.44** (0.20)	**0.05** (0.03)
Southern Enriched High	**30.18** (2.83)	**0.39** (0.18)	**0.01** (0.01)	**35.15** (5.48)	**0.37** (0.03)	**0.12** (0.01)	**29.73** (5.83)	**0.07** (0.05)	**0.10** (0.04)

	NH_4^+:	**NO_3^-:**	**NO_2^-:**
Northern Range (Overall):	21.02 - 51.60 µM	0.09 - 44.4 µM	0.04 - 0.15 µM
Southern Range (Overall):	19.33 - 35.15 µM	0.07 - 11.52 µM	0.01 - 0.12 µM

Table 13: Average (+/- standard error) parameters between two field seasons (Experiment 2)

2016	**Average (+/-SE) Field Conditions**	
	Northern Location	**Southern Location**
Temperature (°C)	**24.4** (0.04)	**19.2** (0.05)
Salinity (ppt)	**29.7** (0.02)	**30.6** (0.01)
pH	**8.04** (0.001)	**8.01** (0.002)
Chla (ug/L)	**25.9** (0.07)	**16.7** (0.92)
DO (mg/L)	**7.44** (0.06)	**7.80** (0.02)
NH_4^+ (µM)	**19.74** (1.31)	**24.02** (1.10)
2017		
	Northern Location	**Southern Location**
Temperature (°C)	**24.21** (0.33)	**21.41** (0.50)
Salinity (ppt)	**29.83** (0.75)	**31.91** (0.11)
pH	**6.91** (0.00)	**X**
Chla (RFU)	**5.16** (0.53)	**1.17** (0.27)
DO (mg/L)	**6.12** (0.45)	**7.69** (0.19)
NH_4^+ (µM)	**27.73** (1.13)	**25.87** (1.45)

Table 14: Results from a Two-Way ANOVA analyzing the effects of incubation temperature and field treatment on rates of Experiment 2, Year 2 (2017)'s denitrification and nitrous oxide production at each field site (Northern & Southern Location)

Experiment 2, 2017 Incubation								
	Northern Location				Southern Location			
	Denitrification Rates		**N_2O Production**		**Denitrification Rates**		**N_2O Production**	
	$F_{8,11}$	p-value	$F_{8,11}$	p-value	$F_{8,11}$	p-value	$F_{8,11}$	p-value
Incubation Temp	-0.45	0.665	-2.81	**0.022**	2.20	0.058	-0.93	0.377
Field Treatment	1.10	0.304	0.36	0.729	0.04	0.970	0.74	0.480
Inc Temp X Field Treatment	-1.38	0.205	-0.30	0.775	-2.87	**0.021**	-0.34	0.745

Table 15: Results from a Two-Way ANOVA testing the effect of N-level and time of exposure on the percentage of mortality of *M. edulis* at each temperature (Experiment 1)

	% Mortality *M. edulis*			
	Testing the effect of N level and time			
	18°C		24°C	
	$F_{40,59}$-value	p- value	$F_{40,59}$-value	p- value
N Level	4.06	**0.001**	6.77	**0.0008**
Time	1.83	0.142	1.10	0.372
N Level X Time	0.69	0.723	0.59	0.840

Table 16: Averaged water quality parameters over the course of *M. edulis'* 3 month laboratory experiment

Treatments:			Average (+/- SE) water quality parameters overall				
Temp (C)	N Level (uM)		**Temperature (°C)**	**DO (mg/L)**	**pH**	**Salinity**	**NH_4^+ (µM)**
18	5		**17.5** (0.04) B	**7.30** (0.05) BC	**7.76** (0.16) A	**34.4** (0.00)	**52.46** (4.24) A
18	10		**17.5** (0.05) B	**7.59** (0.11) AB	**7.57** (0.16) A	**34.4** (0.00)	**55.00** (3.37) A
18	17.5		**17.5** (0.06) B	**7.03** (0.12) C	**7.49** (0.11) A	**34.4** (0.00)	**54.85** (1.64) A
18	25		**17.5** (0.03) B	**7.46** (0.21) ABC	**7.57** (0.16) A	**34.4** (0.00)	**64.23** (6.12) A
21	5		**20.5** (0.15) A	**7.82** (0.04) A	**7.74** (0.15) A	**34.4** (0.00)	**53.64** (3.24) A
21	10		**20.4** (0.15) A	**7.45** (0.11) ABC	**7.79** (0.16) A	**34.4** (0.00)	**58.25** (2.65) A
21	17.5		**20.5** (0.15) A	**7.13** (0.11) BC	**7.74** (0.11) A	**34.4** (0.00)	**59.74** (0.53) A
21	25		**20.4** (0.18) A	**7.18** (0.22) BC	**7.69** (0.10) A	**34.4** (0.00)	**57.47** (3.33) A

Table 17: Two-way ANOVA results, testing the effects of, N-Level and time on water quality parameters at each temperature treatment of *M. edulis* experimental tanks over the course of 3-month experiment

M. edulis, Weekly Water Quality Measurements												
	18°C						21°C					
	DO		**pH**		**NH_4^+**		**DO**		**pH**		**NH_4^+**	
	$F_{88, 131}$	p-value	$F_{56, 83}$	p-value	$F_{32,47}$	p-value	$F_{88,131}$	p-value	$F_{56, 83}$	p-value	$F_{32,47}$	p-value
N Level	4.72	**0.004**	0.46	0.711	1.80	0.167	16.71	**<0.0001**	0.06	0.982	0.77	0.518
Time (Days)	5.19	**<0.0001**	5.18	**0.0003**	1.80	0.166	13.46	**<0.0001**	1.87	0.102	10.64	**<0.0001**
N Level X Time	1.29	0.178	0.21	0.999	1.52	0.184	0.74	0.822	0.04	1.000	3.06	**0.009**

Table 18: Average (+/- SE) pre, post, and overall change in water quality parameters over the course of each *M. edulis* incubation experiment

Water quality parameters over course of incubation (Average +/- SE)											
Treatments:		Incubation 1 (Day 2)									
		Dissolved oxygen (mg/L)			**pH**			**NH_4^+ (µM)**			
Temp (C)	N Level (uM)	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change	
18	5	**10.49** (0.03)	**6.24** (0.09)	**-4.26** (0.12)	**8.03** (0.01)	**7.51** (0.02)	**-0.52** (0.02)	**26.88** (5.07)	**47.14** (12.84)	**20.25** (17.81)	
18	10	**10.53** (0.05)	**6.75** (0.15)	**-3.77** (0.20)	**8.02** (0.01)	**7.57** (0.01)	**-0.46** (0.02)	**61.80** (10.83)	**61.45** (7.02)	**-0.35** (17.75)	
18	17.5	**10.47** (0.11)	**6.44** (0.12)	**-4.03** (0.19)	**8.03** (0.01)	**7.54** (0.02)	**-0.49** (0.01)	**76.11** (11.44)	**70.53** (8.33)	**-5.59** (3.50)	
18	25	**10.65** (0.06)	**6.74** (0.08)	**3.90** (0.12)	**8.04** (0.00)	**7.58** (0.02)	**0.46** (0.02)	**78.21** (6.17)	**94.97** (8.33)	**16.76** (14.24)	
21	5	**10.53** (0.03)	**5.77** (0.35)	**-4.76** (0.37)	**8.03** (0.01)	**7.45** (0.03)	**-0.59** (0.03)	**30.73** (11.94)	**68.78** (14.46)	**38.06** (22.77)	
21	10	**10.59** (0.05)	**5.16** (0.37)	**-5.43** (0.42)	**8.04** (0.01)	**7.40** (0.05)	**-0.64** (0.06)	**47.14** (9.06)	**79.26** (1.51)	**32.12** (7.75)	
21	17.5	**10.51** (0.10)	**5.44** (0.15)	**5.07** **(**0.22)	**8.04** (0.01)	**7.44** (0.02)	**-0.60** (0.03)	**78.56** (12.69)	**79.61** (6.53)	**1.05** (11.16)	
21	25	**10.72** (0.06)	**5.65** (0.08)	**-5.07** **(**0.13)	**8.03** (0.00)	**7.42** (0.02)	**-0.62** (0.02)	**340.77** (31.17)	**486.71** (44.50)	**145.94** (72.90)	

Treatments:		Incubation 2 (Day 53)								
		Dissolved oxygen (mg/L)			**pH**			**NH_4^+ (µM)**		
Temp (C)	N Level (uM)	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change
18	5	**7.07** (0.40)	**4.64** (0.63)	**-2.43** (0.49)	**7.70** (0.10)	**7.53** (0.05)	**-0.18** (0.06)	**84.76** (13.51)	**68.16** (2.99)	**-16.61** (10.63)
18	10	**8.02** (0.13)	**5.03** (0.63)	**-2.99** (0.77)	**7.62** (0.11)	**7.53** (0.08)	**-0.09** (0.04)	**124.74** (18.24)	**97.48** (9.43)	**-27.26** (9.52)
18	17.5	**7.86** (0.68)	**5.24** (0.83)	**-2.62** (0.16)	**7.46** (0.01)	**7.46** (0.05)	**-0.00** (0.06)	**84.84** (4.67)	**101.04** (9.71)	**16.19** (7.60)
18	25	**6.14** (0.52)	**4.72** (0.52)	**-1.41** (0.26)	**7.59** (0.11)	**7.48** (0.06)	**-0.11** (0.05)	**76.17** (4.16)	**95.00** (11.31)	**18.84** (7.77)
21	5	**6.65** (0.37)	**5.32** (0.12)	**-1.33** (0.29)	**7.90** (0.02)	**7.63** (0.02)	**-0.27** (0.00)	**63.12** (9.39)	**78.65** (7.84)	**15.53** (17.22)
21	10	**6.50** (0.69)	**4.64** (0.55)	**-1.86** (0.22)	**7.82** (0.07)	**7.52** (0.09)	**-0.30** (0.02)	**76.25** (6.69)	**82.28** (3.33)	**6.03** (9.93)
21	17.5	**7.31** (0.27)	**4.99** (0.47)	**-2.33** (0.32)	**7.80** (0.10)	**7.60** (0.05)	**-0.20** (0.06)	**95.58** (6.68)	**91.12** (5.79)	**-4.46** (5.48)
21	25	**7.64** (0.25)	**4.72** (1.03)	**-2.92** (0.97)	**7.74** (0.14)	**7.57** (0.09)	**-0.17** (0.07)	**91.12** (5.79)	**94.10** (3.23)	**2.97** (3.56)
Treatments:		Incubation 3 (Day 105)								
		Dissolved oxygen (mg/L)			**pH**			**NH_4^+ (µM)**		
Temp (C)	N Level (uM)	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change	Pre-Incubation	Post-Incubation	Overall Change
18	5	**4.14** (0.93)	**3.24** (0.70)	**-0.91** (0.37)	**no data**			**51.20** (3.3)	**59.58** (3.71)	**8.38** (0.82)

18	10		**4.24** (0.66)	**4.54** (0.49)	**-0.30** (0.46)				**63.00** (1.01)	**63.22** (2.05)	**0.22** (1.37)
18	17.5		**5.63** (0.31)	**3.62** (0.22)	**-2.02** (0.16)				**66.33** (4.00)	**67.52** (1.12)	**1.19** (4.94)
18	25		**4.34** (1.30)	**4.30** (0.57)	**-0.04** (0.74)				**63.29** (2.15)	**65.59** (1.27)	**2.30** (3.41)
21	5		**4.81** (1.26)	**4.27** (0.99)	**-0.54** (0.29)		**no data**		**72.86** (8.61)	**79.62** (7.47)	**6.75** (4.39)
21	10		**5.51** (0.57)	**4.75** (0.42)	**-0.76** (0.16)				**55.20** (1.58)	**57.65** (1.89)	**2.45** (2.90)
21	17.5		**5.56** (0.42)	**4.83** (0.42)	**-0.74** (0.09)				**59.66** (1.52)	**60.10** (1.91)	**0.45** (4.86)
21	25		**5.34** (0.30)	**4.40** (0.42)	**-0.94** (0.12)				**59.43** (1.52)	**59.21** (3.26)	**-0.22** (4.78)

Table 19: Results from Two-Way ANOVAS, testing the effect of temperature and N-level on the *change* of water quality parameters over the course of each *M. edulis* incubation experiment

Change in water parameters over the course of each *M. edulis* incubation						
Testing the effect of Temperature and N-Level						
	Incubation 1 (Day 2)					
	Change in DO (mg/L)		Change in pH		Change in NH_4^+ (µM)	
	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values
Temp	26.51	**<0.0001**	29.24	**<0.0001**	3.36	0.085
N-Level	0.06	0.981	0.13	0.930	2.00	0.154
Temp X N-Level	1.25	0.324	1.23	0.332	1.22	0.334
	Incubation 2 (Day 53)					
	Change in DO (mg/L)		Change in pH		Change in NH_4^+ (µM)	
	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values
Temp	0.33	0.5741	10.58	**0.005**	0.73	0.404
N-Level	0.38	0.767	1.67	0.213	1.21	0.338
Temp X N-Level	1.97	0.160	0.86	0.481	3.05	0.059
	Incubation 3 (Day 105)					
	Change in DO (mg/L)		Change in pH		Change in NH_4^+ (µM)	
	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values	$F_{16,23}$-values	p-values
Temp	0.06	0.811	**no data.**		0.04	0.840
N-Level	2.42	0.104			1.01	0.416
Temp X N-Level	3.08	0.058			0.10	0.959

Table 20: Results from a Two-Way ANOVA, analyzing the effect of N level and time on rates of denitrification for both temperatures, during each *M. edulis* incubation experiment (Day 2, 53, 105).

M. edulis denitrification rates						
Testing the effect of Temperature and N level						
	Incubation 1 (Day 2)		Incubation 2 (Day 53)		Incubation 3 (Day 105)	
	$F_{16,23}$	p value	$F_{16,23}$	p value	$F_{16,23}$	p value
Temperature	0.07	0.789	0.245	0.626	1.53	0.230
N level	0.10	0.954	0.179	0.908	0.34	0.790
Temp X N Level	1.14	0.360	0.072	0.974	2.00	0.150

Table 21: Results from a Two-Way ANOVA, analyzing the effect of N level and time on rates of nitrous oxide production for both temperatures, during each *M. edulis* incubation experiment (Day 2, 53, 105).

M. edulis Nitrous Oxide Production Rates						
Testing the effect of Temperature and N level						
	Incubation 1 (Day 2)		Incubation 2 (Day 53)		Incubation 3 (Day 105)	
	$F_{16,23}$	p value	$F_{16,23}$	p value	$F_{16,23}$	p value
Temperature	0.07	0.786	3.48	0.080	2.00	0.176
N level	0.13	0.934	2.05	0.147	2.50	0.095
Temp X N Level	0.91	0.454	1.89	0.170	0.676	0.579

Table 22: Comparison of *C. virginica* and *M. edulis* denitrification rates measured within in this study to past literature

Denitrification rates		
Environment / species	**Rate N_2 / m^2 / hr**	**Reference**
restored oyster reef	500	Kellogg et al 2013
restored oyster reef	581.9 (164)	Humphries et al 2016
aquaculture operation	346.1 (168.6)	Humphries et al 2016
cultch	60.9 (44.3)	Humphries et al 2016
bare sediment	24.2 (10.1)	Humphries et al 2016
Crassostrea virginica (laboratory)	40.6	Caffery et al 2016
Results presented in this document **avg umol N_2 / m^2 / hr (+/- SE)**		
Crassostrea virginica	86.27 (13.69)	Chapter 1, Experiment 1, Year 1
Crassostrea virginica	426.88 (111.21) *	Chapter 1, Experiment 1, Year 2
Crassostrea virginica	327.86 (82.73) **	Chapter 1, Experiment 1
Mytilus edulis	344.52 (128.28) **	Chapter 2
*reflects only N_2 production values		
**averaged across all timepoints, and reflects only N_2 production values		

Table 23: Comparison of *C. virginica* and *M. edulis* N_2O production rates measured within in this study, to other marine invertebrates measured by Heisterkamp et al 2010

N_2O production in other marine inverts (Heisterkamp et al 2010)		
Species	**(common name)**	**N_2O (nmol /g /hr) (+/- SD)**
Ascidia sp.	tunicates	0.043 (0.024)
Carcinus maenas	European green crab	0.369 (0.137)
Pagurus bernhardus	hermit crab	0.020 (0.018)
Corophium volutator	mud shrimp	0.955 (0.664)
Echinocyamus pusillus	pea urchin	0.040 (0.027
Echinocardium cordatum	sea potato (urchin)	0.069
Scrobicularia plana	marine bivalve	0.302 (0.083)
Cerastoderma edule	cockle	0.126
Mytilus edulis	blue mussel	0.269 (0.280)
Macoma balthica	salt water clam	1.098 (1.066)
Polyplacorphora	chiton	0.471 (0.237)
Littorina littorea	periwinkle	0.237 (0.208)
Hinia reticulata	dog whelk	0.608 (0.265)
Gibbula sp.	small sea snail	0.107 (0.037)
Hydrobia ulvae	mud snail	5.440 (1.822)
Arenicola marina	lug worm	0.045 (0.032)
Lepidonotus squamatus	scale worm	0.666
Nephtys hombergii	catworm	0.082 (0.053)
Nereis diversicolor	ragworm	0.398 (0.319)
Litopenaeus vannamei	whiteleg shrimp	0.183 (0.066)
Results presented in this document avg nmol N_2O / g wet weight /hr (+/- SD)		
Crassostrea virginica	field 2016 manipulation	X

Crassostrea virginica	field 2017 manipulation 18°C	0.013 (0.050)
Crassostrea virginica	field 2017 manipulation 24°C	0.119 (0.107)
Crassostrea virginica	lab experiments 18°C	0.195 (0.377)
Crassostrea virginica	lab experiments 24°C	0.112 (0.313)
Mytilus edulis	lab experiments 18°C	0.217 (0.214)
Mytilus edulis	lab experiments 24°C	0.137 (0.080)

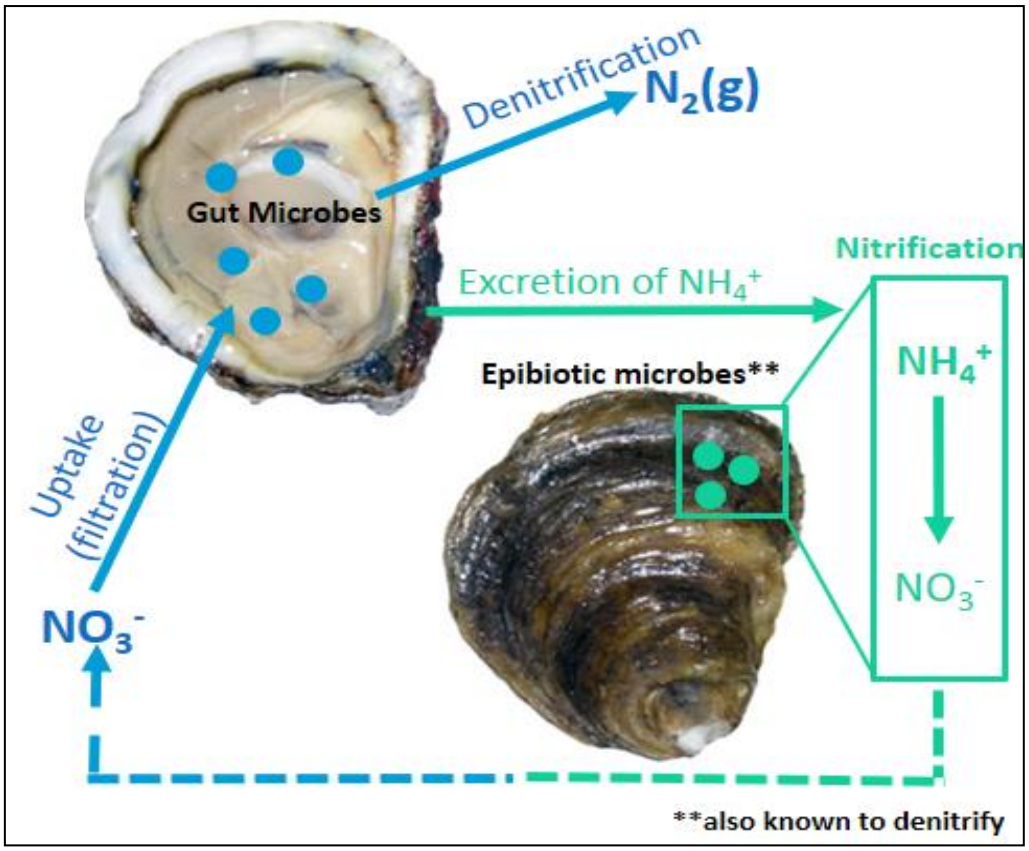

Figure 1: Main aspects of the contribution of the eastern oyster to the marine N cycle. As the organism uptakes NO^-_3 rich water, its microbial gut community intercepts and converts it to an inert N_2 gas through the process of denitrification ($NO_3^- \rightarrow NO_2^- \rightarrow NO + N_2O \rightarrow N_2$). Denitrification is tightly coupled to nitrification ($NH_4^+ \rightarrow NH_2OH \rightarrow NO_2^- \rightarrow NO_3^-$). There are two opportunities for N_2O production: (1) as an intermediate gas in denitrification and (2) as a byproduct during nitrification.

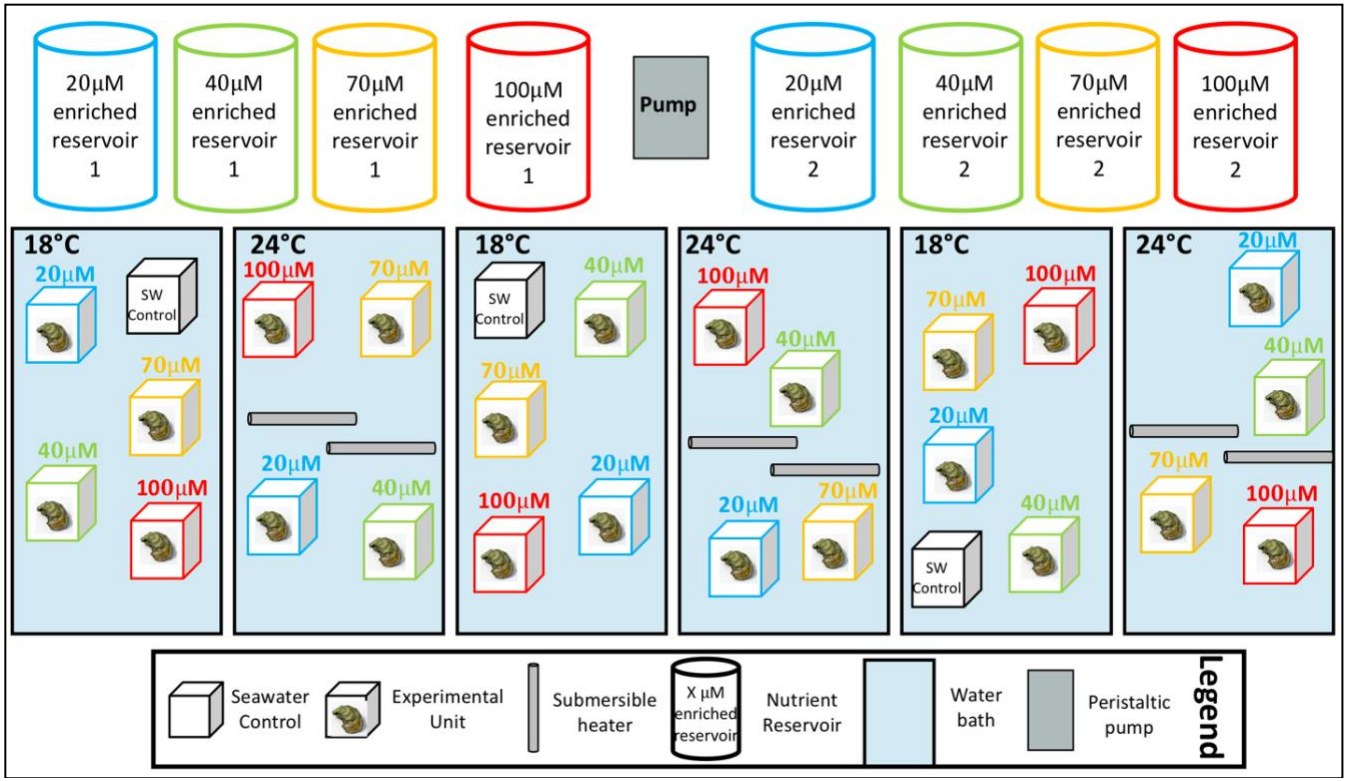

Figure 2: Layout of experimental tanks for laboratory portion (Experiment 1) of the study. Aquaria of varying nutrient levels were randomly placed within a water bath, and maintained at either 18°C or 24°C. Each aquarium contained 10 organisms to start. For the mussel experiments (Chapter 2), the gradient of N levels was reduced to 5µM, 10 µM, 17.5 µM, and 25 µM and maintained at either 18°C or 21°C.

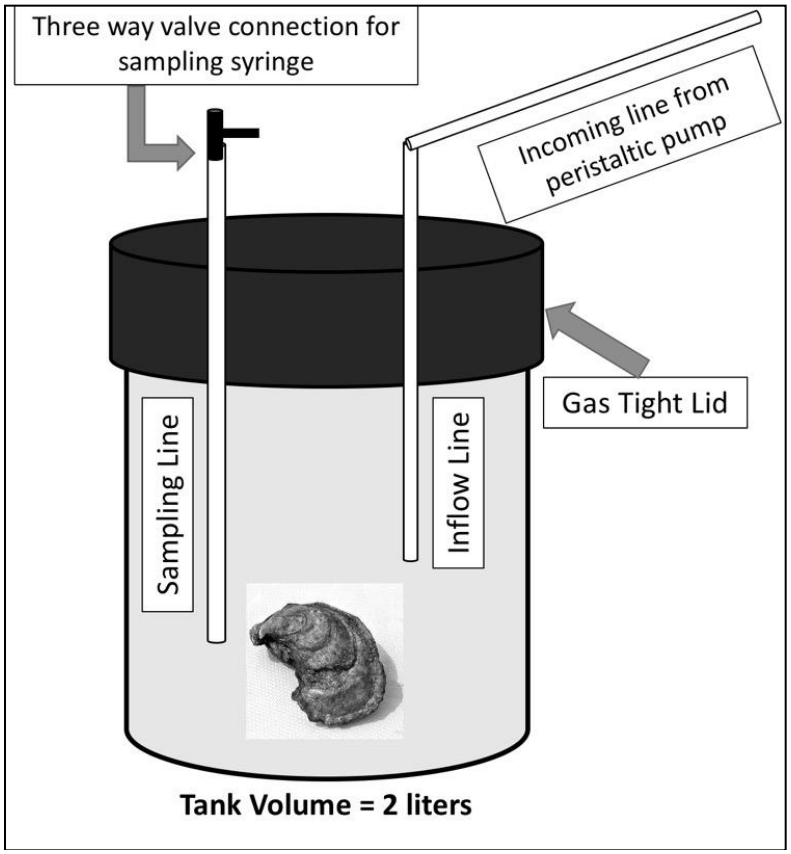

Figure 3: Depiction of incubation tank (same set up for oysters Experiment 1 & 2 and mussels)

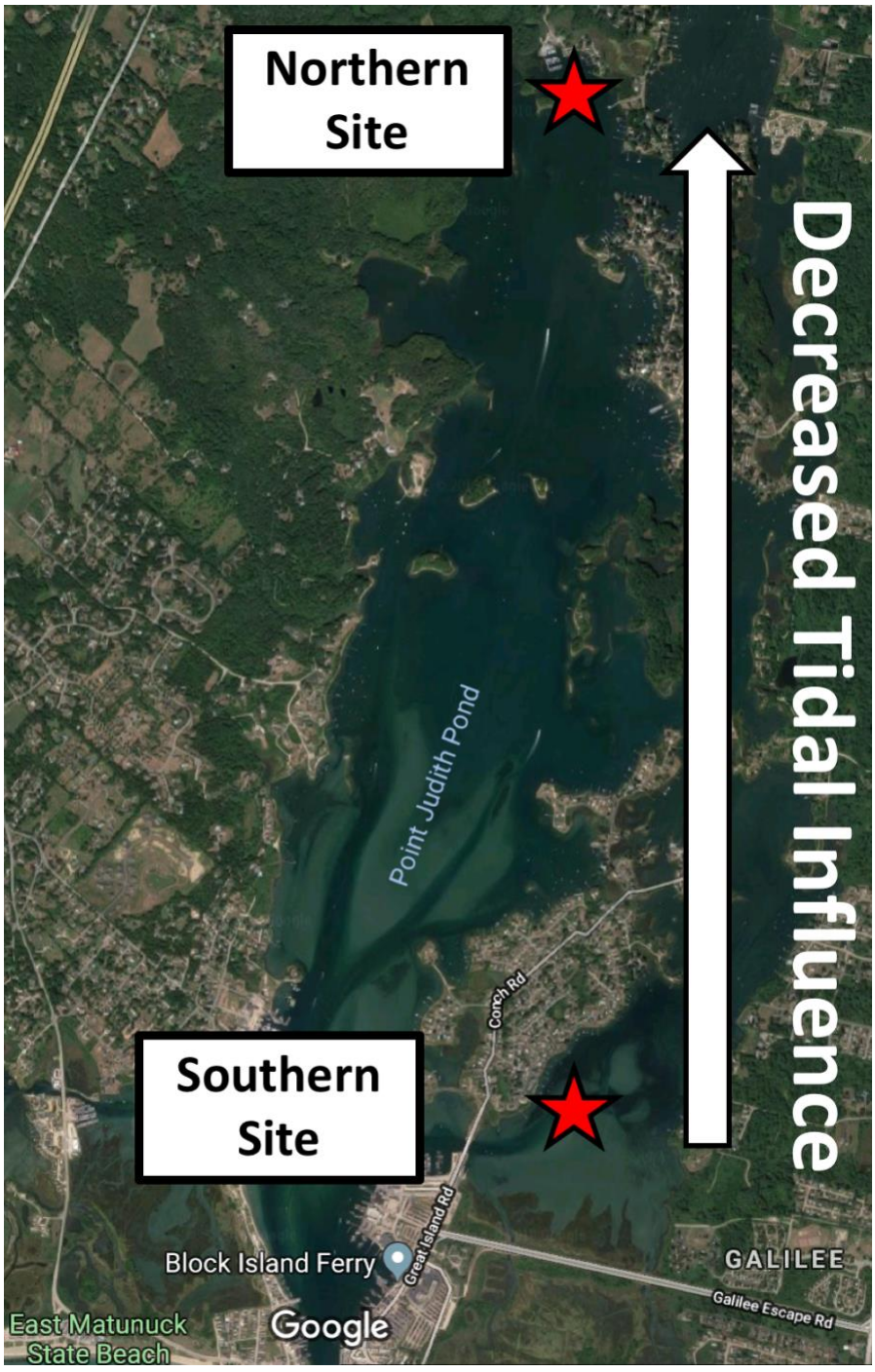

Figure 4: Map depicts Point Judith Pond. Red stars mark the two field locations (Experiment 2), located at contrasting ends of the estuarine gradient of the Pond.

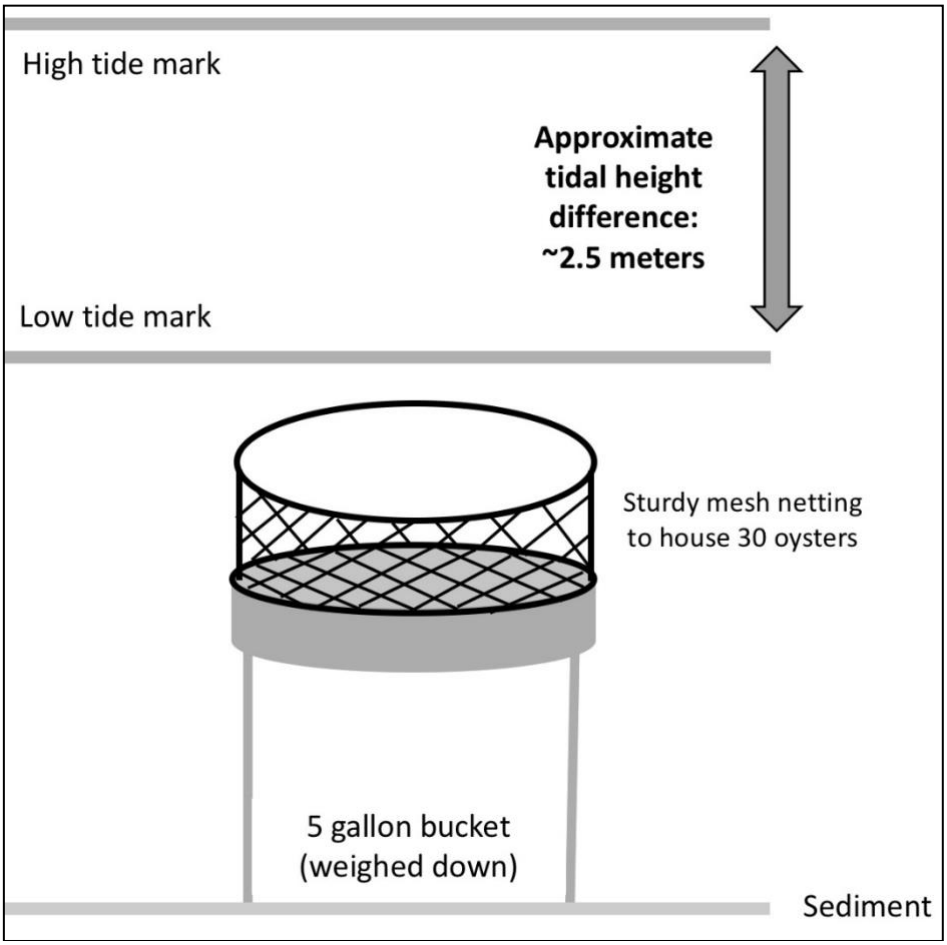

Figure 5: Experimental bucket setup for field component for Experiment 2

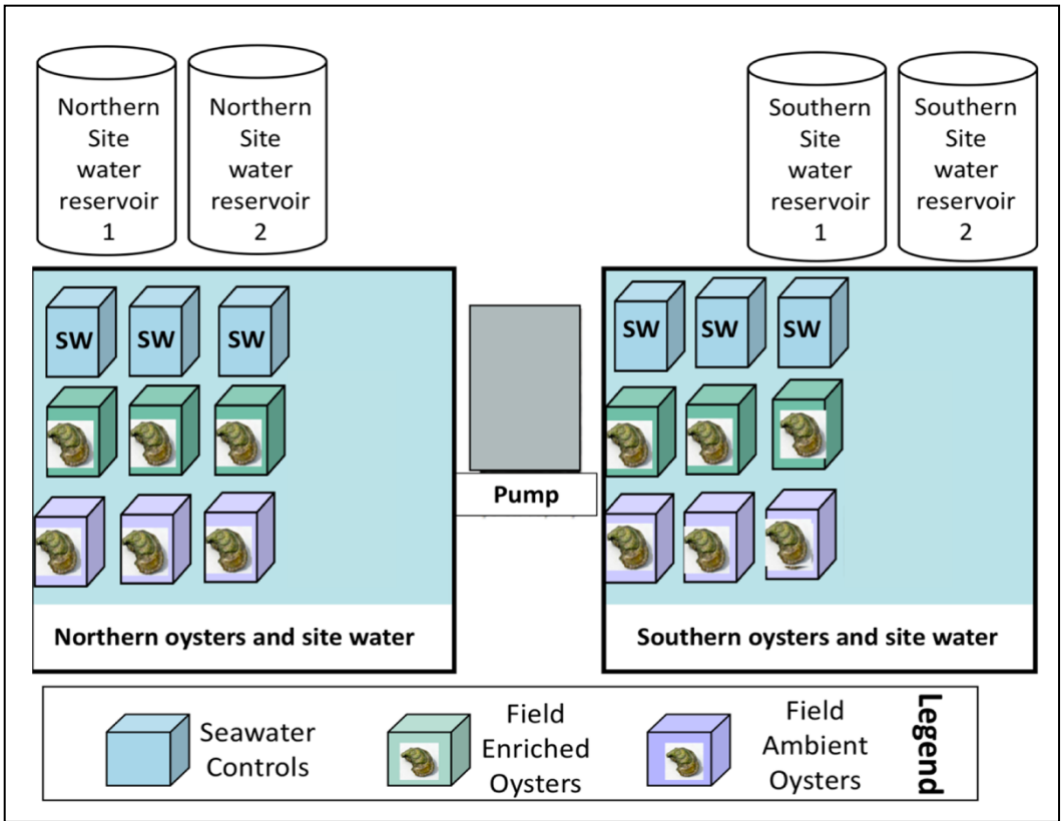

Figure 6: Incubation setup for Field 2016 oysters (Experiment 2, Year 1)

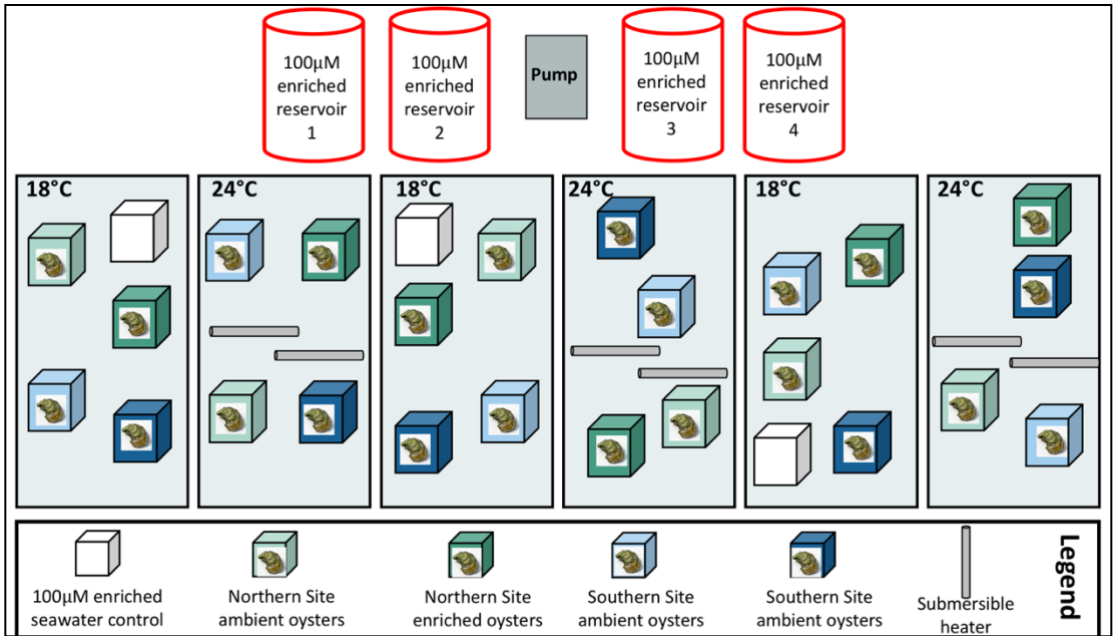

Figure 7: Incubation setup for Field 2017 oysters (Experiment 2, Year 2)

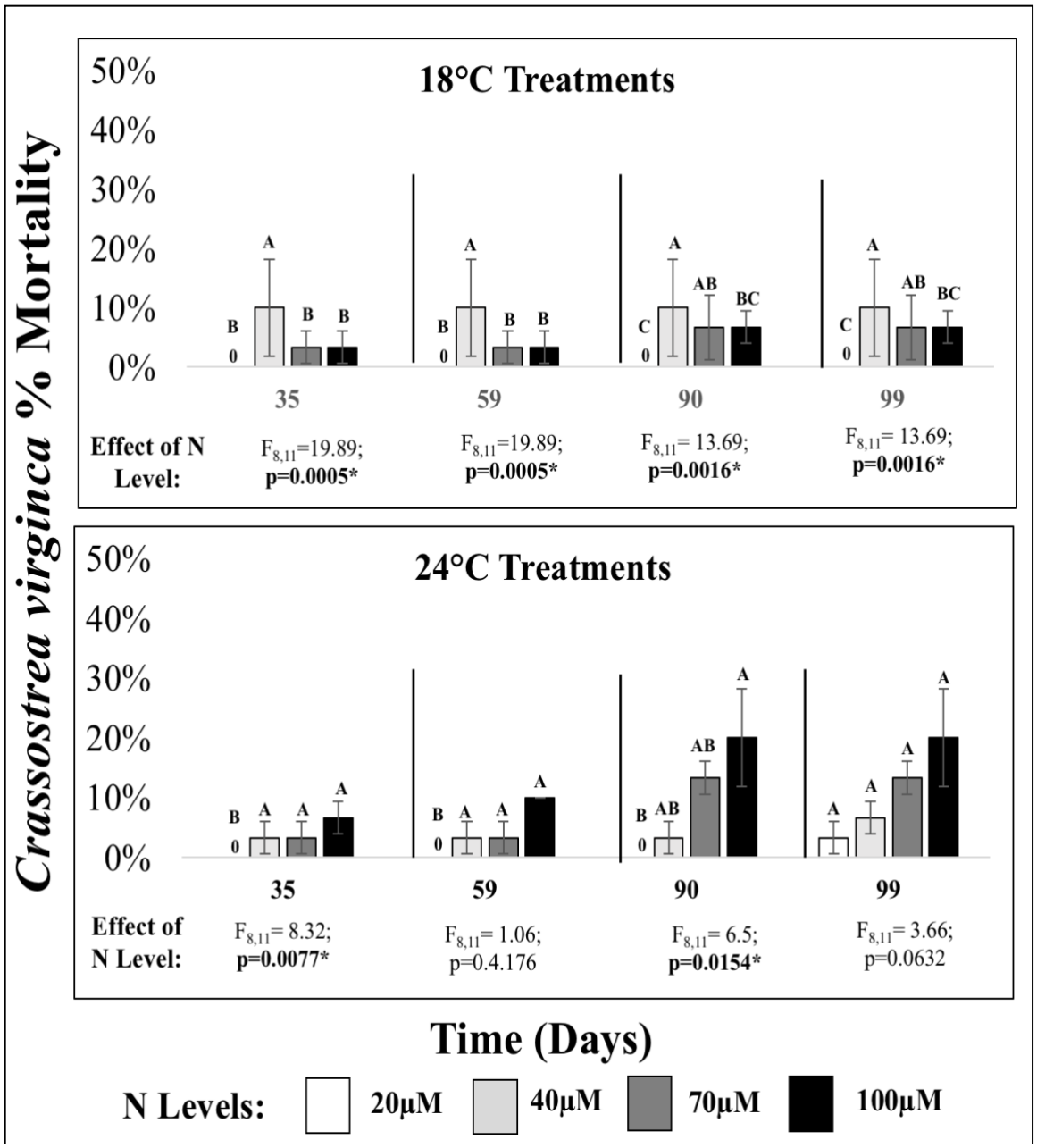

Figure 8: Effect of nitrogen enrichment on oyster mortality at two different temperatures. Percentage of mortality for each measured time period in Experiment 1; both figures show the four N levels and different letters denote significant effect N-level within each observation (independent of temperature and time; letters correspond with p and F values under each observation)

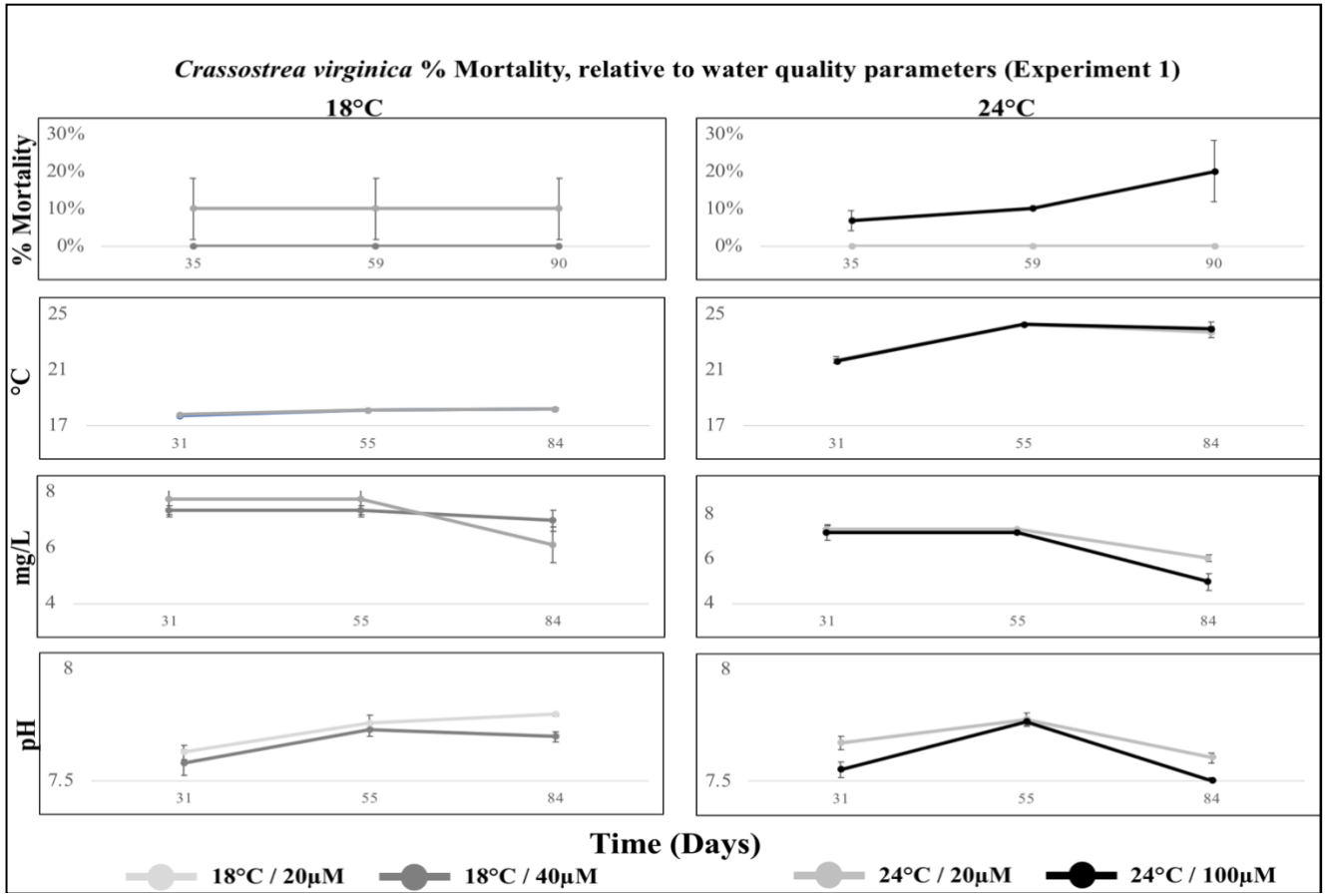

Figure 9: *C. virginica* mortality in relation to water quality parameters of experimental aquaria: Figures to the left represent % mortality and corresponding water quality parameters (temperature, oxygen, pH, respectively), over time within the lowest (20µM) and highest (40µM) mortality levels within the cooler (18°C) treatments. Figures to the right represent the same parameters within the warmer (24°C) treatments; the lowest level of mortality occurred in the 20µM treatments, and the highest in the 100µM

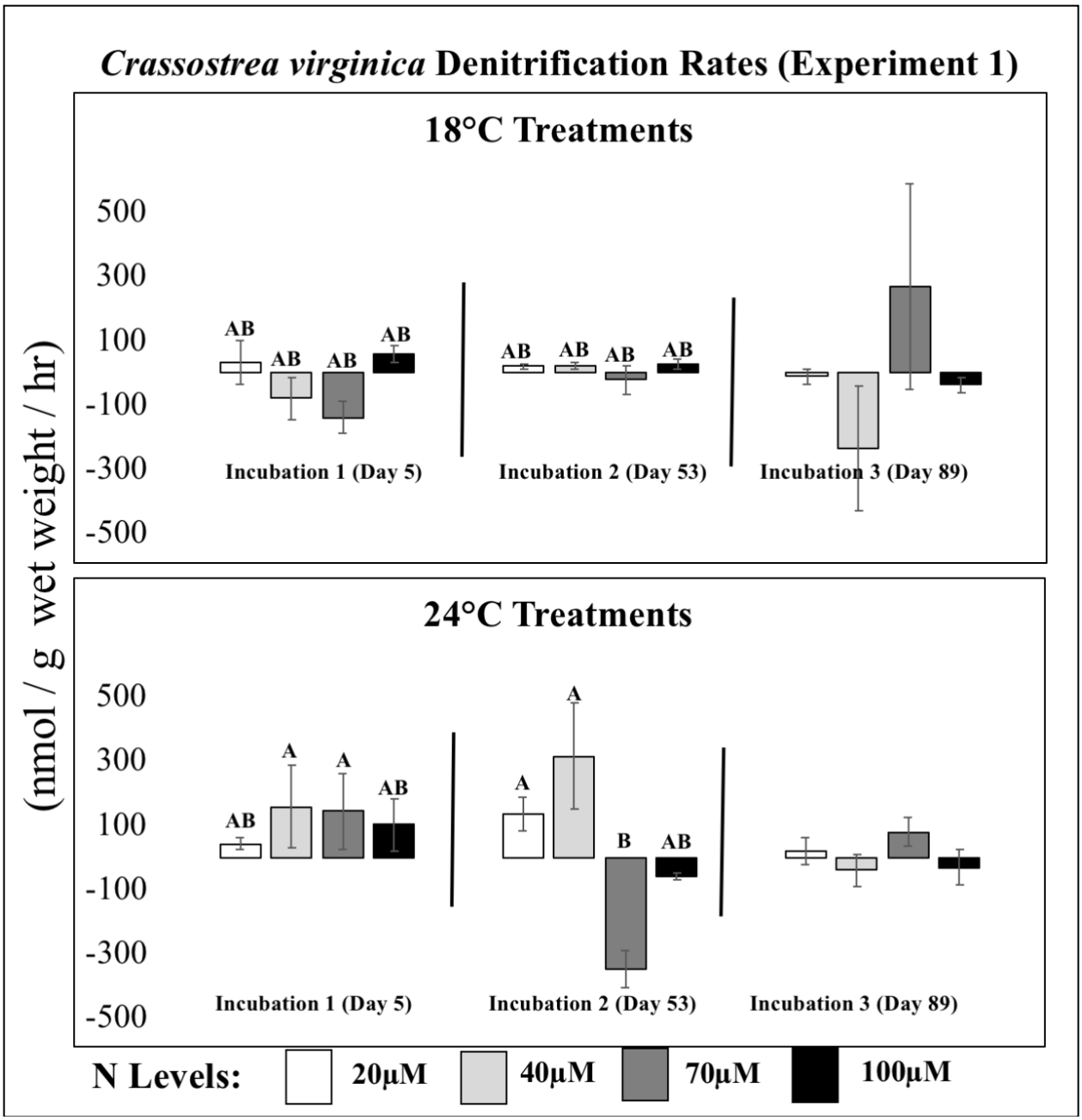

Figure 10: Effect of nitrogen enrichment on denitrification rates in oysters incubated at two different temperatures (T). Denitrification rates were measured after 5, 53, and 89 days of incubation in the different experimental conditions. Different letters denote significant effects of Temperature X N level X Time (days). Incubation 3 (Day 89) was excluded from analysis due to issues with the experimental setup.

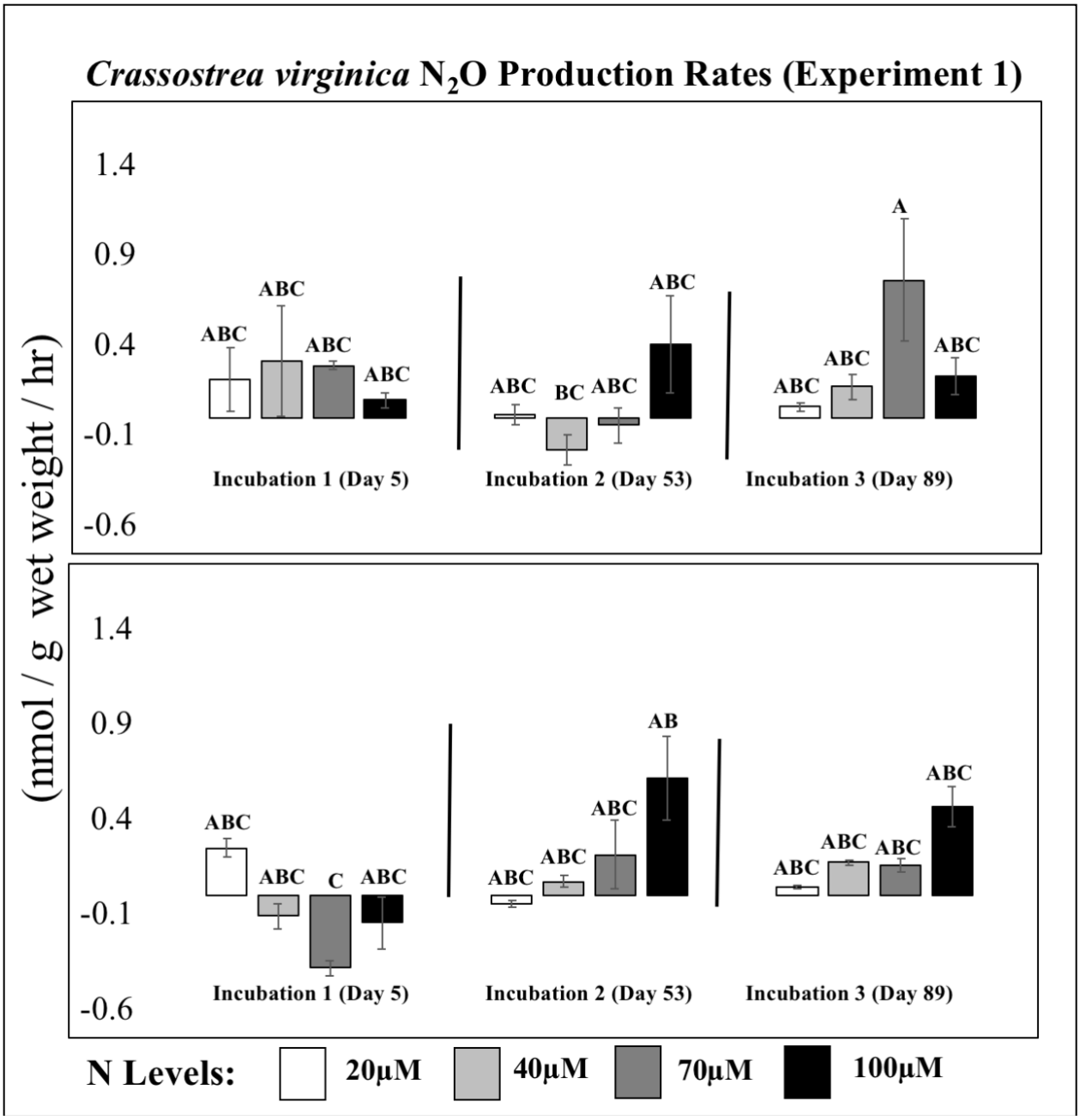

Figure 11: Effect of nitrogen enrichment on N_2O production rates in oysters incubated at two different temperatures (T). N_2O production rates were measured after 5, 53, and 89 days of incubation in the different experimental conditions. Different letters denote significant effects of Temperature X N level X Time (days).

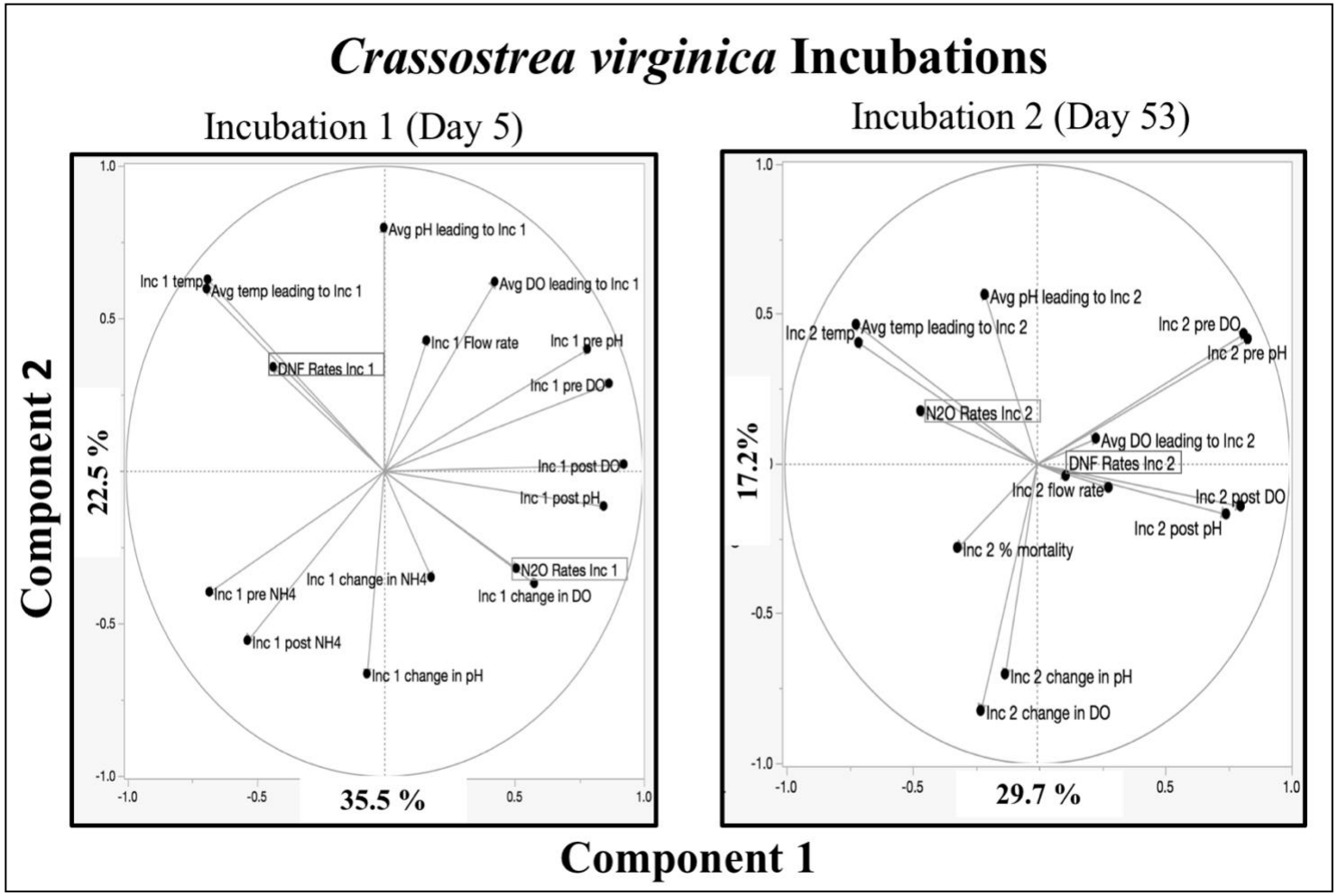

Figure 12: Results from a Principal Component Analysis for Day 0 and Day 48 of the oyster incubation experiments; Day 84 was excluded due to reason believe technical difficulties with experimental setup.

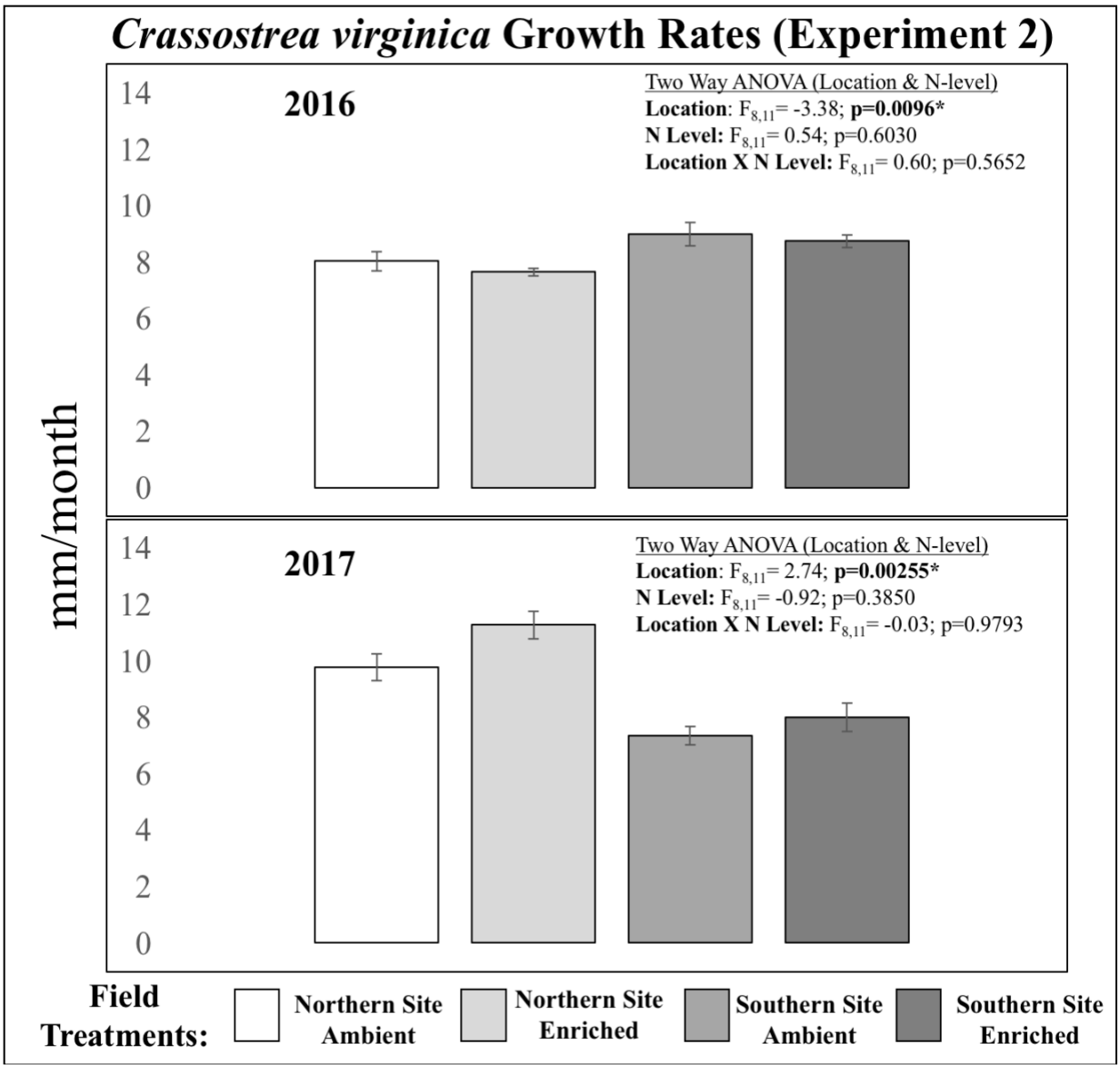

Figure 13: *Crassostrea virginica* growth rates from 2016 & 2017's field experiments. Displayed on each graph are results from a Two-Way ANOVA analyzing the effect of location and N-level on each year's data

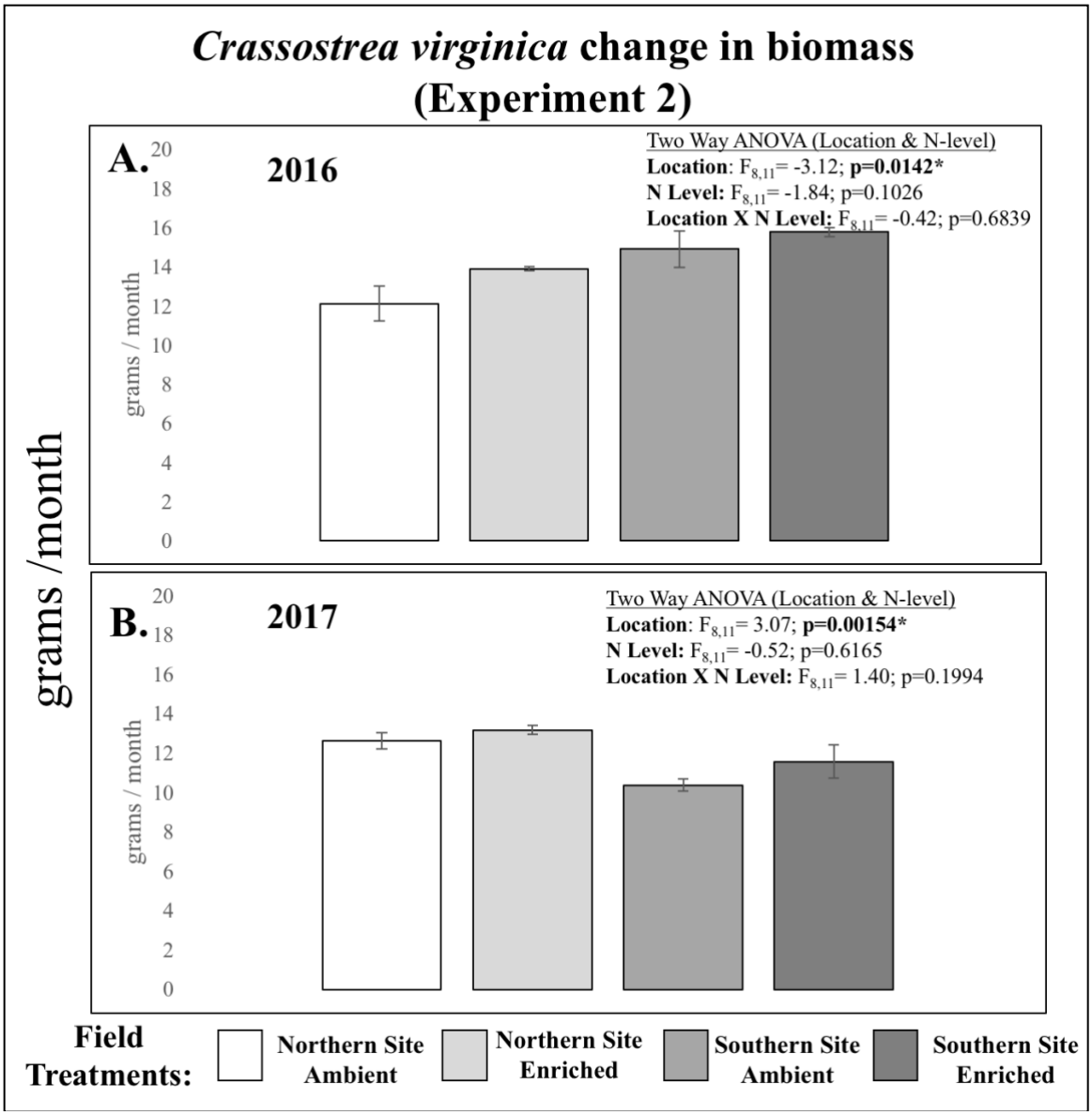

Figure 14: *Crassostrea virginica* biomass rates from 2016 & 2017's field experiments. Displayed on each graph are results from a Two-Way ANOVA analyzing the effect of location and N-level on each year's data

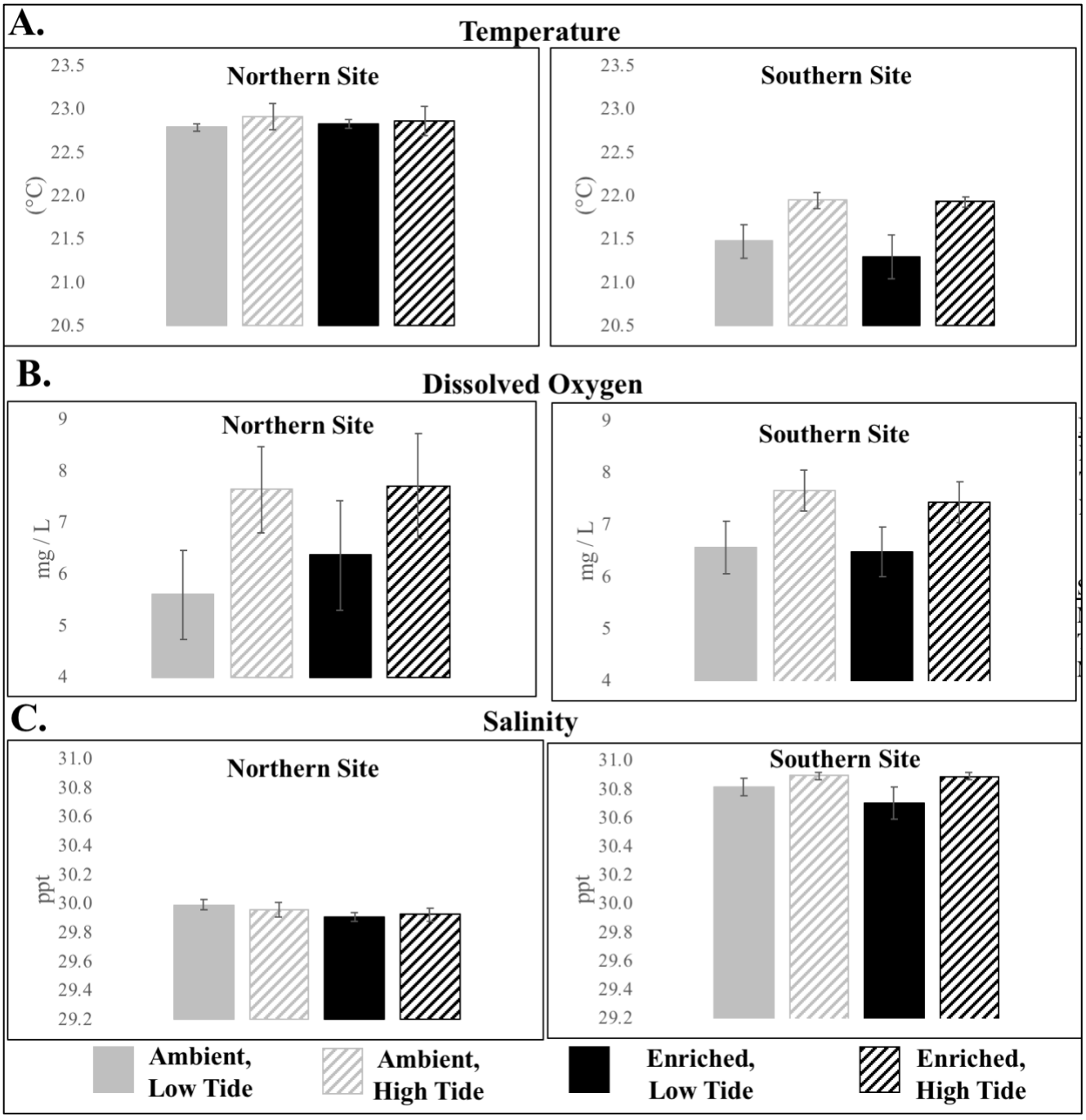

Figure 15: Results from 2016 field "all day profile" – examining the differences in environmental factors between location, N-level, and tidal height

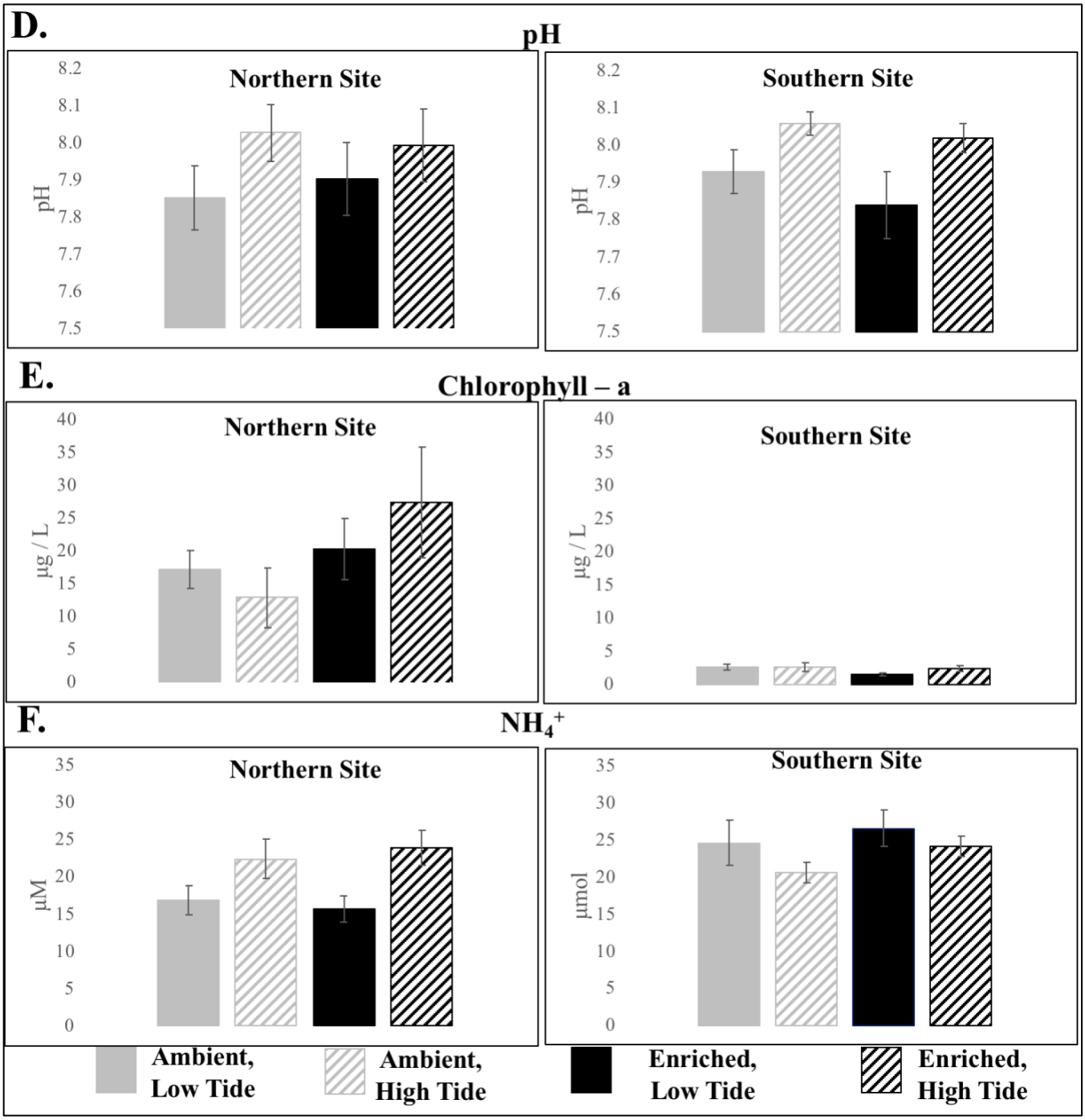

Figure 15 (CONT.): Results from 2016 field "all day profile" – examining the differences in environmental factors between location, N-level, and tidal height

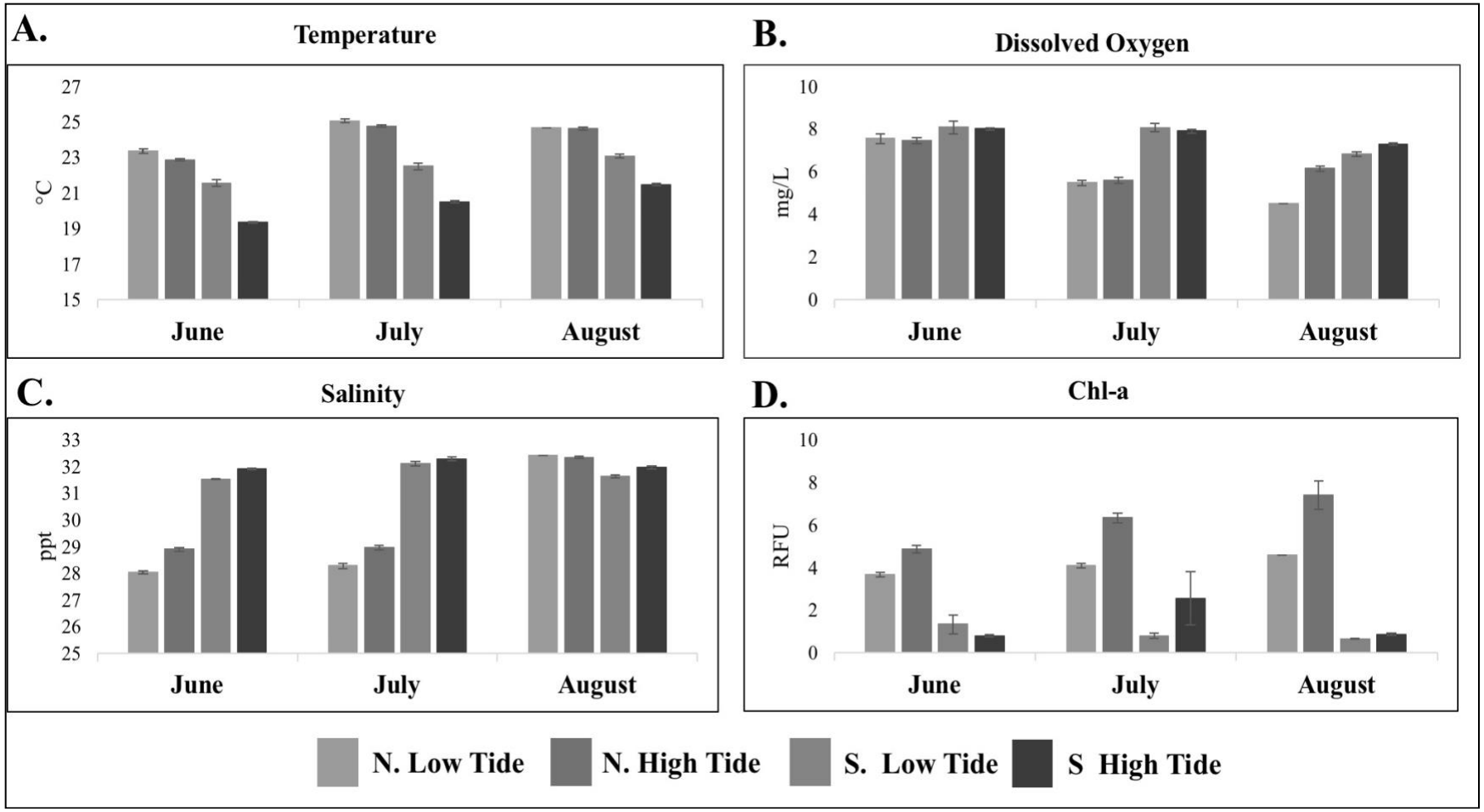

Figure 16: Environmental factors from the 2017 field season, displayed by location and tidal height

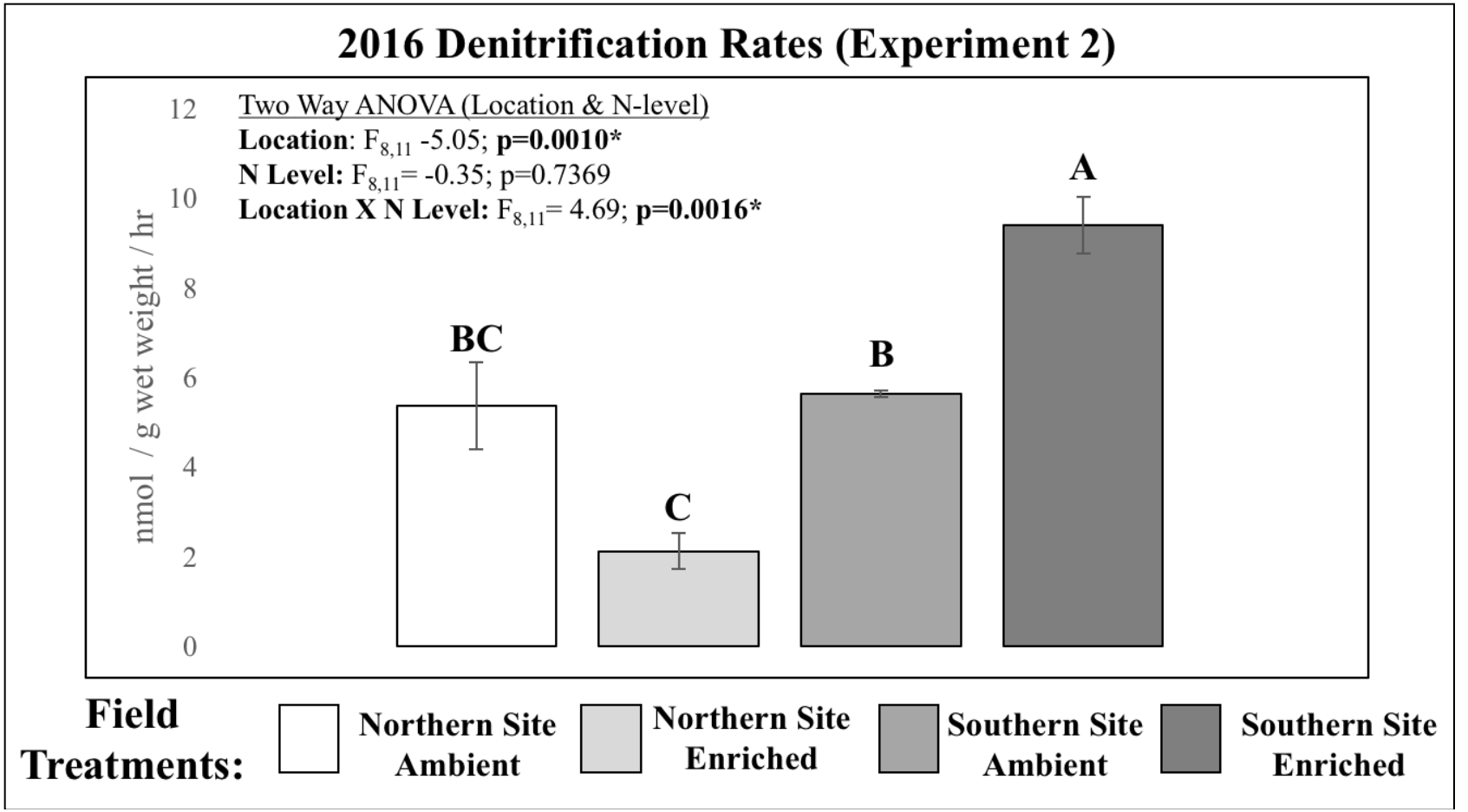

Figure 17: *Crassostrea virginica* denitrification rates from the 2016 field oyster incubation; organisms were incubated at 18°C and received flow from unenriched site water; Results from a Two-Way ANOVA analyzed the effect of field location and field N-level is displayed in the upper left corner; letters denote significant differences among treatments

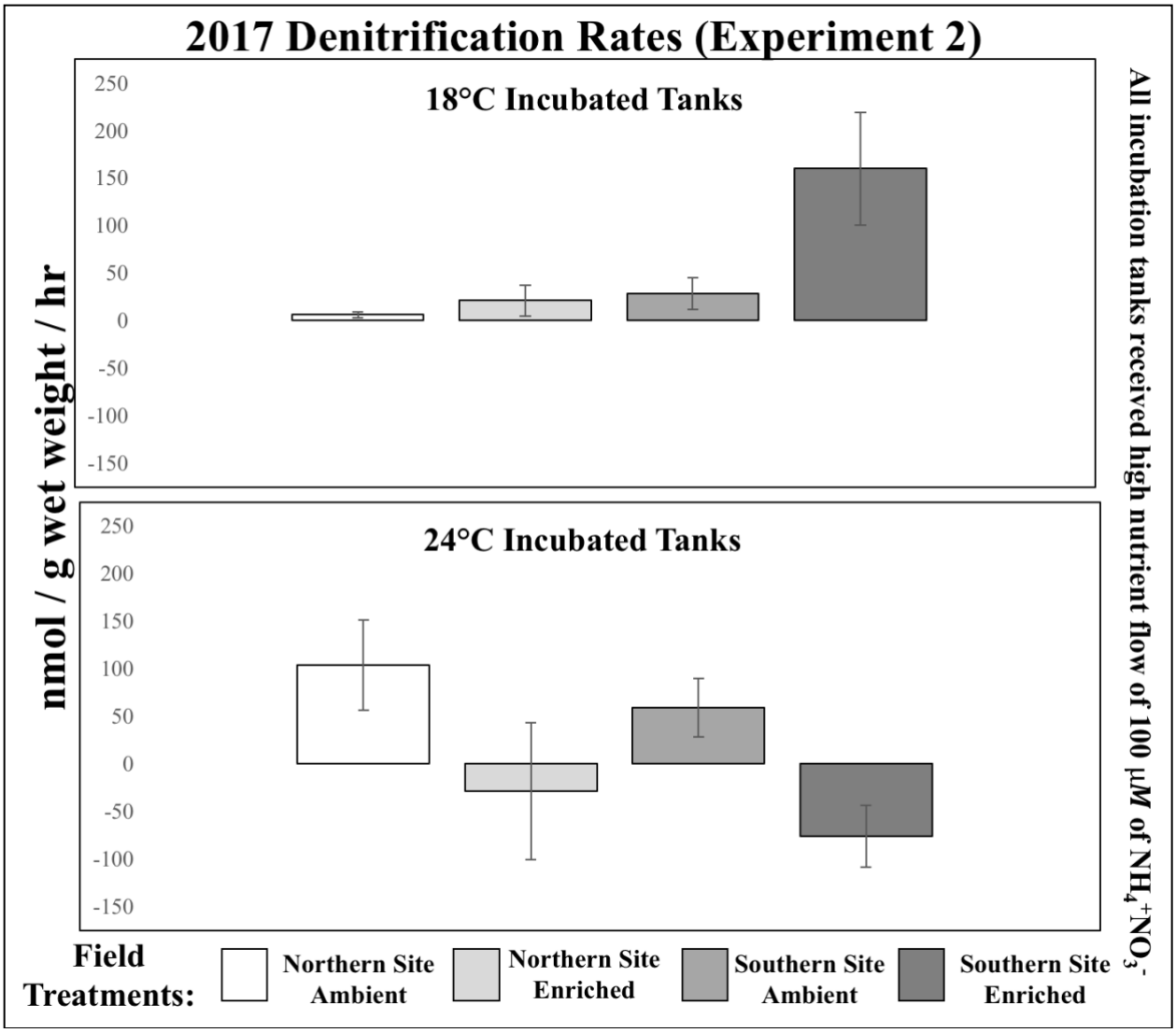

Figure 18: *C. virginica* denitrification rates (2017 field experiment).

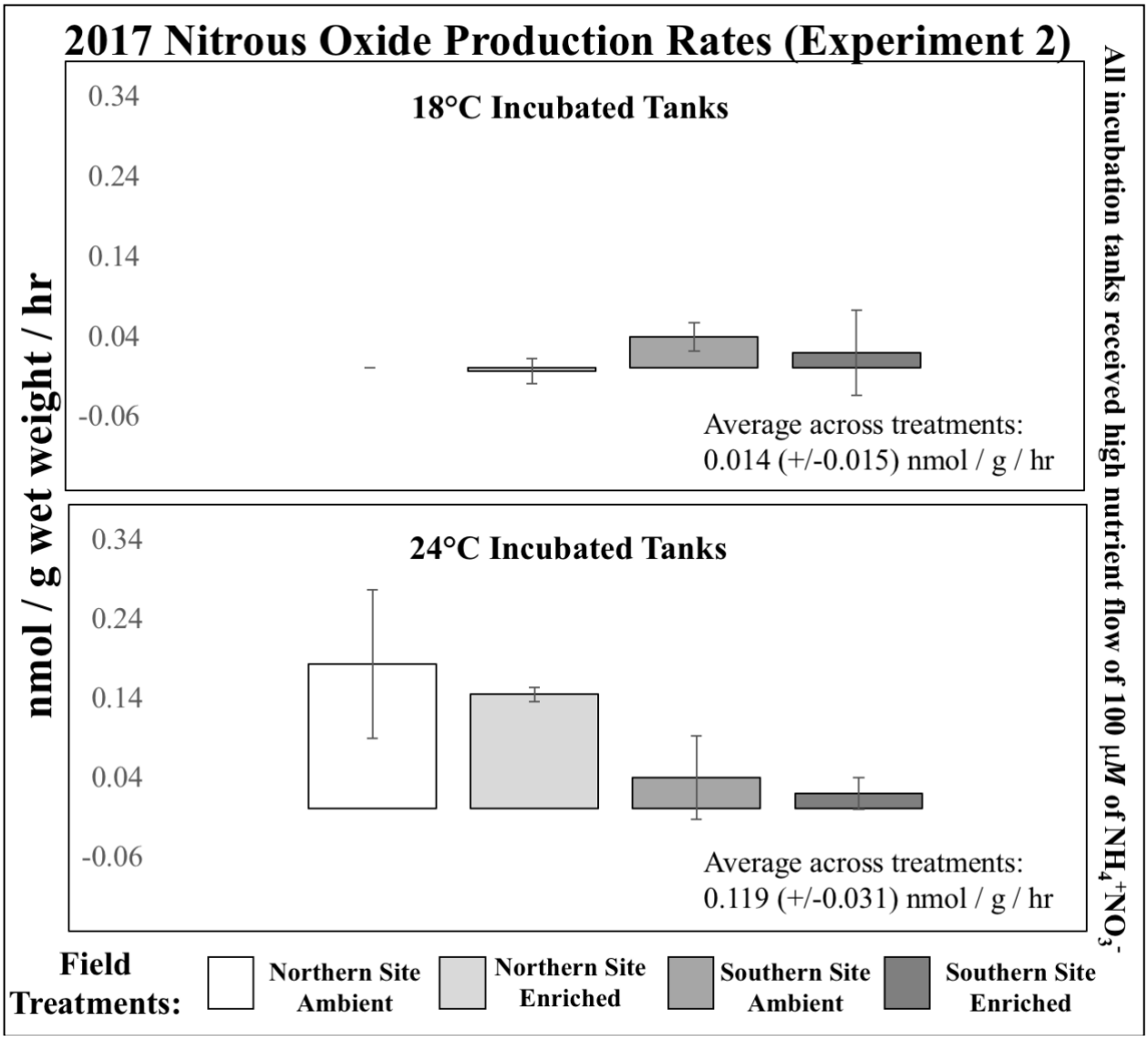

Figure 19: *C. virginica* nitrous oxide production rates (2017 field experiment)

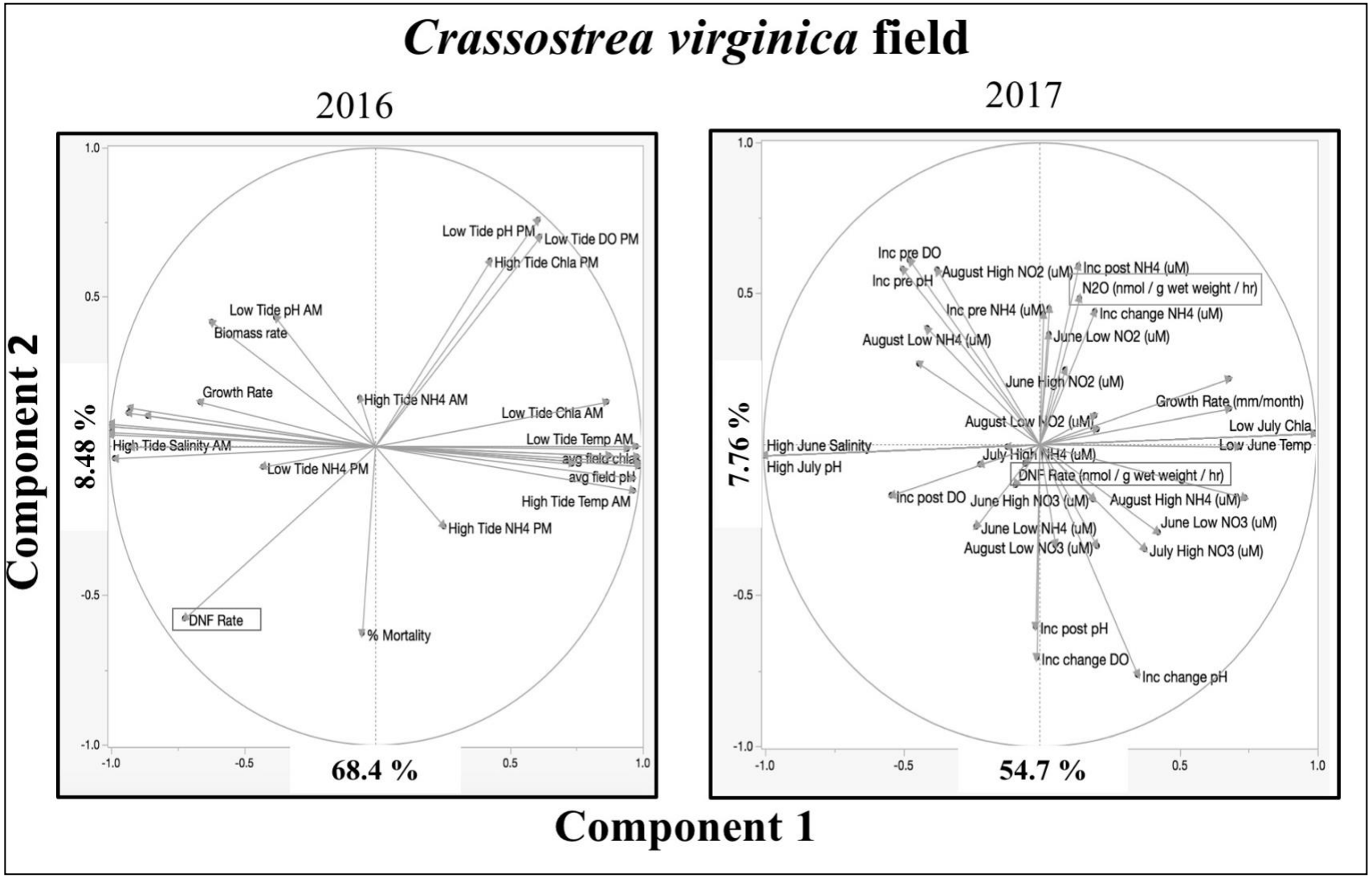

Figure 20: Principal Component Analysis for 2016 & 2017 field oyster incubations (Experiment 2)

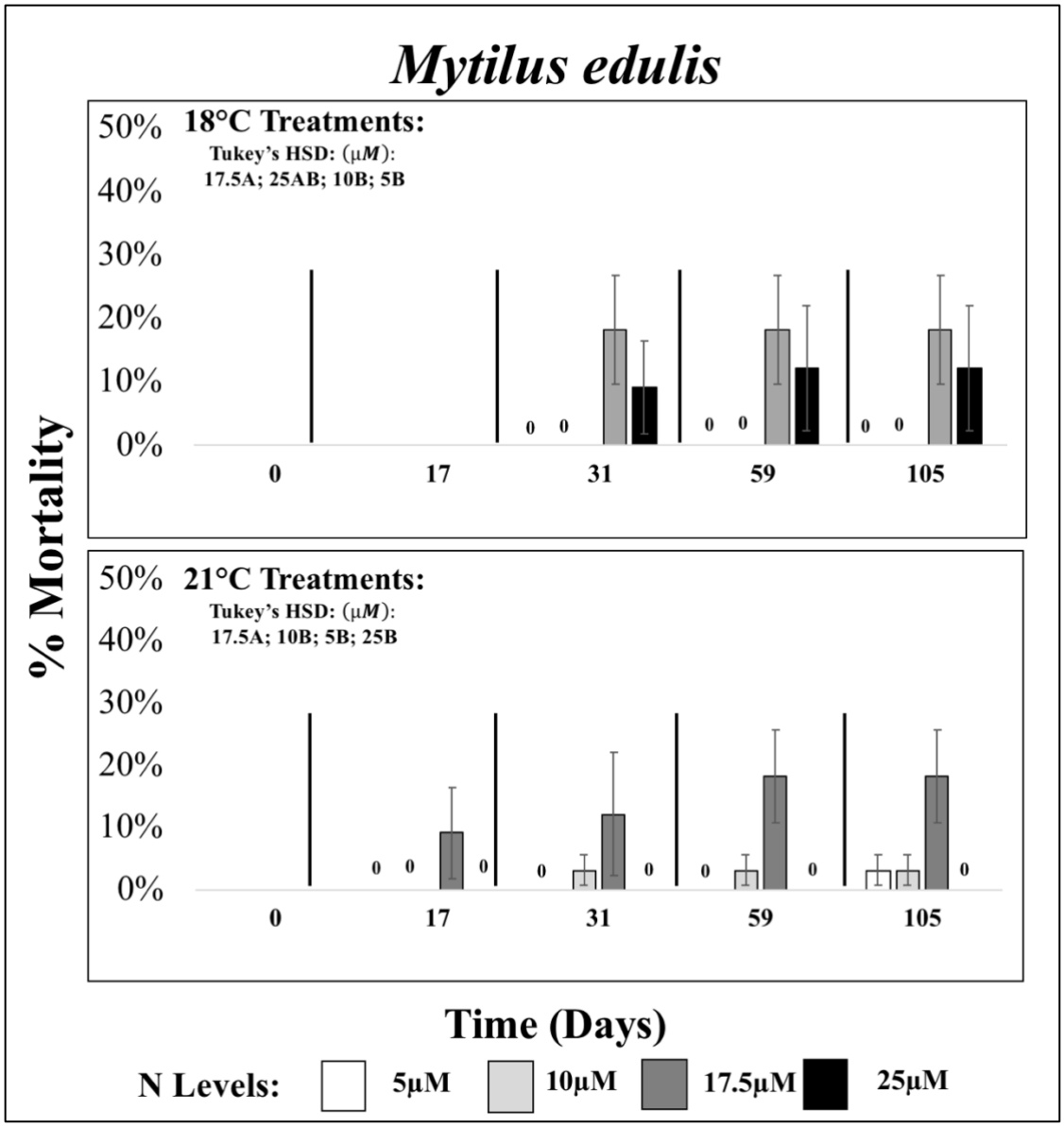

Figure 21: Effect of nitrogen enrichment on mussel mortality at two different temperatures Percentage of mortality over the course of the mussel laboratory experiment; the upper portion represents the 18°C treatments at each N level over time (days), and the lower portion the 21°C treatments. Two-way ANOVAs were used to test the effect of N level and time (independent of temperature)

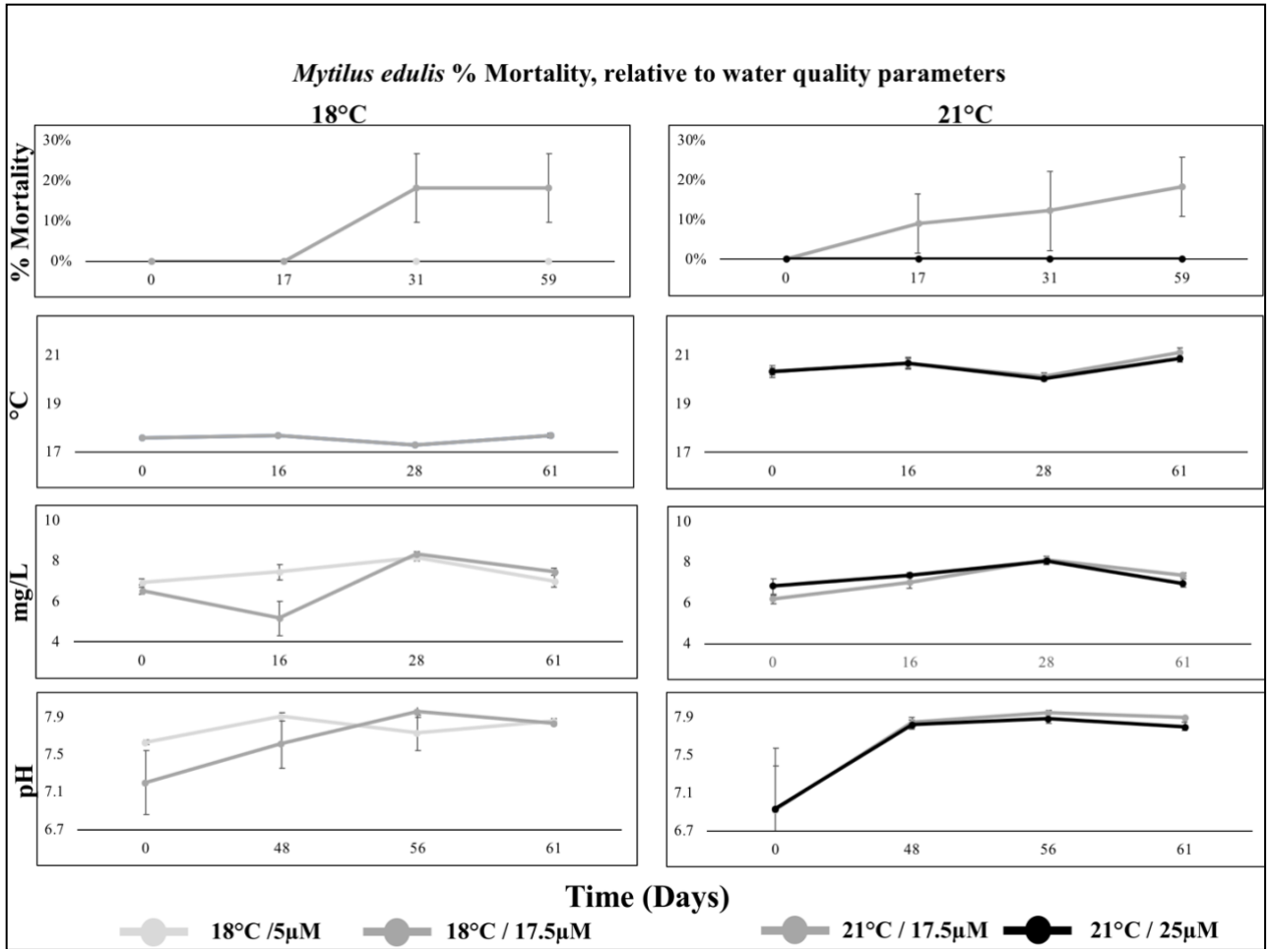

Figure 22: *M. edulis* mortality in relation to water quality parameters of experimental aquaria: Figures to the left represent % morality and corresponding water quality parameters (temperature, oxygen, pH, respectively), over time, within the lowest (5µM) and highest (17.5µM) morality levels within the cooler (18°C) treatments. Figures to the right represent the same parameters within the warmer (21°C) treatments; the lowest level of mortality occurred in the 25µM treatments, and the highest in the 17.5µM

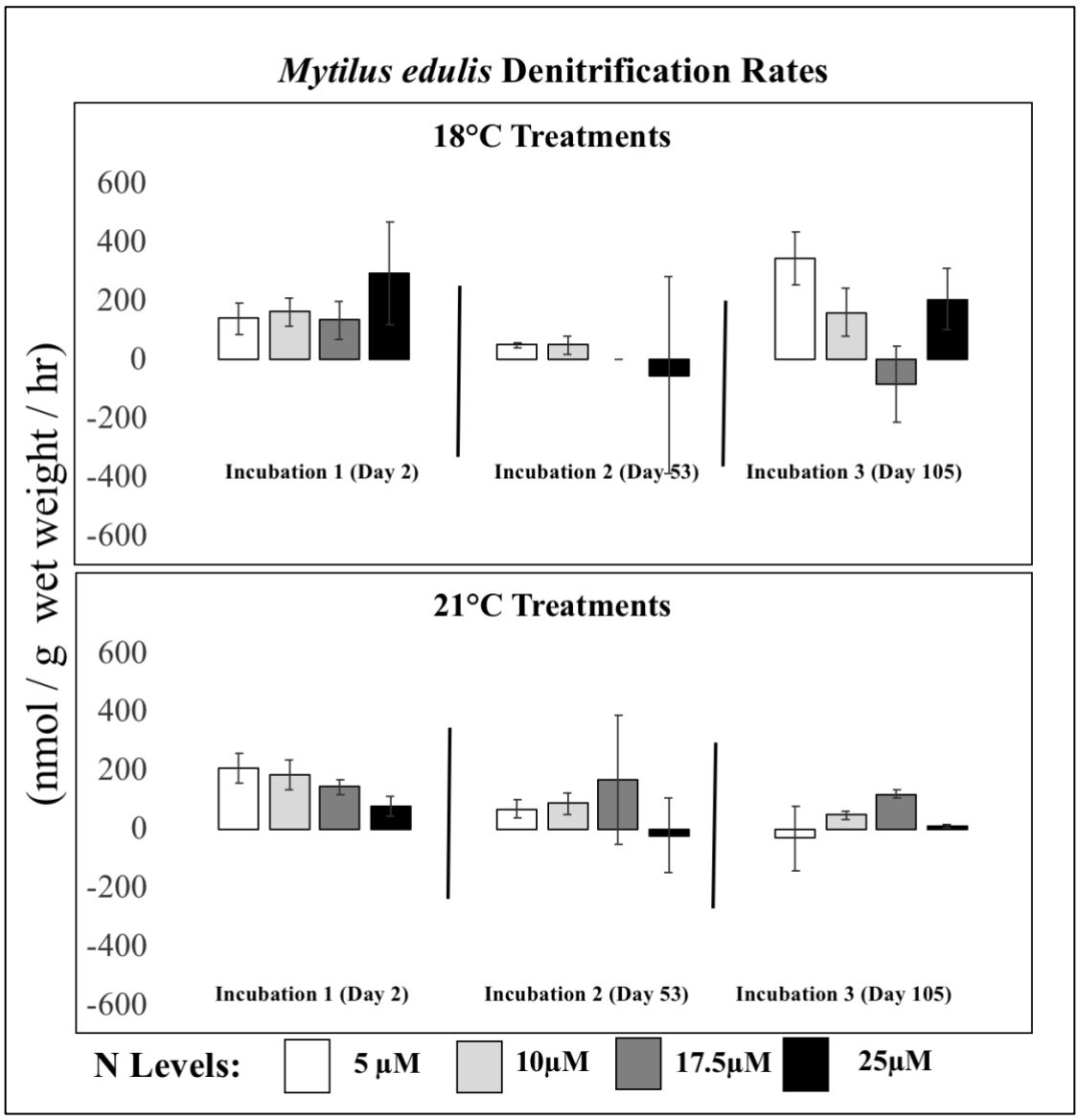

Figure 23: Effect of nitrogen enrichment on denitrification rates in mussles incubated at two different temperatures (T). Denitrification rates for each incubation event (Day 2, 53, 105) for *M. edulis'* laboratory experiment. Each Incubation time point for18°C treatments are represented in the upper portion and 21°C in the lower portion of the figure. N Levels are depicted along the bottom

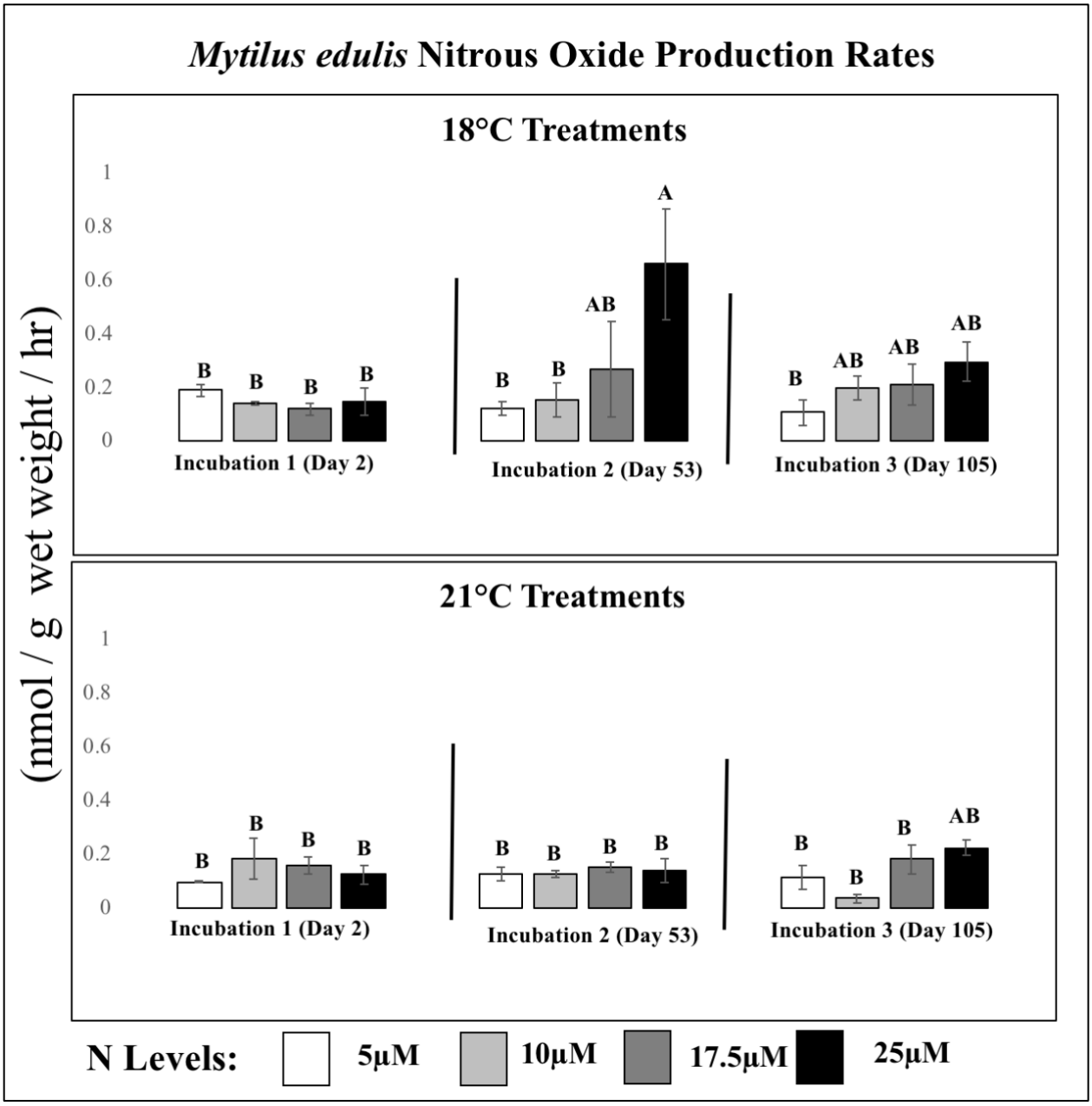

Figure 24: Effect of nitrogen enrichment on N_2O production rates in mussels incubated at two different temperatures (T). N_2O production rates for each incubation event (Day 2, 53, 105) for *M. edulis*. Letters denote significant effects of Temperature X N Level X Time.

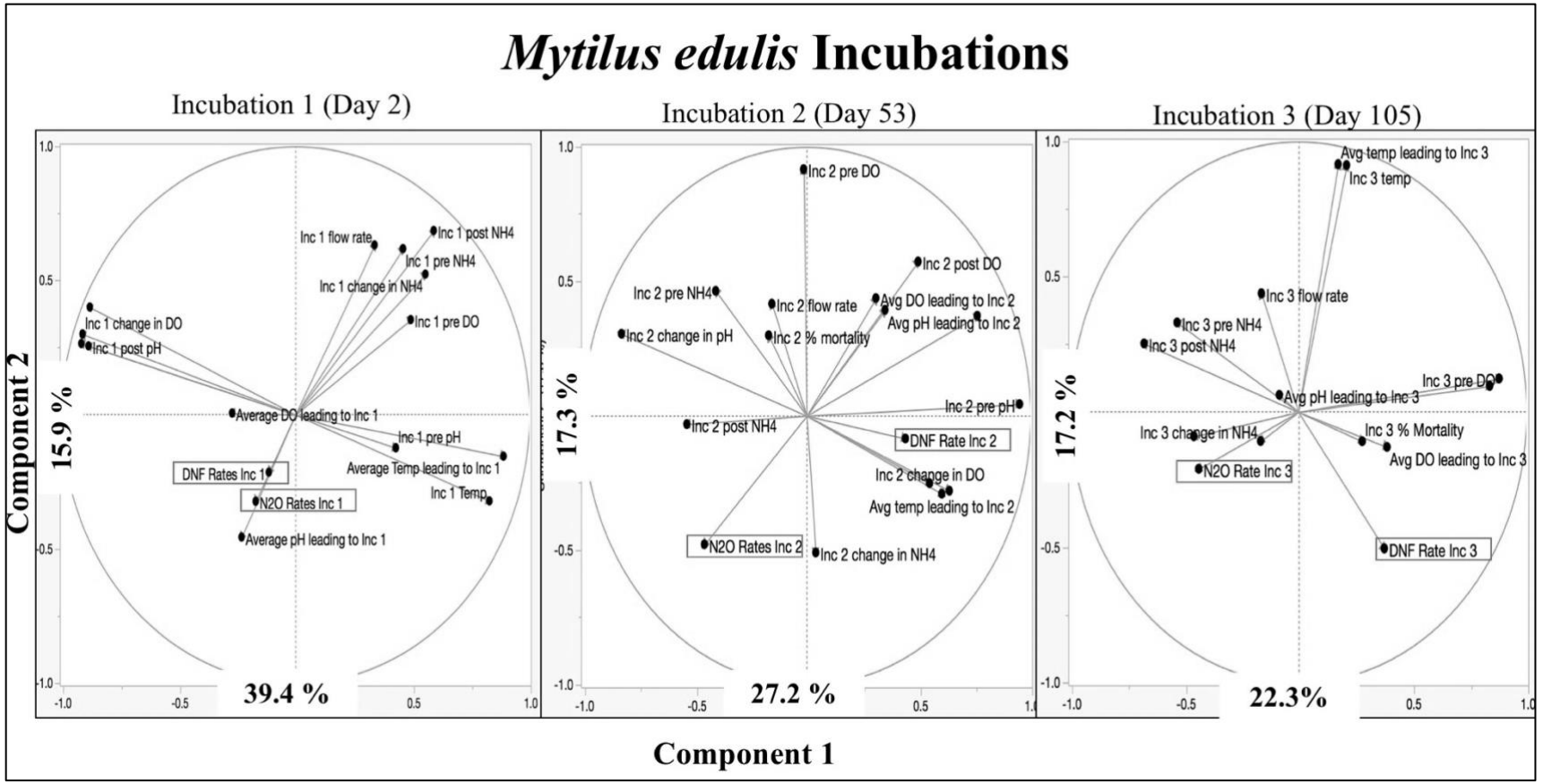

Figure 25: Results from a Principal Component Analysis for each *M. edulis* incubation over the course of the 3 month laboratory experiment.

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APPENDIX

Appendix 1: Results from a Three-Way ANOVA testing the effect of temperature, N-level, and time on cumulative percent mortality of *C. virginica* through the laboratory experiment (Experiment 1, Days 35, 59, 90, 99).

C. virginica percentage of mortality (Experiment 1)			
Testing the effect of Temperature, N Level, & Time			
		$F_{96,134}$-value	p-value
Temp		0.53	0.467
N Level		4.85	**0.004**
Temp * N Level		3.00	**0.035**
Time		3.45	**0.007**
Temp * Time		0.64	0.672
N Level * Time		0.57	0.889
Temp * N Level * Time		0.25	0.998

Appendix 2: Tukey HSD post-hoc results indicating the significant differences which arose from the three-way ANOVA analyzing the effect of Temperature, N-level and Time on *C. virginica* % mortality (Experiment 1). Significant factors were N level, Temperature X N level, and time.

***C. virginica* % Mortality (Experiment 1)**		
Effect of N level ($F_{96, 143}$ =4.85, p=0.0035)		
Level (µM)		**Least Sq. Mean**
100	A	0.07
40	A	0.06
70	AB	0.05
20	B	0.003
Effect of Temp X N level ($F_{96, 143}$ =43.00, p=0.0345)		
Level (°C / µM)		**Least Sq. Mean**
24,100	A	0.10
18,40	AB	0.08
24,70	ABC	0.06
18,100	ABC	0.04
18,70	ABC	0.04
24,40	ABC	0.03

24,20	BC	0.006
18,20	C	0.000
Effect of Time ($F_{96, 143}$ =3.455, p=0.0065)		
Level (days)		**Least Sq. Mean**
99	A	0.08
90	A	0.08
59	AB	0.04
35	AB	0.04
4	AB	0.03
0	B	0.00
*Levels not connected by the same letter are significantly different		

Appendix 3: Three-way ANOVA result, testing the effects of temperature, N-Level, and time on weekly water quality measurements of *C. virginica* experimental tanks over the course of 3-month experiment (Experiment 1)

Weekly water quality for *C. virginica* laboratory experiment (Experiment 1)								
Testing the effect of Temperature, N Level, & Time								
	Temperature (°C)		**DO (mg/L)**		**pH**		**NH_4^+ (µM)**	
	$F_{48,71}$-value	*p-value*	*$F_{48,71}$-value*	*p-value*	*$F_{48,71}$-value*	*p-value*	*$F_{48,71}$-value*	*p-value*
Temp	3372.18	**<0.0001**	0.94	0.334	48.89	**<0.0001**	0.20	0.653
N Level	0.11	0.955	2.07	0.111	9.81	**<0.0001**	3.90	**0.0143**
Temp * N Level	0.15	0.932	5.71	**0.001**	13.56	**<0.0001**	0.36	0.785
Time	25.95	**<0.0001**	2.60	**0.042**	25.28	**<0.0001**	34.79	**<0.0001**
Temp * Time	16.76	**<0.0001**	1.48	0.217	0.94	0.459	0.59	0.559
N Level * Time	0.09	1	0.48	0.918	1.23	0.265	1.23	0.308
Temp * N Level * Time	0.08	1	0.47	0.926	0.75	0.726	0.64	0.700

Appendix 4: Tukey HSD post-hoc results indicating the significant differences which arose from the three-way ANOVA analyzing the effect of Temperature, N-level and Time on the average water quality parameters (Temperature, DO, pH, NH_4^+) over the course of the 3-month, *C. virginica* laboratory-based study (Experiment 1)

C. virginica water quality over the course of 3-month laboratory experiment (Experiment 1)

Temperature

Effect of Temperature ($F_{112, 167}$=3372.18, p=<0.0001)

Level		Least Sq. Mean
24	A	22.92
18	B	17.91

Effect of Time ($F_{112, 167}$ =25.95, p=<0.0001)

Level (days)		Least Sq. Mean
55	A	21.17
68	A	21.16
41	B	20.66
17	C	20.13
10	C	20.02
24	C	19.98
31	C	19.78

Dissolved Oxygen (mg/L)

Effect of Temperature X N Level ($F_{80, 119}$ =16.76, p=<0.0001)

Level (°C, µM)		Least Sq. Mean
18,40	A	7.64
18,20	A	7.62
24,70	A	7.61
24,20	AB	7.41
24,100	AB	7.35
24,40	AB	7.27
18,100	AB	7.08
18,70	B	6.91

Effect of Time ($F_{80, 119}$ =2.60, p=0.0420)

Level (days)		Least Sq. Mean
10	A	7.60

pH

Effect of Temperature ($F_{96, 143}$ =48.89, p=<0.0001)

Level (°C)		Least Sq. Mean
24	A	7.69
18	B	7.61

Effect of N Level ($F_{96, 143}$ =9.81, p=<0.0001)

Level (µM)		Least Sq. Mean
40	A	7.68
20	A	7.68
70	A	7.64
100	B	7.60

Effect of Temperature X N Level ($F_{96, 143}$ =13.56, p=<0.0001)

NH_4^+ (µM)

Effect of N Level ($F_{48, 71}$=3.90, p=0.0143)

Level (µM)		Least Sq. Mean
100	A	93.53
70	AB	87.22
40	AB	83.68
20	B	70.41

Effect of Time ($F_{48, 71}$=34.79, p=<0.0001)

Level (days)		Least Sq. Mean
31	A	105.53
17	B	89.56
41	C	56.04

Effect of Temperature X Time ($F_{112, 167}$=16.76, p=<0.0001)		
Level (°C, days)		**Least Sq. Mean**
24,68	A	24.31
24,55	AB	24.18
24,41	B	23.43
24,17	C	22.40
24,10	C	22.26
24,24	C	22.04
24,31	C	21.78
18,55	D	18.15
18,68	D	18.02
18,24	D	17.91
18,41	D	17.88
18,17	D	17.85
18,10	D	17.78
18,31	D	17.77

41	AB	7.49
17	AB	7.31
31	AB	7.28
24	B	7.14

Level (°C, µM)		**Least Sq. Mean**
24,40	A	23.01
24,100	A	22.90
24,70	A	22.90
24,20	A	22.87
18,100	B	17.91
18,70	B	17.91
18,20	B	17.91
18,40	B	17.90

Effect of Time ($F_{96, 143}$ =25.28, p=<0.0001)		
Level (days)		**Least Sq. Mean**
55	A	21.17
68	A	21.16
41	B	20.66
17	C	20.13
10	C	20.02
24	C	19.98
31	C	19.78

***Levels not connected by the same letter are significantly different**

Appendix 5: Tukey HSD post-hoc results indicating the significant differences which arose from the two-way ANOVA analyzing the effect of Temperature and N level on the *change* of water quality parameters (DO, pH, NH_4^+) for each *C. virginica* incubation timepoint (Day 5, 53, 89) (Experiment 1)

***C. virginica* change in water quality over the course of each incubation event (Experiment 1)**									
Incubation 1 (Day 5)									
Change in DO (mg/L)				Change in pH				Change in NH_4^+ (µM)	
Level (°C / µM)		**Least Sq. Mean**		**Level (°C / µM)**		**Least Sq. Mean**		**Level (°C / µM)**		**Least Sq. Mean**
18,100	A	-1.17		18,100	A	-0.18		18,70	A	19.37
18,20	A	-1.33		24,100	A	-0.18		24,100	A	9.47
18,70	A	-1.73		18,70	A	-0.19		18,20	A	1.37
18,40	A	-1.86		18,20	A	-0.22		18,100	A	-5.05
24,20	A	-1.98		18,40	A	-0.24		18,40	A	-6.53
24,70	A	-1.99		24,70	A	-0.25		24,40	A	-7.69
24,100	A	-2.25		24,40	A	-0.26		24,20	A	-8.42
24,40	A	-2.49		24,20	A	-0.30		24,70	A	-28.11
Incubation 2 (Day 53)									
Change in DO (mg/L)				Change in pH				Change in NH_4^+ (µM)	
Level (°C / µM)		**Least Sq. Mean**		**Level (°C / µM)**		**Least Sq. Mean**		**Level (°C / µM)**		**Least Sq. Mean**
18,100	A	-0.17		18,100	A	-0.05		24,40	A	11.01
24,20	A	-0.66		18,70	A	-0.06		24,20	A	9.20
24,100	A	-0.66		24,40	A	-0.08		18,70	A	8.04
24,40	A	-0.71		24,70	A	-0.08		18,20	A	0.00
18,70	A	-0.74		24,100	A	-0.08		24,100	A	-3.77
18,40	A	-0.76		18,40	A	-0.09		18,100	A	-5.07

24,70	A	-0.81		24,20	A	-0.10		18,40	A	-12.39
18,20	A	-1.11		18,20	A	-0.16		24,70	A	-14.64
Incubation 3 (Day 89)										
Change in DO (mg/L)				Change in pH				Change in NH_4^+ (µM)		
Level (°C / µM)		**Least Sq. Mean**		**Level (°C / µM)**		**Least Sq. Mean**		**Level (°C / µM)**		**Least Sq. Mean**
18,100	A	-0.46		18,100	A	-0.04		24,20	A	13.33
18,70	A	-0.54		24,70	A	-0.09		18,70	A	8.41
18,40	AB	-0.76		18,70	A	-0.09		24,100	A	6.52
24,40	AB	-0.80		24,40	AB	-0.10		18,100	A	-1.81
24,20	AB	-0.85		24,100	AB	-0.11		24,40	A	-3.84
24,70	AB	-1.11		24,20	AB	-0.12		18,20	A	-3.99
24,100	AB	-1.43		18,40	AB	-0.15		18,40	A	-11.96
18,20	B	-1.72		18,20	B	-0.30		24,70	A	-13.84
****Levels not connected by the same letter are significantly different**										

Appendix 6: Results from a Three-Way ANOVA testing the effect of temperature, N level, and time on rates of denitrification and nitrous oxide production of *C. virginica* during Experiment 1

C. virginica rates of denitrification & N_2O Production (Experiment 1)						
Testing the effect of Temperature, N level & Time						
		Denitrification Rates			N_2O Production Rates	
		F-value	p-value		F - value	p-value
Temp		2.53	0.121		1.39	0.243
N Level		3.47	**0.027**		1.79	0.162
Temp * N Level		2.28	0.098		1.59	0.205
Time		0.39	0.535		2.59	0.086
Temp * Time		2.6	0.117		4.07	**0.023**
N Level * Time		2.46	0.081		3.05	**0.013**
Temp * N Level * Time		3.31	**0.032**		1.21	0.317

Appendix 7: Tukey HSD post-hoc results indicating the significant differences which arose from the three-way ANOVA analyzing the effect of Temperature, N level, and time rates of denitrification and N_2O production of *C. virginica* (Experiment 1). Day 89 was excluded from the denitrification analysis due to experimental error

C. virginica Rates of Denitrification and Nitrous Oxide Production (Experiment 1)

Denitrification Rates

Level (°C, µM, days)		Least Sq. Mean
24,40,53	A	314.86
24,40,5	A	157.92
24,70,5	A	143.50
24,20,53	A	135.52
24,100,5	AB	101.561
18,100,5	AB	57.40
24,20,5	AB	41.46
18,20,5	AB	31.70
18,100,53	AB	26.70
18,40,53	AB	20.85
18,20,53	AB	20.76
18,70,53	AB	-22.14
24,100,53	AB	-57.93
18,40,5	AB	-80.01
18,70,5	AB	-139.87
24,70,53	B	-350.00

Nitrous Oxide Production Rates

Level (°C, µM, days)		Least Sq. Mean
18,70,89	A	0.76
24,100,53	AB	0.62
24,100,89	ABC	0.47
18,100,53	ABC	0.41
18,40,5	ABC	0.31
18,70,5	ABC	0.29
24,20,5	ABC	0.25
18,100,89	ABC	0.23
18,20,5	ABC	0.21
24,70,53	ABC	0.21
24,40,89	ABC	0.17
18,40,89	ABC	0.17
24,70,89	ABC	0.16
18,100,5	ABC	0.10
24,40,53	ABC	0.07
18,20,89	ABC	0.06
24,20,89	ABC	0.05
18,20,53	ABC	0.01
24,20,53	ABC	-0.04
18,70,53	ABC	-0.04
24,40,5	ABC	-0.11
24,100,5	ABC	-0.14
18,40,53	BC	-0.18
24,70,5	C	-0.38

***Levels not connected by the same letter are significantly different**

Appendix 8: Tukey HSD post-hoc results indicating the significant differences which arose from the two-way ANOVA analyzing the effect of Temperature and N level at each incubation time point (independently) of denitrification and N_2O production of *C. virginica* (Experiment 1). Day 89 was excluded from the denitrification analysis due to experimental error

***C. virginica* rates of denitrification and N2O production for each incubation event (Experiment 1)**						
Incubation 1 (Day 5)						
Denitrification Rates (nmol / g wet weight / hr)				N_2O production Rates (nmol / g wet weight / hr)		
Level (°C / N level)		**Least Sq. Mean**		**Level (°C / N level)**		**Least Sq. Mean**
24,40	A	157.92		18,40	A	0.31
24,70	A	143.50		18,70	A	0.29
24,100	A	101.56		24,20	A	0.25
18,100	A	57.40		18,20	A	0.21
24,20	A	41.46		18,100	A	0.10
18,20	A	31.70		24,40	A	-0.11
18,40	A	-80.01		24,100	A	-0.14
18,70	A	-139.87		24,70	A	-0.38
Incubation 2 (Day 53)						
Denitrification Rates (nmol / g wet weight / hr)				N_2O production Rates (nmol / g wet weight / hr)		
Level (°C / N level)		**Least Sq. Mean**		**Level (°C / N level)**		**Least Sq. Mean**
24,40	A	314.86		24,100	A	0.62
24,20	A	135.52		18,100	A	0.41
18,100	AB	26.70		24,70	A	0.21
18,40	AB	20.85		24,40	A	0.07
18,20	AB	20.76		18,20	A	0.01
18,70	AB	-22.14		24,20	A	-0.04
24,100	AB	-57.93		18,70	A	-0.04
24,70	B	-350.00		18,40	A	-0.18
Incubation 3 (Day 89)						
Denitrification Rates (nmol / g wet weight / hr)				N_2O production Rates (nmol / g wet weight / hr)		
				Level (°C / N level)		**Least Sq. Mean**
				18,70	A	0.76
				24,100	A	0.47

		18,100	A	0.23
		24,40	A	0.17
		18,40	A	0.17
		24,70	A	0.16
		18,20	A	0.06
		24,20	A	0.05

****Levels not connected by the same letter are significantly different**

Appendix 9: Summary of growth, biomass, and morality over the course of two field seasons (Experiment 2 - 2016 & 2017), anazlyed with a Three-Way ANOVA, testing the effect of location, N level, and field season (year).

Comparison of *C. virginica* growth and mortality rates: 2016 and 2017 field data (Experiment 2)								
Testing the effect of Location, N-level, and Year								
		Growth Rates			**Biomass Gain**		**% Mortality**	
		F-value	*p-value*		*F-value*	*p-value*	*F-value*	*p-value*
Location		0.34	0.571		0.28	0.606	0.19	0.670
N Level		0.25	0.622		5.35	**0.034**	1.79	0.199
Location * N Level		0.08	0.776		0.00	0.996	1.79	0.199
Year		5.43	**0.033**		21.12	**0.0003**	0.09	0.770
Location * Year		16.79	**0.001**		18.95	**0.001**	0.08	0.777
N Level * Year		1.14	0.302		0.32	0.578	0.79	0.386
Location * N Level * Year		0.11	0.742		0.43	0.522	0.78	0.391

Appendix 10: Results from a Three-Way ANOVA which analyzed the effect of location, N-level, and tidal height on the environmental measurements from a "one day profile" sampling event in the 2016 field season (Experiment 2)

Full day profile of 2016 field characteristics (Experiment 2)								
Testing the effect of Location, N Level, & Tidal Height								
	Temperature (°C)			**DO (mg/L)**			**Salinity (ppt)**	
	$F_{40,47}$-*value*	*p-value*		$F_{40,47}$-*value*	*p-value*		$F_{40,47}$-*value*	*p-value*
Location	10.71	**<0.0001**		-0.35	0.726		-20.96	**<0.0001**
N Level	0.48	0.635		-0.22	0.824		1.42	0.163
Location * N Level	-0.43	0.673		-0.50	0.621		0.05	0.961
Tidal Height	-2.85	**0.007**		-2.37	**0.023**		-1.58	0.122
Location * Tidal Height	2.16	**0.037**		-0.57	0.572		1.70	0.097
N Level * Tidal Height	0.15	0.884		-0.36	0.721		0.98	0.331
Location * N Level * Tidal Height	-0.56	0.578		-0.25	0.804		-0.35	0.730
	pH			**Chl-a (µg/L)**			**NH_4^+ (µM)**	
	$F_{40,47}$-*value*	*p-value*		$F_{40,47}$-*value*	*p-value*		$F_{40,47}$-*value*	*p-value*
Location	-0.30	0.769		5.61	**<0.0001**		-2.51	**0.016**
N Level	0.48	0.635		-1.34	0.189		-0.84	0.408
Location * N Level	-0.62	0.540		-1.54	0.132		0.75	0.458
Tidal Height	-2.42	**0.020**		-0.30	0.762		-1.08	0.286
Location * Tidal Height	0.17	0.867		-0.18	0.861		-2.96	**0.005**
N Level * Tidal Height	-0.13	0.900		1.02	0.312		0.67	0.507
Location * N Level * Tidal Height	-0.58	0.568		0.86	0.395		0.19	0.847

Appendix 11: Tukey HSD post-hoc results indicating the significant differences which arose from the three-way ANOVA analyzing the effect of Location, N level, and Tidal height on environmental parameters of Point Judith Pond for the 2016 all day profile (Experiment 2)

Experiment 2: 2016 all day profile of environmental parameters

Temperature (°C)

Level (Location, N level, Tidal Height)		Least Sq. Mean
Northern, Ambient, High	A	22.91
Northern, Enriched, High	A	22.86
Northern, Enriched, Low	A	22.83
Northern, Ambient, Low	A	22.79
Southern, Ambient, High	B	21.95
Southern, Enriched, High	B	21.93
Southern, Ambient, Low	B	21.47
Southern, Enriched, Low	B	21.29

pH

Level (Location, N level, Tidal Height)		Least Sq. Mean
Southern, Ambient, High	A	8.06
Northern, Ambient, High	A	8.03
Southern, Enriched, High	A	8.02
Northern, Enriched, High	A	7.99
Southern, Ambient, Low	A	7.93
Northern, Enriched, Low	A	7.90
Northern, Ambient, Low	A	7.85
Southern, Enriched, Low	A	7.84

DO (mg/L)

Level (Location, N level, Tidal Height)		Least Sq. Mean
Northern, Enriched, High	A	7.71
Southern, Ambient, High	A	7.66
Northern, Ambient, High	A	7.64
Southern, Enriched, High	A	7.44

Chl-a (µg/L)

Level (Location, N level, Tidal Height)		Least Sq. Mean
Northern, Enriched, High	A	27.46
Northern, Enriched, Low	AB	20.24
Northern, Ambient, Low	AB	17.21
Northern, Ambient, High	AB	12.92

Southern, Ambient, Low	A	6.56		Southern, Ambient, Low	B	2.70
Southern, Enriched, Low	A	6.47		Southern, Ambient, High	B	2.59
Northern, Enriched, Low	A	6.37		Southern, Enriched, High	B	2.47
Northern, Ambient, Low	A	5.61		Southern, Enriched, Low	B	1.58

Salinity (ppt)				NH_4^+ (µM)		
Level (Location, N level, Tidal Height)		**Least Sq. Mean**		**Level (Location, N level, Tidal Height)**		**Least Sq. Mean**
Southern, Ambient, High	A	30.90		Southern, Enriched, Low	A	26.57
Southern, Enriched, High	A	30.89		Southern, Ambient, Low	AB	24.67
Southern, Ambient, Low	A	30.81		Southern, Enriched, High	AB	24.18
Southern, Enriched, Low	A	30.70		Northern, Enriched, High	AB	24.00
Northern, Ambient, Low	B	29.99		Northern, Ambient, High	AB	22.38
Northern, Ambient, High	B	29.96		Southern, Ambient, High	AB	20.65
Northern, Enriched, High	B	29.93		Northern, Ambient, Low	AB	16.95
Northern, Enriched, Low	B	29.91		Northern, Enriched, Low	B	15.62

***Levels not connected by the same letter are significantly different**

Appendix 12: Results from 2017's field season (Experiment 2), highlighting the significant differences between location, month, and tidal height on environmental parameters

	2017 field water quality (Experiment 2)							
	Testing the effect of location, month, and tidal height on 2017's field parameters							
	Temperature (°C)		Salinity (ppt)		DO (mg/L)		Chl-a (RFU)	
	$F_{1923,1934}$-values	p-values	$F_{1923,1934}$-values	p-values	$F_{1923,1934}$-values	p-values	$F_{923,1934}$-values	p-values
Location	1349.96	**<0.0001**	1160.55	**<0.0001**	240.71	**<0.0001**	147.30	**<0.0001**
Month	147.74	**<0.0001**	637.37	**<0.0001**	81.38	**<0.0001**	1.56	0.211
Location*Month	26.29	**<0.0001**	816.42	**<0.0001**	22.45	**<0.0001**	5.64	**0.004**
Tidal Height	209.63	**<0.0001**	41.04	**<0.0001**	10.07	**0.0015**	15.15	**0.001**
Location*Tidal Height	118.41	**<0.0001**	2.33	0.127	5.62	**0.018**	5.97	**0.015**
Month*Tidal Height	5.37	**0.0047**	7.38	**0.0006**	21.57	**<0.0001**	1.62	0.198
Location*Month *Tidal Height	0.19	0.825	10.51	**<0.0001**	4.25	**0.014**	1.48	0.228

Appendix 13: Tukey HSD post-hoc results indicating the significant differences which arose from the three-way ANOVA analyzing the effect of Location, Month, and Tidal height on environmental parameters of Point Judith Pond for the 2017 field season (Experiment 2)

Experiment 2: 2017 environmental parameters

Temperature (°C)

Level (Location, Month, Tidal height)		Least Sq. Mean
Northern, July, Low	A	25.04
Northern, July, High	AB	24.75
Northern, August, Low	B	24.66
Northern, August, High	B	24.62
Northern, June, Low	C	23.36
Southern, August, Low	C	23.07
Northern, June, High	CD	22.86
Southern, July, Low	D	22.50
Southern, June, Low	E	21.56
Southern, August, High	E	21.48
Southern, July, High	F	20.51
Southern, June, High	G	19.34

DO (mg/L)

Level (Location, Month, Tidal height)		Least Sq. Mean
Southern, June, Low	AB	8.07
Southern, July, Low	A	8.06
Southern, June, High	AB	8.00
Southern, July, High	AB	7.89
Northern, June, Low	ABC	7.54
Northern, June, High	ABC	7.46
Southern, August, High	B	7.29
Southern, August, Low	C	6.81
Northern, August, High	D	6.14
Northern, July, High	E	5.59
Northern, July, Low	E	5.46
Northern, August, Low	F	4.50

Salinity (ppt)

Level (Location, Month, Tidal height)		Least Sq. Mean
Northern, August, Low	A	32.43
Northern, August, High	A	32.34
Southern, July, High	AB	32.29

Chl - a (RFU)

Level (Location, Month, Tidal height)		Least Sq. Mean
Northern, August, High	A	7.39
Northern, July, High	AB	6.33
Northern, June, High	ABCD	4.88

Southern, July, Low	ABC	32.11		Northern, August, Low	C	4.59
Southern, August, High	BC	31.98		Northern, July, Low	C	4.09
Southern, June, High	ABCD	31.92		Northern, June, Low	BCDE	3.68
Southern, August, Low	D	31.64		Southern, July, High	CDEF	2.56
Southern, June, Low	CD	31.53		Southern, June, Low	DEF	1.35
Northern, July, High	E	28.96		Southern, August, High	F	0.87
Northern, June, High	E	28.90		Southern, July, Low	EF	0.80
Northern, July, Low	F	28.28		Southern, June, High	EF	0.80
Northern, June, Low	F	28.04		Southern, August, Low	F	0.65

***Levels not connected by the same letter are significantly different**

Appendix 14: Results from a Three-Way ANOVA analyzing the effect of incubation temperature, field location, and field N-level on the rates of denitrification and nitrous oxide production of the 2017 field oyster incubation

2017 field results: denitrification and N_2O production rates (Experiment 2)					
testing the effect of incubation temperature, field location, and field N-level on C. virginica rates of denitrification and N_2O production					
	Denitrification			**Nitrous Oxide**	
	F-values	*p-values*		*F-values*	*p-values*
Incubation Temperature	1.11	0.285		-2.76	**0.014**
Field Location	-0.49	0.629		0.76	0.460
Incubation Temperature * Field Location	-1.78	0.093		-1.56	0.138
Field N-level	0.85	0.406		0.75	0.465
Incubation Temperature * Field N-level	-2.92	**0.010**		-0.44	0.665
Field Location * Field N-level	0.80	0.434		-0.20	0.845
Incubation Temperature * Field Location * Field N-level	0.84	0.413		0.01	0.991

Appendix 15: Parameter estimates of denitrification and N_2O production rates (Experiment 2, 2017)

Experiment 2: 2017 Denitrification and Nitrous Oxide Production Rates									
	Denitrification Rates					**Nitrous Oxide Production Rates**			
Term	**Estimate**	**Std Error**	**t Ratio**	**Prob> |t|**		**Estimate**	**Std Error**	**t Ratio**	**Prob> |t|**
Intercept	34.14	17.73	1.93	0.07		0.07	0.02	3.46	**0.003**
Field Location[Northern]	-8.73	17.73	-0.49	0.63		0.01	0.02	0.76	0.46
Field Treatment[Ambient]	15.13	17.73	0.85	0.41		0.01	0.02	0.75	0.47
Field Location[Northern]*Field Treatment[Ambient]	14.24	17.73	0.80	0.43		-0.004	0.02	-0.2	0.85
Incubation Temperature[18]	19.61	17.73	1.11	0.29		-0.05	0.02	-2.76	**0.01**
Field Location[Northern]*Incubation Temperature[18]	-31.63	17.73	-1.78	0.09		-0.03	0.02	-1.56	0.14
Field Treatment[Ambient]*Incubation Temperature[18]	-51.77	17.73	-2.92	**0.01**		-0.01	0.02	-0.44	0.67
Field Location[Northern]*Field Treatment[Ambient]*Incubation Temperature[18]	14.90	17.73	0.84	0.41		-0.0002	0.02	-0.01	0.99

Appendix 16: Results from a three way ANOVA, analyzing the impact of temperature, N level, and time on *M. edulis* mortality rates

M. edulis mortality rates			
testing the effect of temperature, N level, and time			
		$F_{80,119}$	p-value
Temp		0.29	0.592
N Level		8.86	**<0.0001**
Temp * N Level		1.32	0.273
Time		2.79	**0.032**
Temp * Time		0.31	0.870
N Level * Time		0.98	0.478
Temp * N Level * Time		0.32	0.984

Appendix 17: Tukey HSD post-hoc results indicating the significant differences between N levels on *M. edulis* % mortality.

M. edulis % Mortality		
Effect of N level $F_{80,119}$ =8.86, p=<0.0001		
Level (µM)		**Least Sq. Mean**
17.5	A	0.11
25	B	0.03
10	B	0.01
5	B	0.003
*Levels not connected by the same letter are significantly different		

Appendix 18: Results analyzing the effect of temperature, N level, and time on the average water quality parameters of *M. edulis'* aquaria over the course of the 3 month experiment

Average water quality parameters over the course of *M. edulis* laboratory experiment											
Testing the effect of Temperature, N Level, & Time											
		Temperature (°C)			**DO (mg/L)**			**pH**			**NH_4^+ (µM)**	
		$F_{176,263}$-*value*	*p-value*		$F_{176,263}$-*value*	*p-value*		$F_{112,167}$-*value*	*p-value*		$F_{64,95}$-*value*	*p-value*
Temp		6822.38	**<0.0001**		0.48	0.492		1.35	0.248		0.07	0.793
N Level		1.06	0.367		10.44	**<0.0001**		0.26	0.857		1.72	0.171
Temp * N Level		0.24	0.872		6.80	**0.0002**		0.23	0.876		1.12	0.347
Time		10.59	**<0.0001**		12.97	**<0.0001**		4.99	**0.0001**		8.65	**<0.0001**
Temp * Time		3.05	**0.001**		2.77	**0.003**		1.81	0.103		1.49	0.227
N Level * Time		0.07	1.000		1.37	0.111		0.06	1.000		1.62	0.129
Temp * N Level * Time		0.07	1.000		0.86	0.676		0.17	0.999		2.55	**0.014**

Appendix 19: Tukey HSD post-hoc results indicating the significant differences which arose from the three-way ANOVA analyzing the effect of Temperature, N-level and Time on the average water quality parameters (Temperature, DO, pH, NH_4^+) over the course of the 3-month, *M. edulis* laboratory-based study

M. edulis water quality over the course of 3-month laboratory experiment

Temperature (°C)			DO (mg/L)			pH			NH_4^+ (µM)		
Level (°C, µM, days)		**Least Sq. Mean**	**Level (°C, µM, days)**		**Least Sq. Mean**	**Level (°C, µM, days)**		**Least Sq. Mean**	**Level (°C, µM, days)**		**Least Sq. Mean**
21,17.5,61	A	21.10	21,5,28	A	8.56	18,5,75	A	8.08	21,5,48	A	78.51
21,10,61	AB	21.03	21,5,56	AB	8.40	21,10,56	A	8.01	21,10,56	A	76.80
21,5,61	ABC	20.97	18,10,28	AB	8.38	21,5,56	A	8.01	21,17.5,48	A	72.67
21,17.5,75	ABC	20.90	21,10,28	AB	8.38	18,17.5,56	A	7.96	21,25,48	A	69.24
21,25,61	ABC	20.87	18,17.5,28	AB	8.35	21,5,69	A	7.96	18,17.5,48	A	68.94
21,5,75	ABC	20.80	18,5,28	ABC	8.17	21,17.5,56	A	7.95	18,25,61	A	68.84
21,10,75	ABC	20.73	21,10,56	ABCD	8.12	21,5,48	A	7.95	18,25,48	A	68.64
21,25,75	ABC	20.73	21,17.5,28	ABCD	8.11	18,10,56	A	7.94	18,5,56	A	67.43
21,17.5,16	ABC	20.67	21,5,69	ABCD	8.11	21,5,61	A	7.94	18,25,28	A	67.02
21,25,16	ABC	20.67	21,5,16	ABCD	8.06	21,17.5,69	A	7.93	21,10,48	A	64.40
21,10,16	ABC	20.63	21,25,28	ABCD	8.05	18,10,48	A	7.92	18,10,48	A	63.70
21,17.5,69	ABC	20.60	18,10,56	ABCD	8.04	21,10,69	A	7.92	18,17.5,28	A	61.48
21,5,16	ABC	20.57	21,5,3	ABCD	7.99	18,10,61	A	7.91	21,17.5,28	A	61.08
21,17.5,48	ABC	20.57	18,25,28	ABCD	7.97	18,25,56	A	7.91	21,17.5,61	A	57.35
21,17.5,56	ABC	20.53	18,25,56	ABCD	7.97	21,10,61	A	7.91	21,25,28	A	56.34
21,17.5,20	ABC	20.50	21,17.5,56	ABCDE	7.87	18,5,48	A	7.91	18,10,28	A	56.04
21,5,48	ABC	20.50	18,17.5,48	ABCDE	7.83	21,5,75	A	7.901	21,25,56	A	54.43

21,5,56	ABC	20.50		18,10,16	ABCDE	7.81		18,25,69	A	7.90		18,10,61	A	52.51
21,5,69	ABC	20.50		18,25,69	ABCDE	7.79		21,17.5,61	A	7.89		18,25,56	A	52.41
21,10,69	ABC	20.47		21,5,7	ABCDE	7.79		21,25,56	A	7.89		18,5,48	A	52.41
21,10,48	ABC	20.43		21,10,69	ABCDE	7.77		21,25,69	A	7.883		21,10,28	A	50.19
21,25,69	ABC	20.43		18,10,7	ABCDE	7.77		21,10,75	A	7.88		21,25,61	A	49.89
21,10,20	ABC	20.43		21,17.5,69	ABCDE	7.74		18,25,48	A	7.88		21,5,61	A	47.87
21,10,56	ABC	20.43		18,10,48	ABCDE	7.73		21,10,48	A	7.87		21,17.5,56	A	47.87
21,25,48	ABC	20.40		21,5,48	ABCDE	7.69		21,17.5,75	A	7.86		18,10,56	A	47.77
21,5,20	ABC	20.40		21,5,0	ABCDE	7.69		18,10,69	A	7.86		18,5,28	A	47.37
21,25,20	ABC	20.37		21,5,61	ABCDE	7.67		18,5,61	A	7.86		18,17.5,61	A	47.17
21,25,56	ABC	20.37		18,5,56	ABCDE	7.65		18,5,69	A	7.85		21,5,28	A	44.95
21,25,0	ABC	20.33		18,25,48	ABCDE	7.63		21,17.5,48	A	7.84		21,5,56	A	43.24
21,17.5,0	ABC	20.33		18,10,61	ABCDE	7.62		18,17.5,75	A	7.84		18,5,61	A	42.63
21,10,0	ABC	20.30		18,5,20	ABCDE	7.61		18,17.5,69	A	7.83		18,17.5,56	A	41.83
21,5,0	ABC	20.27		18,25,7	ABCDE	7.57		18,17.5,61	A	7.83		21,10,61	A	41.63
21,17.5,3	ABC	20.23		21,25,69	ABCDE	7.56		18,10,75	A	7.83				
21,5,28	ABC	20.23		21,10,16	ABCDE	7.54		21,25,48	A	7.82				
21,25,3	ABC	20.20		18,17.5,56	ABCDE	7.54		21,25,75	A	7.81				
21,17.5,7	ABC	20.20		21,25,56	ABCDE	7.51		21,25,61	A	7.80				
21,10,3	ABC	20.17		18,17.5,61	ABCDE	7.48		18,25,75	A	7.80				
21,5,3	ABC	20.17		18,5,69	ABCDE	7.45		21,17.5,7	A	7.77				
21,17.5,28	ABC	20.13		18,5,16	ABCDE	7.45		18,25,61	A	7.77				
21,5,7	ABC	20.13		18,10,20	ABCDE	7.41		18,5,56	A	7.73				
21,25,28	BC	20.03		18,10,75	ABCDE	7.39		21,25,7	A	7.69				
21,10,28	BC	20.03		18,25,3	ABCDE	7.39		21,10,7	A	7.68				
21,10,7	BC	20.03		21,17.5,61	ABCDE	7.39		18,5,0	A	7.63				
21,25,7	C	20.00		21,25,16	ABCDE	7.37		18,17.5,48	A	7.62				
18,10,61	D	17.73		21,10,3	ABCDE	7.34		21,5,7	A	7.44				
18,5,61	D	17.70		18,10,69	ABCDE	7.34		21,10,0	A	7.29				
18,17.5,61	D	17.67		18,17.5,69	ABCDE	7.32		18,25,0	A	7.29				
18,25,61	D	17.67		18,25,16	ABCDE	7.30		18,5,7	A	7.28				

18,5,16	D	17.67	18,25,0	ABCDE	7.30	18,10,0	A	7.23	
18,17.5,16	D	17.67	21,10,7	ABCDE	7.29	18,17.5,0	A	7.20	
18,25,16	D	17.67	18,5,48	ABCDE	7.28	21,5,0	A	7.03	
18,17.5,69	D	17.63	21,10,61	ABCDE	7.28	21,25,0	A	6.93	
18,10,16	D	17.63	21,5,75	ABCDE	7.27	21,17.5,0	A	6.92	
18,17.5,75	D	17.60	21,17.5,3	ABCDE	7.26	18,25,7	A	6.46	
18,10,69	D	17.60	21,25,3	ABCDE	7.24	18,10,7	A	6.32	
18,25,69	D	17.60	21,10,20	ABCDE	7.23	18,17.5,7	A	6.19	
18,5,0	D	17.60	18,5,7	ABCDE	7.19				
18,17.5,0	D	17.60	21,10,48	ABCDE	7.18				
18,5,69	D	17.60	18,25,61	ABCDE	7.15				
18,17.5,56	D	17.57	18,17.5,75	ABCDEF	7.10				
18,10,0	D	17.57	18,25,20	ABCDEF	7.08				
18,10,56	D	17.57	18,10,0	ABCDEF	7.07				
18,5,56	D	17.57	18,17.5,7	ABCDEF	7.05				
18,17.5,3	D	17.53	21,25,7	ABCDEF	7.05				
18,25,0	D	17.53	21,10,75	ABCDEF	7.05				
18,25,56	D	17.53	18,17.5,20	ABCDEF	7.03				
18,5,75	D	17.50	18,5,61	ABCDEF	7.01				
18,25,20	D	17.50	21,17.5,48	ABCDEF	7.01				
18,5,3	D	17.50	21,17.5,16	ABCDEF	6.99				
18,5,20	D	17.50	21,17.5,75	ABCDEF	6.99				
18,17.5,20	D	17.47	18,10,3	ABCDEF	6.98				
18,10,20	D	17.47	18,25,75	ABCDEF	6.97				
18,10,75	D	17.47	21,25,61	ABCDEF	6.96				
18,10,3	D	17.43	18,5,0	ABCDEF	6.96				
18,17.5,48	D	17.40	21,25,48	ABCDEF	6.89				
18,25,3	D	17.40	21,25,75	ABCDEF	6.85				
18,25,75	D	17.37	21,5,20	ABCDEF	6.83				
18,10,48	D	17.37	21,25,0	ABCDEF	6.82				
18,5,48	D	17.37	18,5,3	ABCDEF	6.81				

18,25,48	D	17.33	18,5,75	ABCDEF	6.74		
18,5,7	D	17.33	21,10,0	ABCDEF	6.71		
18,17.5,7	D	17.30	21,17.5,7	ABCDEF	6.69		
18,5,28	D	17.30	21,25,20	ABCDEF	6.68		
18,17.5,28	D	17.30	18,17.5,0	BCDEF	6.54		
18,25,28	D	17.30	21,17.5,20	CDEF	6.22		
18,25,7	D	17.30	21,17.5,0	DEF	6.19		
18,10,28	D	17.30	18,17.5,3	EF	5.95		
18,10,7	D	17.30	18,17.5,16	F	5.18		

****Levels not connected by the same letter are significantly different**

Appendix 20: Tukey HSD post-hoc results indicating the significant differences which arose from the two-way ANOVA analyzing the effect of Temperature and N level on the *change* of water quality parameters (DO, pH, NH_4^+) for each *M. edulis* incubation timepoint (Day 2, 53, 105)

***M. edulis* <u>change in</u> water quality over the course of each incubation event**								
Incubation 1 (Day 2)								
Change in DO (mg/L)			Change in pH			Change in NH_4^+ (µM)		
Level (°C / µM)		**Least Sq. Mean**	**Level (°C / µM)**		**Least Sq. Mean**	**Level (°C / µM)**		**Least Sq. Mean**
18,10	A	-3.77	18,25	A	7.58	21,25	A	145.95
18,25	A	-3.90	18,10	AB	7.57	21,5	A	38.06
18,17.5	AB	-4.03	18,17.5	ABC	7.54	21,10	A	32.12
18,5	AB	-4.26	18,5	ABC	7.51	18,5	A	20.25
21,5	AB	-4.76	21,5	ABC	7.45	18,25	A	16.76
21,25	AB	-5.07	21,17.5	ABC	7.44	21,17.5	A	1.05
21,17.5	AB	-5.07	21,25	BC	7.42	18,10	A	-0.35
21,10	B	-5.43	21,10	C	7.40	18,17.5	A	-5.59
Incubation 2 (Day 53)								
Change in DO (mg/L)			Change in pH			Change in NH_4^+ (µM)		
Level (°C / µM)		**Least Sq. Mean**	**Level (°C / µM)**		**Least Sq. Mean**	**Level (°C / µM)**		**Least Sq. Mean**
21,5	A	-1.33	18,17.5	A	0.003	18,25	A	18.84
18,25	A	-1.41	18,10	AB	-0.09	18,17.5	A	16.19
21,10	A	-1.86	18,25	AB	-0.11	21,5	A	15.53
21,17.5	A	-2.33	21,25	AB	-0.17	21,10	A	6.03
18,5	A	-2.43	18,5	AB	-0.178	21,25	A	2.97
18,17.5	A	-2.62	21,17.5	AB	-0.20	21,17.5	A	-4.461
21,25	A	-2.92	21,5	AB	-0.27	18,5	A	-16.61
18,10	A	-2.99	21,10	B	-0.30	18,10	A	-27.26
Incubation 2 (Day 105)								
Change in DO (mg/L)			Change in pH			Change in NH_4^+ (µM)		

Level (°C / µM)		Least Sq. Mean	Level (°C / µM)	Least Sq. Mean	Level (°C / µM)		Least Sq. Mean
18,10	A	0.30	**No data.**		18,5	A	8.38
18,25	AB	-0.04			21,5	A	6.75
21,5	AB	-0.54			21,10	A	2.45
21,17.5	AB	-0.74			18,25	A	2.30
21,10	AB	-0.76			18,17.5	A	1.19
18,5	AB	-0.91			21,17.5	A	0.44
21,25	AB	-0.94			18,10	A	0.22
18,17.5	B	-2.02			21,25	A	-0.22

****Levels not connected by the same letter are significantly different**

Appendix 21: Results from a three way ANOVA, analyzing the effect of temperature, N level, and time on rates of denitrification for each *M. edulis* incubation experiment (Day 2, 53, 105).

M, edulis denitrification rates		
testing the effect of Temperature, N Level & Time		
	$F_{48,71}$ - value	p-value
Temp	1.04	0.312
N Level	1.02	0.394
Temp * N Level	1.03	0.387
Time	1.19	0.312
Temp * Time	1.01	0.371
N Level * Time	0.99	0.442
Temp * N Level * Time	0.94	0.477

Appendix 22: Results from a three way ANOVA, analyzing the effect of temperature, N level, and time on rates of nitrous oxide production for each *M. edulis* incubation experiment (Day 2, 53, 105).

M, edulis N_2O production rates		
testing the effect of Temperature, N Level & Time		
	$F_{48,71}$ - value	p-value
Temp	5.17	**0.027**
N Level	3.22	**0.031**
Temp * N Level	1.47	0.224
Time	1.52	0.229
Temp * Time	1.71	0.191
N Level * Time	1.31	0.272
Temp * N Level * Time	1.62	0.162

Appendix 23: Tukey HSD post-hoc results indicating the significant differences which arose from the three-way ANOVA analyzing the effect of Temperature, N level, and time rates of denitrification and N_2O production of *M. edulis*

***M. edulis* Rates of Denitrification and Nitrous Oxide Production**						
Denitrification Rates				**Nitrous Oxide Production Rates**		
Level (°C, µM, days)		**Least Sq. Mean**		**Level (°C, µM, days)**		**Least Sq. Mean**
18,25,2	A	295.76		18,25,53	A	0.66
18,5,105	A	219.14		18,25,105	AB	0.30
21,5,2	A	207.29		18,17.5,53	AB	0.27
18,25,105	A	206.25		21,25,105	AB	0.22
21,10,5	A	184.28		18,17.5,105	AB	0.21
21,17.5,53	A	168.81		18,10,105	AB	0.20
18,10,3	A	163.48		18,5,2	B	0.19
18,10,105	A	161.86		21,10,2	B	0.18
21,17.5,2	A	145.31		21,17.5,105	B	0.18
18,17.5,2	A	134.17		21,17.5,2	B	0.16
21,17.5,105	A	120.93		21,17.5,53	B	0.15
18,5,2	A	93.54		18,10,53	B	0.15
21,10,53	A	88.82		18,25,2	B	0.15
21,25,2	A	78.57		18,10,2	B	0.14
21,5,53	A	70.09		21,25,53	B	0.14
18,10,53	A	51.36		21,5,53	B	0.13
18,5,53	A	49.93		21,10,53	B	0.13
21,10,105	A	48.34		18,5,53	B	0.12
21,25,105	A	9.44		21,25,2	B	0.12
18,17.5,53	A	0.00		18,17.5,2	B	0.12
21,25,53	A	-21.21		21,5,105	B	0.12
21,5,105	A	-30.94		18,5,105	B	0.11
18,25,53	A	-52.59		21,5,2	B	0.10
18,17.5,105	A	-83.64		21,10,105	B	0.03
***Levels not connected by the same letter are significantly different**						

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