ASSESSMENT OF METHODS AND ENVIRONMENTAL INFLUENCE ON THE *NEMATOSTELLA VECTENSIS* MICROBIOME

by

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ABSTRACT

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Assessment of Methods and Environmental Influence on the *Nematostella Vectensis* Microbiome
(Under the direction of ADAM M. REITZEL)

Cnidarians are a diverse clade of marine invertebrates that inhabit a variety of environments. The conditions of these habitats are becoming more extreme with the progression of time. These organisms associate with bacteria, which are composed of a larger community, known as the microbiome. To better understand the interactions between individual bacterial isolates and the model cnidarian *Nematostella vectensis*, it is imperative to investigate and develop methodologies. Here, the impacts of antibiotics were quantified throughout the life stages with a variety of methods. Antibiotic treatment effectively eliminates the resident bacteria of *N. vectensis,* though the anemone experiences transcriptional changes, even after removal of the antibiotics. Additionally, two methods to vector bacteria to the terminal host were quantitatively compared: Prey Feeding Method (PFM), and Solution Uptake Method (SUM). The PFM resulted in higher sustained concentrations through two weeks, indicating its potential as a viable method to vector bacteria. Lastly, part of the culturable microbiome was assessed for viability through thermal and saline stressors. Investigation of these methods is imperative to quantifying the interactions between bacteria and the host organism. Together, the assessment of common methodologies in a cnidarian model contributes directly to understanding individual bacteria from the microbiome of *N. vectensis*.

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Introduction

Microbiomes

Microbes are present in all ecosystems and the summation of these microorganisms create the microbiome. Individuals of the microbial community can exhibit greater growth under certain conditions, which can differ greatly between closely related species. The current and recent states an ecosystem can be a predictive model for the composition of the microbiome (Wagg et al. 2019), which will influence the interactions that a microbial community can have with each other and the environment (Posadas et al. 2022). The environment will have large effects on the ability for bacteria to be part of a microbial community, including the microbiome associated with a particular host. If a bacteria species is outside of its survivable range, mortality will eliminate any possible interactions unless the associations it has with other bacteria or the host extend the range of conditions due to a mutualism. Some organisms evade these difficult and potentially deadly conditions through various mechanisms such as sporulation (Nicholson et al. 2000) or entering a Viable But Non-Culturable (VBNC) state (Ramamurthy et al. 2014). These cells enter a dormant state, to preserve the integrity of cellular systems until the stressors have been removed (McKenney et al. 2013). Alternatively, if an organism is found in its optimal condition, it can interact with other microbes with similar niches and the surrounding environment. Considering these factors we can assess the composition of the microbiome, and potentially determine their associations and interactions with the environment and their hosts.

Individual bacteria can combat other bacteria and eukaryotic organisms through various mechanisms. *Vibrio* species, among others, have acquired different secretion systems to send effectors directly into target cells. Type III Secretion System (T3SS) can transport effectors into eukaryotic cells, potentially causing cell death (Coburn et al. 2007). Various members of the *Vibrio* genus contribute to pathogenicity in a variety of marine invertebrates including corals (Munn 2015), oysters (Destoumieux-Garzón et al. 2020), and fish (Egidius et al. 1986; Frans et al. 2011). Additionally, some *Vibrio* species have acquired through horizontal gene transfer another secretion system that targets prokaryotic cells. Type VI Secretion System (T6SS) can limit competing sensitive bacterial populations by directly injecting effectors into adjacent cells (Navarro-Garcia et al. 2019). Alternatively, other organisms, such as *Bacillus velezensis* has proven to be an effective probiotic that can protect against pathogenic challenges via control of oxidative stress or production of antibiotics and antifungal compounds (Emam & Dunlap 2020; Thurlow et al. 2019). Additionally, other bacteria such as species within the *Pseudoalteromonas* genus have antagonistic properties against other bacteria via antibiotic production and lytic mechanisms (Hamid 2020; Holmström & Kjelleberg 1999; Long & Azam 2001; Tang et al. 2020). Bacteria that have different methods of controlling bacterial populations may be ideal candidates for usage in host organisms.

The holobiont, aggregate host and its respective microbiome, can interact with each other and the environment. The interactions between the 'native' microbiome and the respective host organism have proven useful in describing behavior and other mechanisms in the host (Dirksen et al. 2016; Peixoto et al. 2017; Weiland-Bräuer et al. 2020). Many factors can influence the composition of the microbiome including

temperature (Ketchum et al. 2021), salinity (Röthig et al. 2016), and geographic distribution (Faddetta et al. 2020; Williams et al. 2022). While the microbiome can be specific to an individual, their microbiome can be distinct from its environment (Carrier & Reitzel 2019). Many organisms in the environment experience modulation of the microbiome through these variable biotic and abiotic factors. These effects can be difficult to parse, especially in the context of marine systems.

Marine invertebrate microbiomes

Short-term fluctuations and trends of temperature and salinity across time can make environments difficult to survive. Many corals inhabit these extreme environments, and typically have distinct life stages to allow for movement, settlement, and growth phases (Vermeij et al. 2006). Gametes, embryos, and larvae disperse with water circulation patterns, while many adult species are incapable of movement after settlement and establishment and become immobile after development from the juvenile stage (Kaufman et al. 1992). As cnidarians transition from larvae to juveniles, the organism develops tissue layers and cell types with different functions that serve as a habitat for differential microbial communities that serve vastly different functions. For example, the mucosal surfaces influence the bacterial communities on corals, which is a significant factor when responding to stress (Glasl et al. 2016). In many marine invertebrates, some microbes are pathogenic to host organisms which can harm or induce mortality (Brennan et al. 2017; De O Santos et al. 2011; Diaz & Restif 2014; Sweet et al. 2014; Yang et al. 2021; Zhenyu et al. 2013).

Microbes that are active in the host organism are potentially capable of extending the survivable threshold of the host organism they reside in. The microbiome of marine invertebrates continues to change over time and reacts to stressors such as temperature (Connelly et al. 2022; Meron et al. 2020; Santoro et al. 2021), salinity (Röthig et al. 2016), and invading pathogens (Fraune et al. 2015; Peixoto et al. 2017; Tang et al. 2020). If bacteria can influence the conditions a host experiences to eliminate or reduce mortality, it is vital to describe what potential organisms' capabilities are to extend the hosts environmental tolerance. The relative abundance of bacteria in a host organism modulates with various stressors such as heat and can serve as an indicator for the overall health of the host (Gardner et al. 2019; Meron et al. 2020; Ziegler et al. 2017). As the temperature of the environment increases, the microbiome of the host modulates to acclimate by modifying the phylogenetic structure to adapt to the conditions (Santoro et al. 2021). Additionally, the host can initiate an immune response to modulate the microbiome (Augustin et al. 2010; Miller et al. 2007; Parisi et al. 2020; Poole et al. 2016; Sampath 2018). The cumulative response the host and its respective microbiome has to stress has been characterized in some marine invertebrates (Connelly et al. 2022; Meron et al. 2020; Santoro et al. 2021). Assessment of the bacterial individuals that constitute the microbiome is imperative to implement effective interventions for animals under stress.

Traditional approaches for microbiome control

Antibiotics are a common approach for the elimination of microorganisms in a host animal. Historically antibiotics have been a common prescribed method for

maintaining microbial populations in aquaculture (Chen et al. 2020). White Band Disease (WBD) found in corals can be attributed to *Vibrio* sp., where antibiotic application has been a common method utilized to prevent the spread of necrotic tissue (Sweet et al. 2014). Antibiotics have been effective in some systems, but bacteria that persist may survive the treatment. A variety of organisms have been detected after antibiotic treatment including members of the families Rhodobacteraceae and Alteromonadaceae in *Pocillopora* (Connelly et al. 2022). Additionally in *Euphyllia,* members found in the alphaproteobacteria and gammaproteobacterial were most prevalent after treatment (Meron et al. 2020). The persistence of bacteria in combination with little characterization of off target effects on the host introduces skepticism in implanting antibiotics in aquaculture.

Antibiotics are a common intervention method for inhibition of microorganisms, but the known effects on host organisms are limited (Connelly et al. 2022; Glasl et al. 2016; Sweet et al. 2014; Yang et al. 2020). While effective, there is limited research regarding the implications in the host organisms. More specifically in invertebrates, the direct effect on the host has limited scope in current literature (Connelly et al. 2022; Hartman et al. 2022; Meron et al. 2020; Neely et al. 2020; Salgueiro et al. 2021; Sweet et al. 2014; Watson et al. 1997). While antibiotics can successfully prevent mortality in diseased corals, the short-term and potentially long-term functional changes to the host are not known. Additionally, typical treatment regimen for infected individuals involves utilizing high concentrations of antibiotics, and occasionally combining multiple compounds to target various processes distinct to prokaryotic organisms. As these compounds are directly affecting prokaryotic organisms, there may be additional targets

in eukaryotic cells, such as in the mitochondria (Miller & Singer 2022). To determine the overall impact of antibiotics on a host organism, it is necessary to determine the changes in the host at the transcriptional level, since antibiotics do not typically result in mortality. With the rise of antibiotic resistance, an alternative in the context of aquaculture is imperative for pathogen control. Antibiotic resistance can result in bacterial species surviving and thriving in hosts that have been treated, while other microbes may be eliminated and can no longer assist in protecting the host. In the environment, antibiotic application is limited in feasibility, especially in aquatic systems, as dilution and dissipation are a major risk to generating antibiotic resistance. With these drawbacks, a more effective and long-term solution is necessary. Probiotics may be the solution to solving pathogenesis in aquaculture and cnidarian applications.

Emerging remedy, probiotics

An alternative to antibiotic methods to control pathogens is the application of beneficial microorganisms to the host. Some organisms have been established as 'probiotic' in nature, such as *Pseudoalteromonas*, which exhibits antagonistic properties such as antibiotic production, provides settlement cues for a variety of organisms (Sneed et al. 2014; Tebben et al. 2011; Unabia & Hadfield 1999), and have redox mechanisms (Yu et al. 2013). These probiotic organisms can potentially outcompete the pathogenic microbes and become a sustained source of pathogen defense. Additionally, the probiotic bacteria that are transferred to diseased organisms require a high enough concentration to effectively combat pathogens (Vega & Gore 2017). The stoichiometric dependence and persistence of bacteria in the environment indicates the need to assess transmission

dynamics of bacteria to the terminal animal. The viability of these probiotic organisms may be dependent on the conditions of the system they inhabit. This indicates the interactions that would occur between a virulent microbe and a probiotic is also dependent on the system's conditions.

Several members of the *Bacillus* genus have been identified as probiotics (Emam & Dunlap 2020; Soltani et al. 2019). Specific members of the *Bacillus* genus can associate with these hosts and have been found to exhibit several functions. In *Artemia franciscana*, *Bacillus subtilis* has shown to effectively increase redox activation against *Vibrio anguillarum*, which increased the survival of the host (Giarma et al. 2017). Additionally, *Bacillus velezensis* has been characterized as a probiotic in *Ictalurus punctatus*, where it increased the growth rate of the host and controlled the intestinal microbiome (Thurlow et al. 2019). While these organisms can be effective probiotics in similar systems it is important to understand how they interact with the host under different conditions and how they can be successfully vectored into the terminal host. Current literature investigating these effects on an engineered microbiome is limited in the context of marine environments (Ahmed et al. 2019; Costa et al. 2021; Damjanovic et al. 2019; Doering et al. 2021; Dungan et al. 2022). Many interactions have been hypothesized via genomic inquiry, but many bacteria are uncultured (Huggett & Apprill 2019). While probiotic applications have been previously researched, including via genomic investigation, the interactions that occurred in the system have not been fully addressed. Utilizing environmental isolates will be vital to elucidate the interactions between individual or combinations of bacteria. Previous work has described interactions of *Vibrio corallilyticus* and *N. vectensis* (Brennan et al. 2017), and *Aiptasia pallida*

challenged with *Serratia marcensens* (Poole et al. 2016). While both studies described pathogenicity in their respective models, neither of the bacteria are ecologically relevant. Alternatively, other studies utilize the lab acclimated microbiome to conduct research, which can differ from the environmental microbiome (Baldassarre et al. 2022) (preprint). When investigating bacterial interactions, it is vital to utilize environmentally relevant isolates, to better characterize what occurs between the host and its microbiome. While *S. marcescens* is a pathogen, it is not an environmentally relevant organism to *A. pallida*, thus reducing the interpretability of the study. The utilization of ecologically relevant bacteria in model hosts can extend the applicability and accessibility of a wider range of hosts.

The model organism: *Nematostella vectensis*

Cnidarians are a sister group to the supergroup Bilateria and serve as a model group for evolutionary analysis. The ancestor to the cnidarians and bilaterians likely had a well-developed neural, innate immune, and stress response systems (Miller et al. 2007; Reitzel et al. 2008b). The neural system manages functions such as prey capture, stinging, and peristalsis (Marlow et al. 2009). Additionally, antibiotics have been described to target the hair bundles of *N. vectensis* (Menard 2018). The model sea anemone *Nematostella vectensis* does not possess an adaptive immune system, which provides a rare opportunity to probe how immunity developed and evolved in modern organisms. For example, *N. vectensis* has a toll-like receptor NF-κB, which is utilized for pathogen detection (Brennan et al. 2017). Additionally, the anemone has a STING mechanism, 2’3’cGAMP, that effectively mounts an antiviral response (Margolis et al.

2021). These two mechanisms have been characterized, with dozens more identified such as bZIP and zinc finger proteins through genomic inquiry (Reitzel et al. 2008b). *Nematostella vectensis*, like other organisms, can respond to stress through various mechanisms. The anemone can respond to hypoxia through HIF-1α, oxidative stress via catalase and bZIP, and heat stress through heat shock protein pathways (Reitzel et al. 2008b). The variety of stress responses make *N. vectensis* a viable model for investigating these pathways.

Nematostella vectensis is an established model for cnidarians, as it is easy to culture and propagate, the first cnidarian to have a sequenced genome, and one of a few species where the full life cycle can be studied under laboratory conditions (Darling et al. 2005; Hand & Uhlinger 1992). These attributes make it a useful model for assessing questions related to how animals respond to their environment, including studies for the interactions between the host and the microbiome. *Nematostella vectensis* inhabits a wide range of locations across the Eastern and Western coasts of the United States, and along the coast of the United Kingdom (Darling et al. 2004; Darling et al. 2005). The microbiome of *N. vectensis* has been found to vary across both time and geographic population (Har et al. 2015; Mortzfeld et al. 2016). Due to the wide geographic range of these populations, additional probing of adaptation through acclimation of differing environments can be assessed. These population level distinctions are important for discovering how individuals of the same species can survive differently from each other. Bacteria can serve a variety of functions throughout the life span of the organism. Additionally, the microbiome of *N. vectensis* is distinct through each of the life stages (Mortzfeld et al. 2016). Some Operational Taxonomic Units (OTUs) in *N. vectensis*

fluctuate throughout the day, following a diel cycle (Leach et al. 2019). Prior bacterial experimentation in *N. vectensis* investigated the effects at the microbiome level. The microbiome of *N. vectensis* has a broad level of assessment for a small quantity of isolates (Har et al. 2015), but their interactions *in vivo* have yet to be described. Alternatively, the effect of bacteria when colonizing axenic organisms *Rugeria* sp. and *Vibrio* sp. (competitive), *Acinetobacter* sp. (cooperative), *Aeromonas* sp. and *Pseudomonas* sp. (neutral), in addition to the natural microbiome, was lost after a short time period (Domin et al. 2018). Though the effects of the potential pathogen were investigated with *Vibrio coralliilyticus* (Brennan et al. 2017). The number of studies that investigate the effects of individual bacteria are limited in number.

To identify the effects of individual bacteria on the host *Nematostella vectensis*, it is important to investigate the methods to conduct such research. The implications of antibiotics on marine organisms are limited and requires additional assessment. Literature that describe the impacts of antibiotics are limited (Huggett et al. 2006b), but many studies have characterized microorganisms that promote and induce settlement in a vast array or marine invertebrates (Dobretsov & Rittschof 2020; Freckelton et al. 2022; Freire et al. 2019; Sneed et al. 2014; Tebben et al. 2011; Unabia & Hadfield 1999). Though studies have investigated the persistent bacteria through antibiotic treatment, none have described this community in *N. vectensis*. These persistent bacteria are likely the first organisms to repopulate the microbiome, and thus need to be identified. Additionally, quantification methods to efficiently transplant bacteria into the terminal host is poorly characterized. Previous literature has described solution mediated methods that yielded success for many marine invertebrates. Alternatively, prey vectoring has been described

in predatory organisms to mediate the transfer of bacteria from prey to the terminal host organism. Finally, the effects of individual bacteria and their impact on survivability of *N. vectensis* is limited. Previous research investigated the longevity and interactions of single isolates in conjunction with the natural microbiome (Domin et al. 2018). The microbiome of *N. vectensis* exposed to long term thermal and saline variation differs significantly (Mortzfeld et al. 2016). The specific capabilities of individual isolates remains understudied in this model organism (Har et al. 2015).

Dissertation Research Structure and Aims

The aims of this dissertation are to (1) determine the impacts of antibiotics on *Nematostella vectensis* at various life stages, (2) develop a method for transplanting and quantifying microbes within *Nematostella vectensis*, and (3) assess the influence of a structured microbiome on the survivable range of *Nematostella vectensis*, and vice versa. This research is divided into three data chapters:

Chapter 1: This research assessed the developmental and transcriptional impact of antibiotics on *N. vectensis.* Antibiotics were found to have a variety of effects throughout the life stages of the anemone. Larvae had extended settlement time when exposed to antibiotics, a variety of bacteria persisted through treatment and were readily culturable, and revealed differential transcription both through, and beyond treatments. These changes across the life stages of *N. vectensis* show that while antibiotic treatment does not typically result in mortality in a host, the organism is impacted on a transcriptional level.

Chapter 2: To properly implement the use of probiotics, a repeatable and efficient method to quantify the number of bacteria within *N. vectensis* is needed. Three methods were compared for the efficiency and longevity of transplanted bacteria. The two prey items tested, *Artemia salina*, and *Brachionus plicatilis*, had differential uptake of bacteria, but similar transplantation to the terminal predator. The Solution Uptake Method, while comparable to the Prey Feeding Method, shows lower retention of microbes over time for three transplanted isolates in the terminal organism, *N. vectensis*. Over a short time course, these data suggest a repeated regiment of probiotic applications for applicable treatments.

Chapter 3: This work assessed the survivability of environmental isolates associated with *N. vectensis,* and the influence of three bacterial species on host survival at elevated temperatures and variable salinities. While temperature can be a factor determining survivability, for many bacteria tested the combination of temperature and salinity limited growth for many organisms. Of the three bacterial species, the concentration of cells per individual fluctuated with the changes in environmental conditions. Additionally, an increase in temperature destabilizes the concentration of culturable bacteria in *N. vectensis.*

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CHAPTER 1

ANTIBIOTICS ALTER DEVELOMENT AND GENE EXPRESSION IN THE MODEL CNIDARIAN *NEMATOSTELLA VECTENSIS*

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Abstract

Background. Antibiotics are used for controlling microbial growth in diseased organisms. In aquatic environments, antibiotic treatments are difficult to implement due to dilution, and thus the effectiveness of antibiotic applications can be more difficult to quantify. However, antibiotic treatments, particularly of early developmental stages, can have negative impacts on development and physiology that could negate the positive effects of reducing or eliminating pathogens. Similarly, antibiotics can shift the microbial community due to differential effectiveness of antibiotics on the susceptible bacterial community. Though antibiotic application does not typically result in mortality of marine invertebrates, little is known about the developmental and transcriptional effects. Here, we quantify the impact of antibiotic treatment on development, gene expression, and persistent bacteria through the life cycle of a model cnidarian, *Nematostella vectensis*. Methods. Four antibiotics (ampicillin, streptomycin, rifampicin, and neomycin) were used to compare how individual and combined antibiotics impact *N. vectensis* and its microbial community. We quantified the relative sensitivity of developmental stages for this species when exposed to each antibiotic treatment to determine differential impacts of each on the development and metamorphosis. We used 16S rRNA gene sequencing to

compare the culturable bacteria that persist after each antibiotic treatment to determine how antibiotic treatments may differentially select against the native microbiome. Third, we determined how acute (3-day) and chronic (8-day) antibiotic treatments impact gene expression of adults to identify the transcriptional response, which is essential to understand the molecular response of animals with engineered bacterial communities.

Results. Embryonic settlement was impacted by antibiotic exposure, where settlement time extended as the concentration of antibiotics increased. Mean settlement time was delayed three days (50% increase) from control for the highest concentrations of antibiotics. *Alteromonas* sp., *Vibrio* sp. and other bacteria were found to persist post-antibiotic treatment in juvenile anemones. *Micrococcacae* dominated some conditions DMSO200, A50 and 200, and S200, (+82% relative frequency (RF)), while was present across N200 and N50 (40-80% RF) *Pseudomonadaceae* was present in some controls and ampicillin treatments (+92% RF). *Rikenellaceae* was prevalent in S50/200, N200, A200 (46-62% RF) The 3-day acute and 8-day chronic exposures resulted in 51 and 305 differentially expressed genes (DEGs) versus the control, respectively. The acute and chronic conditions shared 116 DEGs between these treatments. Gene Ontology (GO) reveals that constant antibiotic exposure resulted in enrichment of metabolic processes, while neuronal function and development are depleted. Additionally, acute antibiotic exposure yielded no enriched terms, while 222 were depleted including development and metabolism.

Discussion. Antibiotic application resulted in a significant increase to settlement time of larvae and gene expression of *N. vectensis*. Bacteria survived and were culturable from juveniles after each antibiotic treatment up to seven days after antibiotic removal.

Additionally, genes related to diverse cellular and physiological processes were impacted, which may indicate sublethal stress on the anemone. Our research suggests that impacts of antibiotics beyond the reduction of pathogenic bacteria may be important to consider when they are applied to aquatic invertebrates including reef building corals.

Introduction

Animals and bacteria interact with one another in numerous ways depending on the specific bacteria (Chan et al. 2019; Diaz & Restif 2014; Vega & Gore 2017), host (Berg et al. 2016), and environment (Dirksen et al. 2016; Samuel et al. 2016). These microbial communities associated with the animal host are typically diverse, remain distinct in composition from the surrounding environment (Carrier & Reitzel 2019), and are, to some extent, specific to each species (O'Brien et al. 2020). Additionally, the microbiomes vary between geographically distinct populations, which may also be dependent on the environmental conditions (Faddetta et al. 2020; Ketchum et al. 2020; Williams et al. 2022). Animals initially associate with bacteria through a combination of vertical and horizontal transmission. Bacteria can be transferred from parents to offspring vertically via intracellular bacteria in eggs or extracellularly associated bacteria with eggs or sperm (Giraud et al. 2022; Unzueta-Martínez et al. 2022). Alternatively, horizontally transferred bacteria from the environment (Sullam et al. 2012), prey items or conspecifics, is common in animals, including aquatic invertebrates (Carrier et al. 2022). These associations can vary functionally and temporally over the developmental stages of aquatic organisms, including inducing settlement of *Hydrodies elegans* (Unabia & Hadfield 1999). Additionally, some bacteria such as *Pseudoalteromonas* spp. (Sneed et

al. 2014; Tebben et al. 2011), *Alteromonas* spp. (Freire et al. 2019), and *Pseudomonas* spp. (Huggett et al. 2006a) are essential for providing settlement cues to planktonic invertebrates (Dobretsov & Rittschof 2020). Most bacteria that are part of a complex microbial community have not been characterized with respect to how they influence the life history of a host. The importance of determining the impact of specific bacterial species is critical for not only identifying the beneficial bacteria that may facilitate resilience but also for controlling pathogenic bacteria that are lethal to the host. Antibiotics have been used commonly in aquaculture in efforts to control pathogenic bacteria and/or promote growth in various animals (Chen et al. 2020; Landers et al. 2012; Olafsen 2001; Sweet et al. 2014). These compounds are similarly used in the field of gnotobiology, where germ-free individuals are studied to determine the roles of specific bacteria or the microbiome for numerous species (Nass & Hamza 2007; Provasoli & Shiraishi 1959; Tinh et al. 2006; Xiang et al. 2013). Studies of direct toxicity from antibiotics suggest that negative effects are more common for plants and microbes than for animals (Connelly et al. 2022; Lanzky & Halting-Sørensen 1997; Nass & Hamza 2007). However, exposure of some animals to antibiotics can also result in negative impacts to development and can impact molecular processes that are likely independent of targeted bacteria (Kohanski et al. 2010). For example, aminoglycoside antibiotics are a class of antibiotics used to treat infections caused by gram-negative bacteria but also result in ototoxicity and nephrotoxicity by damaging hair cells during development (Huth et al. 2011; Rizzi & Hirose 2007). *Branchiostoma belcheri*, a cephalochordate, exposed to antibiotics resulted in sublethal effects including immunosuppression and a reduction in eicosanoids (Yuan et al. 2015). While antibiotics can reduce and potentially kill

adverse bacteria in a host, the direct implications of this approach on the host are understudied (Baralla et al. 2021; Bojarski et al. 2020; Yang et al. 2020). Investigating the responses the bacterial cohort and its respective host have to antibiotics is necessary to understand the effects when treated together.

In marine systems, antibiotics are used to reduce or remove potential pathogenic bacteria from organisms of interest, such as fish (Cabello 2006; Salgado-Caxito et al. 2022; Yukgehnaish et al. 2020), oysters (Baralla et al. 2021; Salgueiro et al. 2021), and corals (Aeby et al. 2019; Hartman et al. 2022; Neely et al. 2020). Antibiotic use in aquaculture is projected to grow by more than 30% by 2030 to increase food production around the globe (Schar et al. 2020). Non-specific effects of antibiotics are poorly characterized in aquatic host organisms (Yang et al. 2020). Corals are particularly susceptible to rapid environmental changes and may become susceptible to opportunistic pathogens (Gardner et al. 2019). White Band Disease (WBD) is prominent in coral populations, which may be associated with pathogenic bacterium (Sweet et al. 2014). The applications of antibiotics arrested the progression of WBD, but necrotic tissues were unable to recover (Sweet et al. 2014). This indicates that antibiotic application alone is inadequate to save diseased individuals in this system. Antibiotic treatment of *Euphyllia paraivisa* resulted in differentially expressed genes related to development, cell communication and cell signaling as part of a larger heat stress study (Meron et al. 2020). Some of the persistent bacteria from antibiotically treated *E. paraivisa* include members from the alphaproteobacteria and gammaproteobacteria classes. In *Pocillopora*, the elimination of the microbiome in combination of heat stress results in elevated expression to heat response, and increased modulation of the immune response (Connelly et al.

2022). Additionally, Rhodobacteraceae and Alteromonadaceae were the most prevalent bacteria that persisted through antibiotic treatment of this coral. Further assessment of other host impacts such as development, persistent bacteria, and transcriptional responses in a model organism will be beneficial to understand sublethal effects of antibiotics across marine invertebrates.

Nematostella vectensis is a model species to determine the composition, dynamics, and potential functions of cnidarian bacterial communities (Artamonova & Mushegian 2013; Costa et al. 2021; Fraune et al. 2016; Har et al. 2015; Mortzfeld et al. 2016). Due to the ease of collection in estuaries and culturing in the laboratory, the accessibility of genomic and transcriptomic resources, and ability to generate the full life cycle in the lab, *N. vectensis* has been effective for determining how environmental variation, natural or chemical, can impact physiology and gene expression (Fraune et al. 2016; Layden et al. 2016; Putnam et al. 2007; Reitzel et al. 2013; Reitzel et al. 2008a; Reitzel et al. 2008b) of the anemone and the composition of the bacterial community. Individual bacteria in the microbiome can fluctuate hourly (Leach et al. 2019), localize to specific areas of the anemone (Bonacolta et al. 2021), and contrasts in microbiome structure across geographic locations (Har et al. 2015; Mortzfeld et al. 2016). The microbiome, which can differ from the surrounding environment (Carrier & Reitzel 2019), can be vertically transmitted (Baldassarre et al. 2021), and change throughout the life stages of *N. vectensis* (Mortzfeld et al. 2016). When enriched bacteria were added to axenic *N. vectensis* concurrently with the native microbiome, all enriched species were lost after seven days (Domin et al. 2018). While no naturally-associated bacteria has been identified as a pathogen, *Vibrio coralliilyticus* has been found to have temperature

dependent pathogenicity and results in higher rates of mortality (Brennan et al. 2017). Previous research has shown that aminoglycosides have toxicity through the deterioration of hair bundles after exposure in *Haliplanella luciae* (Watson et al. 1997) and *N. vectensis* (Menard 2018). The mechanistic implication of antibiotics on *N. vectensis* is otherwise unknown.

Here, we use three complementary approaches to measure the impact of antibiotic exposure on *N. vectensis*. First, we determined the impact of different antibiotics and their combination on embryonic development and metamorphosis to the juvenile stage. Second, we measured and identified the bacteria capable of growing after application of four individual antibiotics at two concentrations and in combination using 16S rRNA gene sequencing. Lastly, we measured the impact of an acute and chronic exposure to the antibiotic cocktail on the gene expression of adult *N. vectensis*. Together, this research reveals a complex developmental and transcriptional response to antibiotics in a cnidarian model that are important for interpreting future research into the interactions of cnidarians and bacteria.

Materials & Methods

Spawning of *Nematostella vectensis*

The animals used in this experiment were from a lab acclimated population of *Nematostella vectensis* in the Reitzel Lab at the University of North Carolina at Charlotte. Adult anemones were maintained in glass dishware in 15 parts per thousand (ppt) Artificial Seawater (ASW) (Instant Ocean) and fed freshly hatched *Artemia salina* three times per week. Anemones were cultured at room temperature prior to experimentation.

Culturing bowls were cleaned weekly and fresh artificial seawater was replaced at the same time.

Adult *N. vectensis* were spawned following a standard weekly protocol to procure embryos for antibiotic exposures. Anemones are cultured for two days at 16°C in an incubator without light. Anemones are moved to room temperature, fed cubes of dissected mussel gonads or mantle, and then incubated at 25°C overnight in an incubator under constant light. Gametes are shed in the morning and fertilization occurs in the bowl. Fertilized egg masses were moved to clean artificial seawater and maintained at 20°C in the dark for 24-36 hours. Adults were then maintained under standard laboratory conditions for three days before returning to the 16°C incubator.

Antibiotic exposure

Antibiotics

Ampicillin (Acros Organics Cat. No.: 61177), Streptomycin (Alfa Aesar, Cat. No.: J61299), and Neomycin (Alfa Aesar, Cat. No.: J61499) were all dissolved in deionized water to a working concentration of 20 mg mL-1. Rifampicin (Tokyo Chemical Industry, Cat. No.: 236-312-0) was dissolved in dimethyl sulfoxide (DMSO) to 20 mg mL-1. To ensure there is no effect of this additional solvent, an additional control equivalent to the DMSO concentration in each concentration of antibiotic treatment (0.1 and 0.4%, volume/volume). Experiments to measure effects on development and culturable bacteria utilized two concentrations of antibiotics and DMSO, 50 µg mL-1 and 200 µg mL-1. The “antibiotic cocktail” is a mix of all antibiotics at a concentration of 50 µg mL-1 each.

Embryonic settlement

Embryos had dissociated from the gelatinous egg mass after the 24-36 hours and were then moved to 24-well plates containing 1 mL of 15 ppt seawater (control) or 1 ml of seawater containing an antibiotic treatment. Groups of five embryos were placed into each well and four replicate wells were set up per treatment (ampicillin, streptomycin, rifampicin, neomycin, DMSO, and a cocktail mix). Developing embryos were scored daily for survival and stage of development (embryo, larva, juvenile). Observations ceased once all embryos reached the juvenile stage or died.

Culturing bacteria following antibiotic treatment

Juvenile *N. vectensis* (n = 6) were exposed to the respective antibiotic treatments per condition for 24 hours. We washed three sets 2 juveniles from each treatment condition and controls in sterile 15 ppt ASW to remove residual antibiotics and incubated in 10 mL of Estuarine Broth (EB) to determine if culturable bacteria remained associated with the anemones. Broth cultures containing the juveniles were shaken at 200 rpm at 25°C and checked daily over 7 days for growth of bacteria via turbidity. We measured the turbidity of cultures with a spectrophotometer (ThermoScientific Genesys 30) and plated turbid cultures using the standard spread plate method. Turbid cultures were split, 2 mL were spun down in a microcentrifuge at 8000 x g for 5 minutes, and the pellet was frozen for 16S rRNA gene sequencing. The turbid cultures were diluted with 1X Phosphate Buffered Saline (PBS) and spread onto EB agar plates. Morphologically distinct colonies

of bacteria were isolated on subsequent EB plates, grown in EB broth overnight, and then identified with 16S rDNA sequencing.

Generation and maintenance of axenic *Nematostella vectensis*

Adult anemones (n = 4) were incubated in three conditions to determine how acute and chronic exposure to antibiotics impacts gene expression: (1) No Treatment (NT): 15 ppt ASW with no application of antibiotics, (2) Antibiotic Acute (ABA): antibiotics applied for three days, and removed from antibiotics for five days before animal preservation, (3) Antibiotic Constant (ABC): continuous application of antibiotics (8 days) until preservation. The antibiotic solution consisted of 50 µg mL-1 of Ampicillin, Streptomycin, Neomycin, and Rifampicin. All antibiotics were mixed into 15 ppt ASW.

Anemones in all treatments were fed freshly hatched *Artemia salina* three times per week. In order to eliminate the transfer of bacteria from the *A. salina* to the anemones, cysts were cultured in a 1% w/v solution of thiomersal prepared in 30 ppt ASW prior to their isolation for feeding (Verschuere et al. 1999). *Artemia salina* were washed in filter sterilized 30 ppt ASW and confirmed to not harbor culturable bacteria (turbidity test in EB) prior to their use as food.

Molecular Approaches

16S rRNA Gene Amplification

For the broth cultures and bacteria isolated from the antibiotic exposed juveniles, DNA was isolated with ThermoScientific GENEJet Genomic Purification kit from a

bacterial pellet and 16S rRNA gene amplicons were generated via PCR (Q5 polymerase, New England Biolabs). Samples were spot-checked for amplification using gel electrophoresis. The abundance of amplified DNA was quantified with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and normalized to 15 ng μL-1 prior to sequencing. Amplicons were directly sequenced with Illumina MiSeq using the forward PCR primer 5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCTACGGGNGGCWGCAG and reverse primer 5'GTCTCGTGGGCTC GGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC.

RNA Extraction

RNA was isolated from samples (4 biological replicates × 2 treatment groups × 1 control group) using the RNAqueous kit (Ambion) following the manufacturer's protocol. The RNAlater was discarded from each sample, and animals were lysed through pipetting in a lysis buffer for <2 min, and subsequently washed 2–3 times and eluted on a column. Genomic DNA was removed using the DNA-free kit (Invitrogen), and RNA quantity assessed using a NanoDrop 2000 (Thermo Fisher Scientific). RNA was shipped for tag-based library prep at the University of Texas at Austin's Genomic Sequencing and Analysis Facility (GSAF) as described in Meyer, Aglyamova, and Matz (2011) and adapted for Illumina HiSeq 2500. GSAF performed the steps for generation of the sequencing library. RNA was transcribed to cDNA and subsequently purified with AMPure beads. Each sample was then amplified with 18 PCR cycles. Lastly, unique Illumina barcodes were added to the amplicons for indexing. After an additional purification step, the libraries were pooled, then spot-checked for quality on a

Bioanalyzer (Agilent and Pico) and size-selected using BluePippin (350–550 bp fragments).

Bioinformatic Approaches

16S rRNA Gene Identification

The amplified sequences were imported into QIIME2 for taxonomic quantification and identification (Bolyen et al. 2019). The demultiplexed sequences were trimmed to 250 bases using the quality scores generated in QIIME2. Next, Shannon's alpha and Bray-Curtis beta diversities were calculated with a sampling depth of 10884 counts, based on the sample with the least sampling depth (Table S1.1). A PCA plot of the respective communities were imported and constructed as an emperor plot, and the taxonomic distributions were graphed into bar plots using default parameters. Lastly, the taxonomies were assigned with a Naive Bayes classifier with the SILVA database (Quast et al. 2012) trained for the 16S V3/V4 primers used to amplify the gene.

Differential Gene Expression

The raw sequences resulting from the Illumina sequencing were adapter clipped and trimmed using Trimmomatic (Bolger et al. 2014). Next, the trimmed sequences were aligned to the *N. vectensis* Vienna transcriptome (Fredman et al. 2022) using Bowtie2 (Langmead & Salzberg 2012). The alignment sam file was converted to the bam file type with the package samtools (Li et al. 2009). The trimmed and indexed reads and alignment file were used for Trinity with the --genome_guided_bam standard options (Grabherr et al. 2011). Significant features were identified using $p \leq 0.05$ and log-fold change above

1.5. Genes were annotated using Trinotate with the UniProt Database (Consortium 2020). Differentially expressed genes were compared across treatments with the R package ggVennDiagram. The rlog R function transformed counts and PCA based on Manhattan distances. Statistical significance calculated using the adonis, through the vegan package. Principal Coordinate Analysis of the DEGs was analyzed and viewed with the R packages prcomp and ggplot2. Gene Ontology (GO) terms were assigned to transcripts with the Trinotate pipeline. The GOSeq package annotated these terms and assigned enrichment and depletion values to the differentially expressed transcripts. GO terms across the control and treatments were compared with the R package ggVennDiagram.

Results

Antibiotics Extend Larvae Settlement Time

Embryos and larvae exhibited no morphological changes due to antibiotics. Across the two separate trials of antibiotic application the embryos exhibited similar settlement times. Developing larvae took longer to settle and enter the juvenile stage when exposed to most of the antibiotic treatments. The Development Time (DT50) shows the amount of time required for settlement for half of the population (Figure 1.1A). Additionally, the DT100 shows the time required for 100% of the larvae to settle (Figure 1.1B). For larvae DT50, DMSO at 50 µg mL-1 was the only condition that was not statistically different from control (one-way ANOVA, $p = 0.666$). All remaining statistical results can be found in Table 1.1. When comparing the settlement time for 100% of larvae, ampicillin at 200 µg mL-1, was the only condition that was not

statistically significant against controls (one-way ANOVA, p = 0.125), while the remaining treatments were significantly longer (Table 1.1).

Bacteria are cultivatable following relief of antibiotic stressors

Juveniles post exposures resulted in cultivated organisms in most conditions over the following week. Turbidity measurements were taken through seven days (Figure 1.2). The fastest recovering cultures were controls (n = 8), DMSO controls (50 µg mL-1, n = 9, 200 µg mL-1, n = 6), and ampicillin treated cultures (50 µg mL-1, n = 9, 200 µg mL-1, n = 8), which all became turbid within 3 days. The antibiotic cocktail (Mix) yielded growth in four of the replicates but was not statistically different from the control conditions time to turbidity (one-way ANOVA, p = 0.1693). Rifampicin at 200 µg mL-1 (n = 2) and neomycin at both 50 µg mL-1 (n = 8) and 200 µg mL-1 (n = 5) resulted in significantly longer times to turbidity compared to the control (p = 0.0293, p = 0.0183, p = 0.0027, respectively). The unweighted Unifrac plot shows the communities that persisted across treatments (Figure 1.3). Two groups cluster along with the first component. When conditions are grouped by treatment, the unweighted UniFrac distances are significant between neomycin and streptomycin, when compared against the control microbial community (Figure 1.4). The alpha diversity (Faith's Phylogenetic Diversity) across antibiotics revealed statistical differences between controls and neomycin (p = 0.033, Figure S1.1). The dominant class across all communities was gammaproteobacteria, where many marine pathogens are classified (Figure S1.2). *Pseudomonas* sp. was the most prevalent gammaproteobacteria member (Figure 1.4). Conversely, the next most prevalent organism was *Pseudoalteromonas* sp. Bacteria

associated with the juveniles that are capable of growth following antibiotic exposure include *Micrococcale, Pseudomonadales, Bacteroidales,* and *Alteromonadales,* and others.

Differential Gene Expression with Antibiotic Exposure

The Principal Component Analysis revealed clustering of all three groups, No Treatment, Antibiotic Acute, and Antibiotic Constant, where the first two components are responsible for 31.7% of the variation (Figure 1.5). Additionally, the Manhattan distances are statistically different (PERMANOVA, $p = 0.002$), and Condition explains 30.9% of the variation ($R^2 = 0.309$)

The three-day antibiotic acute exposure (ABA) resulted in 30 (21 upregulated, nine downregulated) genes compared to controls (Figure 1.6). Of these 30 DEGs, eight had some annotation based on similarity to other genes. One of these genes, a gene related to NF-X1-type zinc finger containing protein was upregulated 2.23-fold ($p = 0.007$) (NVE20000). Additionally, a Cytochrome C heme binding site, and zinc ion binding (NVE217122), was upregulated 2.01-fold ($p = 0.007$) when compared to the control. Ubiqitin (NVE217764) was upregulated 1.77-fold ($p = 0.002$) in the acutely treated anemones. Of the nine downregulated genes in acute treated anemones, only one gene matched in the database, which functions as a ribonuclease reductase (-1.62-fold, $p = 0.013$).

Constant application of antibiotics resulted in 176 (83 up-regulated, 93 down-regulated) genes differentially expressed transcripts compared to the controls. Of these 176 genes, 13 were shared with the DEGs expressed in the acute versus control

comparison (Figure 1.6), and 50 returned annotations from UniProt. Cytochrome C (NVE180397) was significantly downregulated 2.85-fold (p = 7.94E-06) compared to controls. A nematocyst expression protein was down-regulated 2.43-fold (p = 1.17E-04). Several transcripts related to chymotrypsinogen were downregulated relative to controls. Actin and tubulin transcripts were upregulated. Cubilin was found to be significantly downregulated, which facilitates uptake of iron and vitamins and iron storage upregulated. Interferon regulatory factor 2-binding protein 2 related to *Mus musculus* (NVE183914, no annotation) was upregulated 5.08-fold (p = 5.51E-07) when compared to control anemones.

Comparatively, acute antibiotic and the constant antibiotic treatment resulted in 80 (29 upregulated, 51 downregulated) differentially expressed genes. Cytochrome C was downregulated in the acute treated anemones 2.67-fold (NVE180397, p = 4.04E-06), and a gene related to 2-oxoisovalerate dehydrogenase subunit alpha (NVE183894) was also down-regulated 2.02-fold (p = 0.02). Several tubulin alpha chain genes were upregulated in the acute treated anemones (NVE182198, 1.52-fold, p = 9.11E-04), (NVE182198, 1.63-fold, p = 0.001), and (NVE234521, 1.75-fold, p = 1.03E-05). Additionally, a gene that matched NRX4 in *Drosophila melanogaster* was significantly upregulated (3.32-fold, p = 1.08E-06), but does not return an annotation in the *N. vectensis* transcriptome. Some DEGs were shared between comparisons (Figure 1.4). The largest number (n=32) was shared between NTC_ABC and ABA_ABC. Here, six of the 32 genes were annotated, including hydrogen sulfide metabolism (NVE127536), basic leucine zipper (NVE80243), Cytochrome C (NVE180397), (NVE119418), tubulin alpha chain (NVE182198), collagen alpha 3 chain (NVE228208), and NRX4. Thirteen DEGs were

shared between No Treatment versus Constant and No Treatment versus Acute, four of which returned annotations. Ribonuclease reductase (NVE165579), Glycine-rich RNA-binding protein (NVE246169), ubiquitin (NVE217764), and low-density lipoprotein receptor (NVE107027, *Homo sapiens*). In the final comparison No Treatment versus Acute and Acute versus Constant, three genes were shared between these comparisons NVE245623 (no known annotation), NVE247395 (sacsin, *Rattus norvegius*, no *N. vectensis* annotation), and NVE165579 (ribonucleotide reductase). No differentially expressed genes were shared across all three comparisons.

A total of 222 GO terms (222 depleted, 0 enriched) were found in the acutely treated animals compared to the control animals (Figure 1.7). One-hundred and fifty-seven of the depleted GO terms were categorized as Biological Process (BP), 44 as Cellular Component (CC), 6 in Molecular Function (MF), and 15 were unclassified (Table 2). Eighteen of these depleted terms were related to metabolism, ten terms were related to neuronal processes, and 24 were found to be involved in development. Comparatively, 247 GO terms (192 depleted, 55 enriched) were found in animals that were maintained under constant antibiotic stress relative to controls. Thirty-seven of the enriched terms were categorized as BP, seven as CC, four as MF, and eight unclassified. Of the 37 terms categorized as Biological Processes, 16 were related to metabolism, and an additional six were related to biosynthesis. Three of the seven Cellular Components were organelle related. For the depleted GO terms, 138 were classified as BP, 30 as CC, 10 as MF, and four were unclassified. Many depleted GO terms were related to neuronal processes (16 total terms, BP – 10, CC – 6), metabolism (BP – 13 terms), development (BP – 33 terms), mitochondrial function (7 total terms, CC – 3, MF – 4) and iron

oxidation/binding (MF – 4 terms). When comparing ABA and ABC treatments, a total of 63 GO terms (30 depleted, 33 enriched) were found. Of the 30 depleted terms, 14 are categorized as Biological Process, 11 are Cellular Component, one as Molecular Function, and four unclassified terms. Seven of the 14 BP terms were directly related to metabolic processes. Seven of the 11 CC terms are organelle components. Twenty-two of the 33 enriched terms were categorized as Biological Processes, eight were Molecular Function, and the remaining three were unidentified. Five of the total enriched GO terms were related to sulfur oxidization, and seven were directly associated with different forms of catabolism.

Across both comparisons (control and antibiotic constant), the antibiotic acute treatment resulted in no enriched GO terms. Alternatively, the controls and antibiotic constant treatment resulted in 55 enriched terms, 19 of which are shared with the acute versus constant treatment where seven are depleted metabolism terms and three are depleted organelle terms (Figure 1.7A).

Interestingly, when comparing the depleted GO terms across treatments and control, a total of 92 terms were shared across the two treatments and the control (Figure 7B). These depleted terms related to developmental processes, metabolism, transport, and neural processes. The remaining depleted terms were unique to acute versus control (n = 100) and acute versus the constant treatment (n = 33).

Discussion

We found that exposure to antibiotics of different classes and concentrations impacts *Nematostella vectensis* and the associated bacteria. In larvae, we found an

increase of 50% settlement time. The impact of certain microbes and their settlement cues have been characterized in several systems (Tran 2022a). For example, *Pseudoalteromonas* spp. produces tetrabtomopyrrole (TBP) which can induce settlement in a wide range of marine invertebrates including *Heliocidaris erythrogramma* (Huggett et al. 2006b)*, Porites astreoides,* and *Orbicella franksi,* and *Acropora* spp. (Sneed et al. 2014; Tebben et al. 2011). While longer settlement times does not induce mortality alone, the increase in time may restrict the larvae from proper settlement. For example, some organisms receive settlement cues from various sources to begin the transition from the pelagic to the benthic stage of their life cycle. *Hydrodes elegans* depends on the cues of bacterial biofilms to induce settlement (Unabia & Hadfield 1999). Additionally, *H. elegans* can sense bacterial lipopolysaccharides as a cue to settle and metamorphose (Freckelton et al. 2022). In *N. vectensis*, the bacterial community shifts through metamorphosis (Mortzfeld et al. 2016), indicating the interactions with the host may change concurrently. Additionally, when the bacterial community of *N. vectensis* larvae is inoculated into juveniles, over time the microbiome becomes similar to the control juveniles (Domin et al. 2018). This indicates that there is a strong interaction between life stage of the anemone and its respective microbiome. Considering the complex interactions anemones have with microbes, it may not be a viable approach to remove bacteria from the system. Additional investigation into these associations between the microbes and their respective host to understand these interactions.

Bacteria have acquired many methods to evade mortality via antibiotic stressors. In other systems, the bacterial community that persisted through antibiotic exposures have been identified. Researchers using *Pocillopora* as an experimental system observed

that bacteria from families Rhodobacteraceae and Alteromonadaceae were the most prevalent following that were detectable after antibiotic treatment (Connelly et al. 2022). Here, we found related organisms across several of the treatments. Additionally, in *Euphyllia* alphaproteobacteria contributed to the largest group in post-treatment organisms, followed by Bacteriodes (Meron et al. 2020). Additional probing into the gammaproteobacterial clade persistent in *Euphyllia* reveals organisms such as *Alteromondales*, *Oceanspiralles*, and *Vibrionales*, all of which were detected in this study. Here, we found gammaproteobacteria were most prevelant in control and DMSO conditions, indicating their presence and ability to grow quickly when antibiotics were absent. Bacteria from the gammaproteobacteria class were also prevalent in most treatment conditions (ampicillin, rifampicin, and streptomycin). This method we utilized permits insight into readily culturable bacteria following antibiotic treatment and allowed us to detect and identify organisms that are readily able to grow and potentially populate the organism with a depleted microbiome. Several different bacteria were readily culturable, indicating that antibiotics may not be a viable solution to marine applications. We cultivated bacteria following a short-term antibiotic exposure, a factor that should be considered when producing axenic anemones. The potential for bacteria to survive treatments via mechanisms such as antibiotic resistance (Uddin et al. 2021), biofilm formation (Antunes et al. 2018; Armstrong et al. 2001; Schillaci et al. 2010), or dormancy (Mu et al. 2020; Wang et al. 2021a; Zhang et al. 2021). These mechanisms may hinder efforts to eliminate pathogens or create axenic individuals for microbiome manipulations.

Antibiotic exposures revealed distinct transcriptional responses in adult *N. vectensis* that effected a large array of cellular processes and functions. Differentially

Expressed Genes (DEGs) ranged from mitochondrial functions, metabolism, and cellular components such as tubulin and actin. One of the transcriptionally downregulated genes in both antibiotic treatments was Cytochrome C, which is imperative for mitochondrial function (Miller & Singer 2022). While this has not been described in *N. vectensis,* researchers described similar targets of antibiotics in other marine organisms (Benedetti et al. 2022; Rodrigues et al. 2019). Additionally, chymotrypsinogen, an enzyme that aids in digestion, was downregulated in the antibiotic constant condition. Under antibiotic stressors, the bacteria and the host may reduce function of metabolism. In addition to the downregulation of metabolism, another mechanism that was downregulated was nematocyst gene 6. Nematocysts are a cnidarian specific cell type and is related to prey paralysis and capture (Beckmann & Özbek 2012). Down regulation of specific genes related to prey capture and metabolism may reduce the fitness of the organism. Following removal of antibiotics, differentially expressed transcripts are still quantifiable five days after the stress has been removed from the animal. A zinc finger protein, which is responsible for viral RNA duplication prevention (Esposito et al. 2022; Wang et al. 2019), was upregulated in this condition. The increased expression of a viral defense gene is one indicator that the native microbiome may play a role in viral pathogen defense of the host. This, in addition to other DEGs such as metabolism upregulation, indicates that the animal is still recovering from the treatment and may reduce overall fitness of the organism.

Gene Ontology revealed many terms were depleted with these treatments (n = 414) while the number of enriched terms was minimal compared to the controls (n = 55). Many of these processes that are depleted are related to developmental pathways, which

is supported with results generated from the extension of settlement in larvae. *Nematostella vectensis* has several stages of development, each of which can be described by distinct gene expression of a subset of genes (Levitan et al. 2015). Downregulation of processes related to development may result in arrest of metamorphosis in *N. vectensis*. Alternatively, endoribonuclease and immunity related genes in *Pocillopora* were upregulated with antibiotic treatment, as was found in *N. vectensis*. The combination of increased metabolism and depletion of various processes such as neural, ion transport, and organelle cellular components indicates the animal experiences a variety of sublethal effects that have been described in other systems (Connelly et al. 2022; Meron et al. 2020). In both systems processes related to metabolism, neural, and development were all downregulated, as we demonstrated here, including in the acutely treated organisms. The combination of bacterial persistence and differentially expressed genes gathered with this study suggest that *N. vectensis* could be a model that lacks an algal symbiont for describing coral microbiome work and transcriptional assessment under stress.

Conclusions

This work sought to investigate the off-target effects of antibiotics in the model cnidarian *Nematostella vectensis*. We found in adult anemones downregulated expressed genes (i.e., mitochondrial, metabolism, and development) and many GO terms were depleted with the two treatment conditions. If these processes are reduced in capacity, it may reduce the fitness of the organism. Additionally, some bacteria survive after antibiotic treatment, thus indicating antibiotic treatments may not successfully create

axenic organisms in some cases. This method favored bacteria that are capable of quick recovery, which may not reflect all the organisms that can survive antibiotic stressors. Interestingly, the settlement time of the larvae increased under many of the differing antibiotic conditions. While some of the development DEGs and GO terms found in adults were downregulated, we hypothesize the increase in settlement is related to this transcriptional response, in addition to the removal of bacteria. Here, we describe a method for generating axenic *N. vectensis*, and the various sublethal effects antibiotics have on the model cnidarian. To further assess the different intervention methods, transcriptional studies should be investigated to determine the impact on the host.

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Table 1.1: Statistical results (p-values) from the DT50 and DT100 settlement assays (one-way ANOVA).

Settlement p-values	DT50	DT100
Control vs. DMSO50	0.6655	0.0039
Control vs. DMSO200	0.0002	0.0039
Control vs. Amp50	<0.0001	<0.0001
Control vs. Amp200	0.0352	0.1251
Control vs. Strep50	<0.0001	0.0039
Control vs. Strep200	0.0002	0.0039
Control vs. Rif50	<0.0001	0.0069
Control vs. Rif200	<0.0001	<0.0001
Control vs. Neo50	<0.0001	<0.0001
Control vs. Neo200	<0.0001	<0.0001
Control vs. Combined	<0.0001	<0.0001

Table 1.2: Gene Ontology distribution of Biological, Molecular Functions, and Cellular Components across treatment types.

	Enriched				Total
GO terms	Biological Process	Molecular Function	Cellular Component	Unknown	Enriched
ABA vs ABC	22	8	0	3	33
NTC vs ABA	0	0	0	0	0
NTC vs ABC	37	4	7	8	55
	Depleted				Total
GO terms	Biological Process	Molecular Function	Cellular Component	Unknown	Depleted
ABA vs ABC	14	1	11	4	30
NTC vs ABA	157	44	6	15	222
NTC vs ABC	138	10	30	14	192

GO terms	Total
ABA vs ABC	63
NTC vs ABA	222
NTC vs ABC	247

Table S1.1: Sampling depth for alpha and beta diversities.

Sample	Count	Sample	Count
D200-2-E3	27080	D50-3-E2	19899
C3-E2	26923	S200-1-E1	19718
D50-2-E3	24493	D200-3-E2	19634
A200-3-E1	24101	D50-2-E2	19546
C2-E2	24053	S200-2-E2	19427
S50-1-E1	23775	S200-1-E3	19192
S200-2-E1	23604	D50-1-E1	19149
MIX-2-E1	23402	D200-1-E3	19082
S50-2-E3	23132	A50-2-E3	19061
C2-E3	23042	N50-1-E1	18805
R200-2-E1	22987	A200-2-E2	18736
R50-2-E3	22712	A50-2-E1	18685
S200-1-E2	22603	N200-1-E3	18652
A200-1-E1	22504	A200-2-E1	18516
A50-1-E3	22462	C3-E3	18341
S50-3-E3	22439	S50-3-E1	18068
MIX-1-E1	22292	R200-1-E1	17818
D50-1-E3	22107	A200-1-E2	17720
A50-1-E1	21820	R50-1-E1	17251
N200-1-E1	21486	A50-3-E3	16778
D200-1-E2	21470	S200-3-E1	16584
C1-E1	21195	S200-2-E3	16288
D50-1-E2	21120	R50-1-E3	15998
C2-E1	20964	N50-2-E3	15911
R50-3-E3	20646	A50-3-E1	15229
N200-2-E2	20480	S50-1-E2	15186
D200-3-E3	20462	N200-2-E1	15105
A200-1-E3	20429	A200-3-E3	14363
S50-2-E1	20312	N50-2-E1	13906
D200-2-E2	20304	MIX-1-E2	13590
N50-1-E3	20289	C1-E3	12768
N50-3-E3	20131	A200-2-E3	12395

C1-E2	20128	D50-3-E3	11889
D50-2-E1	19990	N200-3-E1	10884
S200-3-E3	19959		

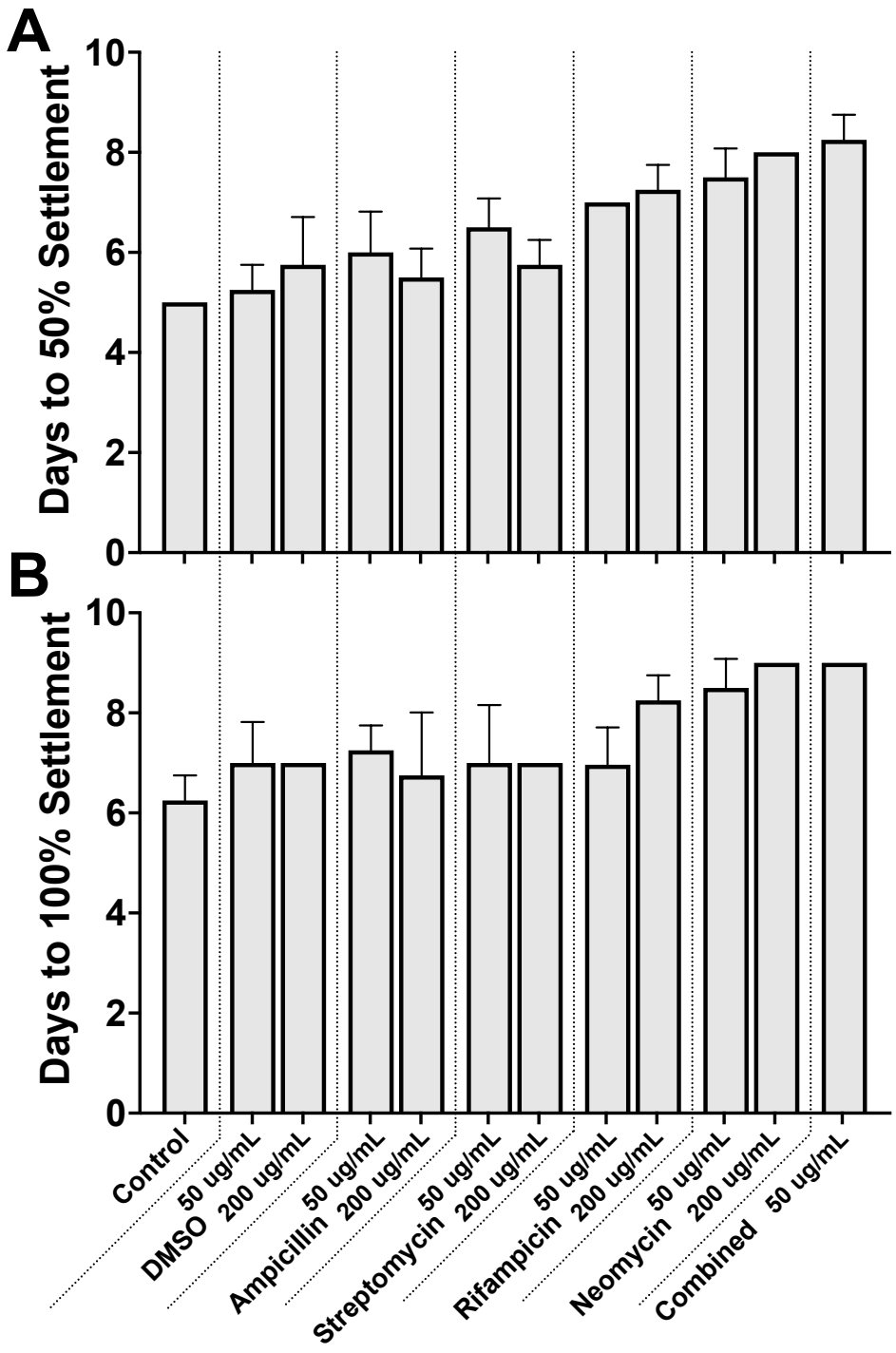


Figure 1.1: Settlement time of newly spawned *N. vectensis*. (A) The number of days required for 50% of the population (n = 20) to settle. (B) The number of days required for 100% of the population (n = 20) to settle. Bars represent the means and error bars standard deviation. Statistical values can be found in Table 1.1.

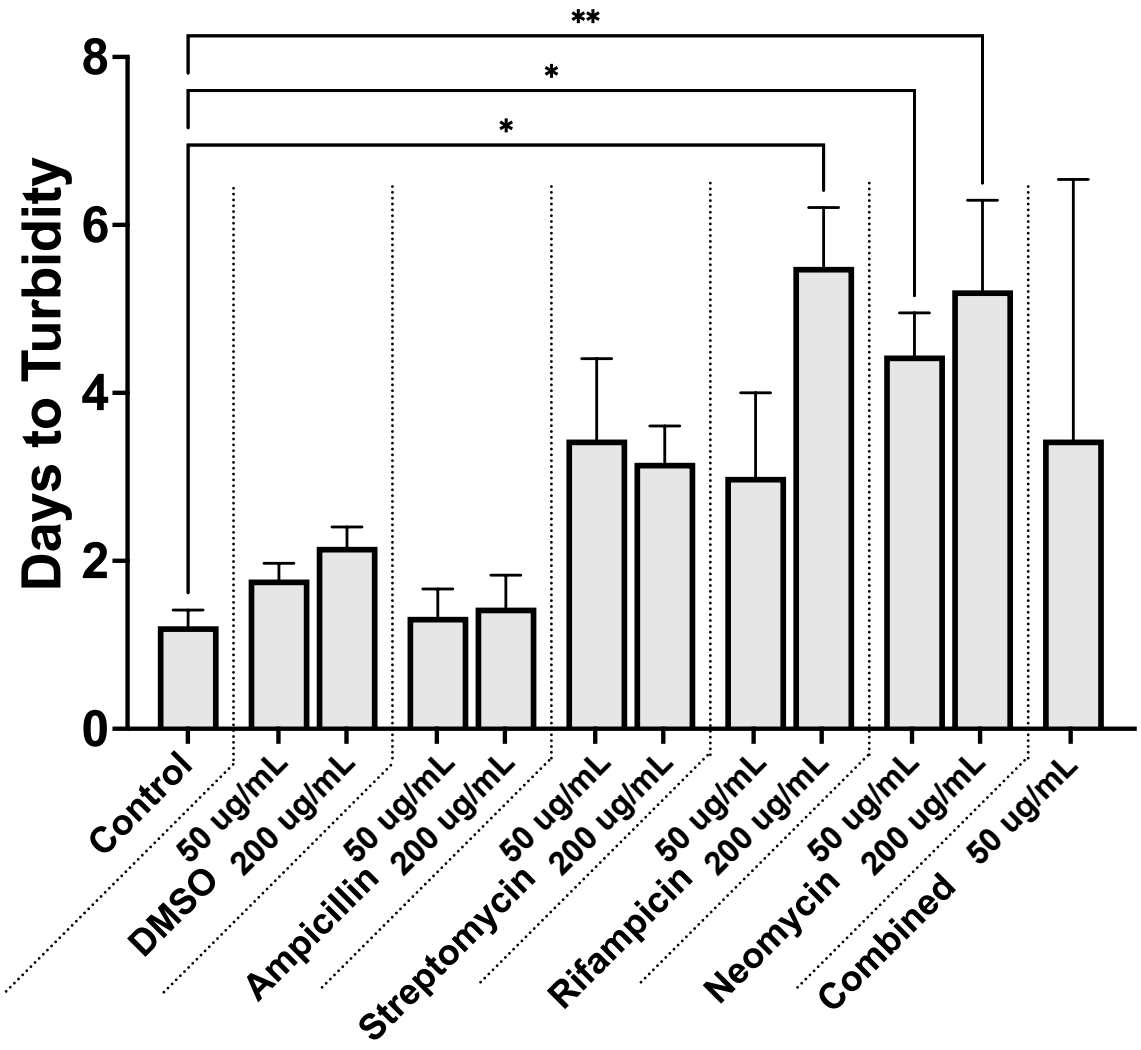


Figure 1.2: Days to turbidity. Bars represent the mean and error bars represent the standard deviation. Statistical values can be found in Table 1.1.

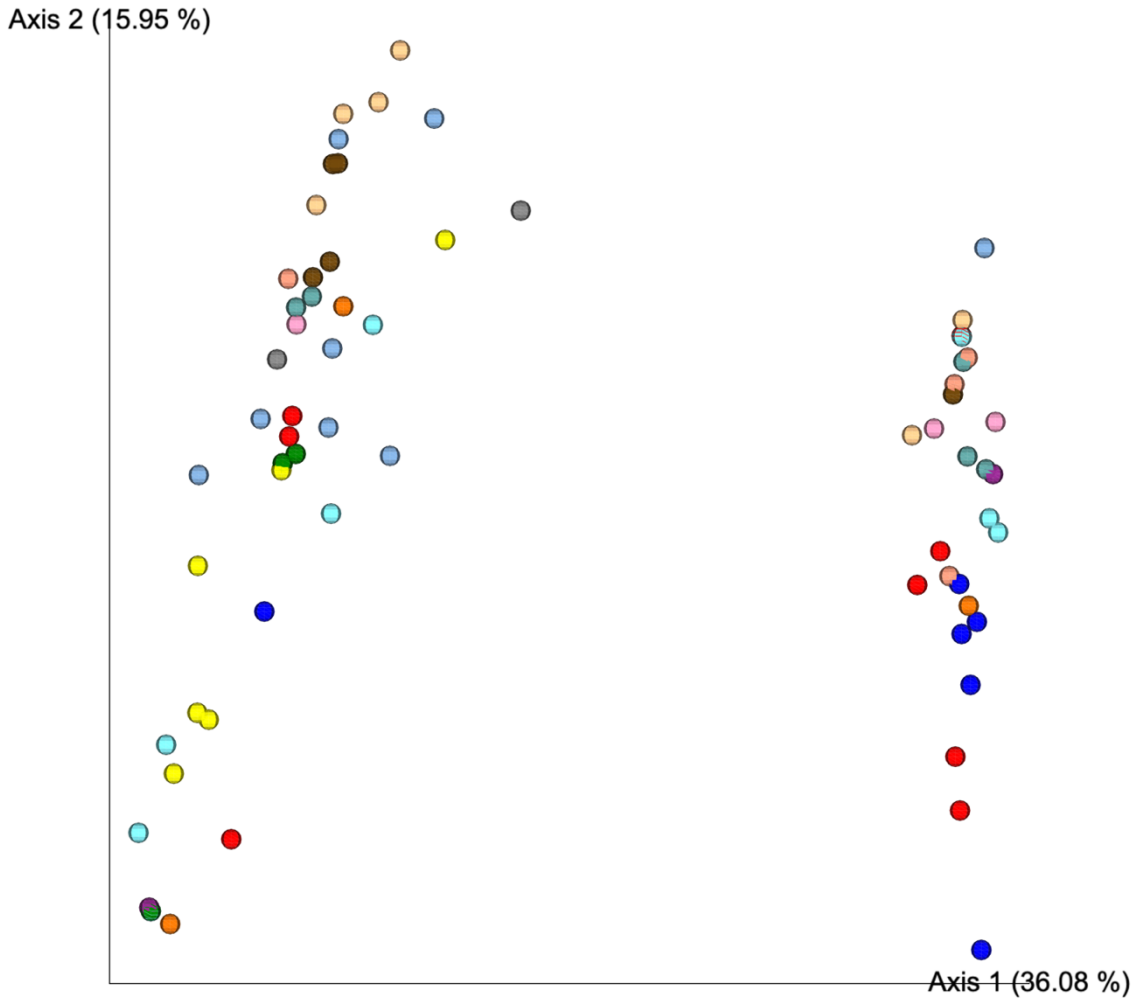
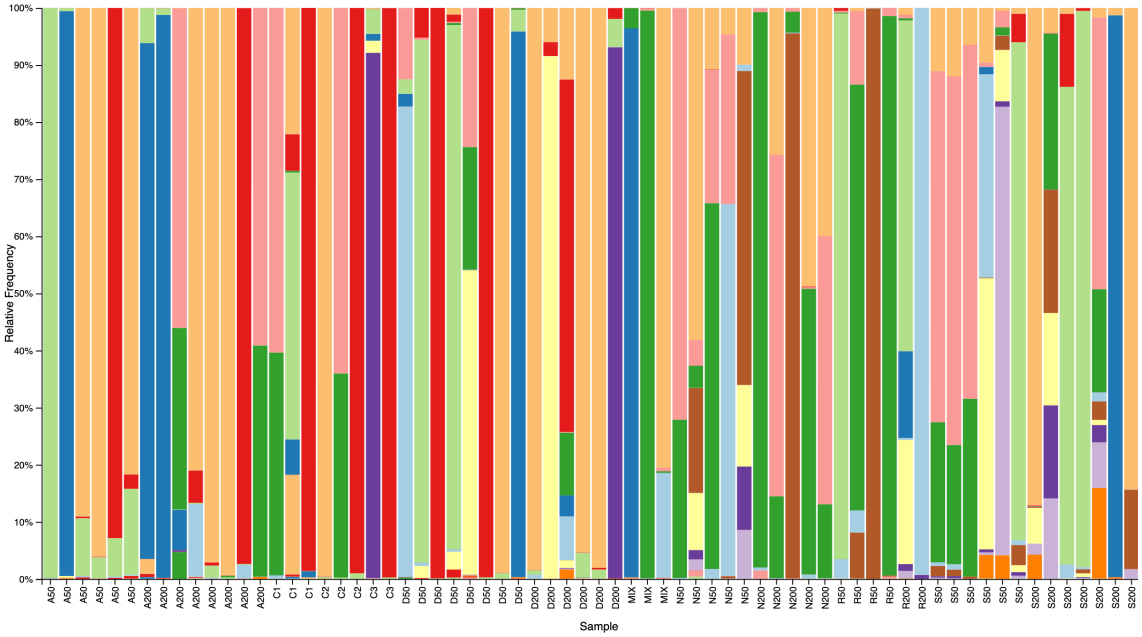


Figure 1.3: Unweighted Unifrac for 16S rDNA gene sequences of persistent bacteria following various antibiotic treatments.



d__Bacteria;p__Actinobacteriota;c__Actinobacteria;o__Micrococcales
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Pseudomonadale
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Bacteroidales
d__Bacteria;p__Bacteroidota;c__Bacteroidia;__
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Alteromonadales
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;__
d__Bacteria;p__Bacteroidota;c__Bacteroidia;o__Flavobacteriales
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Sphingomonadales
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhodobacterales
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Burkholderiales
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;o__Rhizobiales
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Oceanospirillales
d__Bacteria;p__Proteobacteria;c__Gammaproteobacteria;o__Vibrionales
d__Bacteria;__;__;__
Unassigned;__;__;__
d__Bacteria;p__Proteobacteria;c__Alphaproteobacteria;__
d__Bacteria;p__Firmicutes;c__Bacilli;o__Lactobacillales
d__Bacteria;p__Firmicutes;__;__
d__Bacteria;p__Proteobacteria;__;__

Figure 1.4: Persistent bacteria in juvenile *N. vectensis* following antibiotic exposure. Taxonomic classification of Order was selected and sorted by treatment.

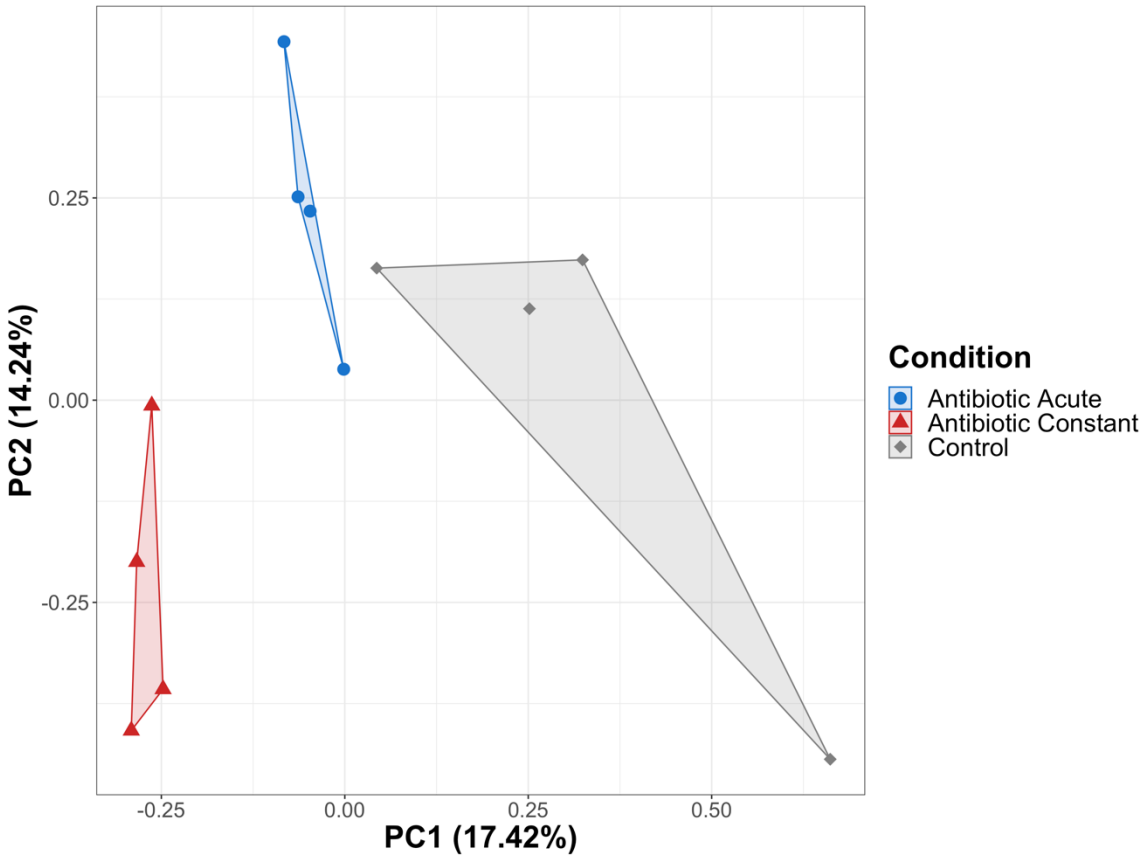


Figure 1.5: PCA of differentially expresses genes across control and both treatments.

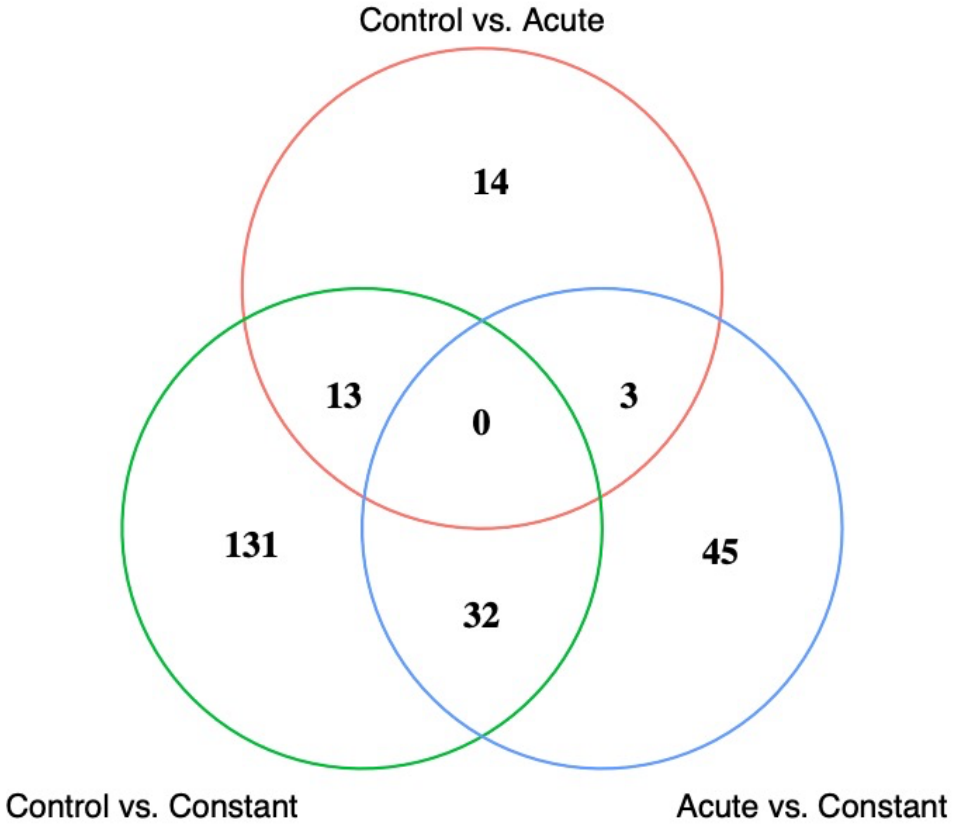


Figure 1.6. Venn Diagram of Differentially Expressed Genes.

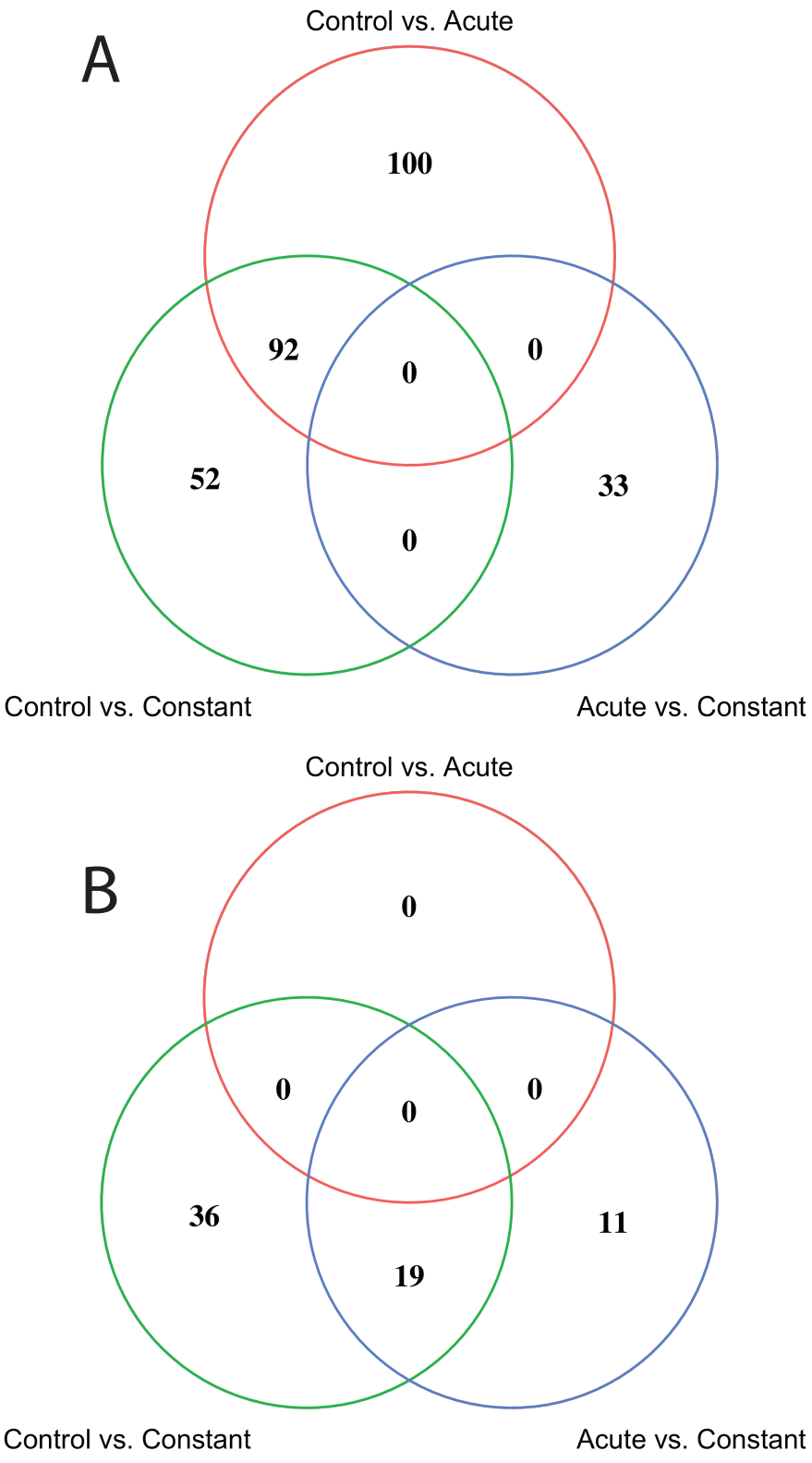


Figure 1.7: Venn Diagram of Gene Ontology terms. (A) Venn Diagram of enriched GO terms, (B) Venn Diagram of depleted GO terms.

CHAPTER 2

COMPARATIVE TRANSMISSION OF BACTERIA FROM *ARTEMIA SALINA* AND *BRACHIONUS PLICATILIS* TO THE CNIDARIAN *NEMATOSTELLA VECTENSIS*

Quinton A. Krueger, Madisun H. Shore, Adam M. Reitzel

Abstract

The microbial community associated with animals (microbiome) is essential for development, physiology, and health of host organisms. A critical step to understand the assembly of microbiomes is to determine how effectively bacteria colonize and establish within the host. Bacteria commonly colonize hosts through vertical transmission, passively from the environment, or through food consumption. Using the prey feeding method (PFM), we test transmittance of *Bacillus velezensis*, *Pseudoalteromonas spiralis*, and *Vibrio alginolyticus* to *Nematostella vectensis* using two prey, *Artemia salina* and *Brachionus plicatilis*. We compare PFM to a solution uptake method (SUM) to quantify the concentration of bacteria in these host organisms, with plate counts. Larvae had a similar uptake with SUM at 6 hours but had greater concentrations at 48 hours versus PFM. Juveniles acquired similar concentrations at 6 hours for SUM and PFM using *B. plicatilis* and *A. salina*. At 2 days, the quantity of bacteria vectored from PFM increased. After 7 days the CFUs decreased 2-fold with *B. plicatilis* and *A. salina* relative to the 2-day concentrations, and further decreased after 14 days. Therefore, prey-mediated methods provide greater microbe transplantation than SUM after 24 hours, supporting this approach as a more successful inoculation method of individual bacterial species.

Introduction

Bacteria and other microorganisms (microbiome) commonly associate with multicellular organisms (host) (McFall-Ngai, 2002; Artamonova & Mushegian, 2013; McFall-Ngai *et al.*, 2013; Zhang *et al.*, 2017; Simon *et al.*, 2019). These associations have a broad range of impacts on the physiology and health of the host (Glasl *et al.*, 2016). The host provides surfaces to colonize (Wein *et al.*, 2018), while also supplying nutrients to the microbes for growth. The native microbiome can protect the host from pathogenic invaders (Berg *et al.*, 2016), assist in the digestion of prey (Herndl & Velimirov, 1985; Parker *et al.*, 2018), and increase availability of essential nutrients to the host organism through diverse metabolic pathways (Heinken & Thiele, 2015; Chan *et al.*, 2019). The establishment and maintenance of an individual's microbiome results from a combination of processes ranging from initial colonization (Nyholm *et al.*, 2000; Ceh *et al.*, 2013), environmental factors (Har *et al.*, 2015), host immune responses (Augustin *et al.*, 2010; Parisi *et al.*, 2020), and microbe-microbe interactions (Domin *et al.*, 2018). Thus, the residing microbiome can influence the diversity and concentration of subsequent acquired bacterial species, including those that would be beneficial to the host.

Bacterial species from the genus *Bacillus*, *Pseudoalteromonas*, and others have demonstrated positive impacts on host health (Holmström & Kjelleberg, 1999; Jamali *et al.*, 2015; Soltani *et al.*, 2019). Developing methods to increase the efficacy of microbial transplantation are necessary to facilitate microbiome manipulations. For example, the addition of probiotic organisms has become a recommendation for the protection and preservation of reef building corals (Assis *et al.*, 2020; Voolstra & Ziegler, 2020), fish,

and other organisms susceptible to climate change and anthropogenic pollutants (Reshef *et al.*, 2006; Santos *et al.*, 2015; Peixoto *et al.*, 2017; Rosado *et al.*, 2019). Alternatively, marine pathogens such as *Vibiro alginolyticus* can also be transmitted to hosts, resulting in disease and potentially death. To facilitate effective delivery of beneficial microorganisms, and quantify transmission of potential pathogens, it is imperative to determine the efficiency of potential methods of colonization to understand how individual bacteria associate with the host. Development of methods for efficient bacterial seeding that could be paired with complementary approaches (i.e., visualization and gene expression) will be important in elucidating functional relationships between bacteria and hosts.

Experimental models for the uptake, retention, and loss of bacteria in aquatic habitats remain rare in comparison to terrestrial species. For example, *Caenorhabditis elegans* is an insightful experimental organism for quantifying and modeling the acquisition of microbial communities (Dirksen *et al.*, 2016). *Caenorhabditis elegans*, a bacterivorous organism, has several known bacterial colonization methods including vertical transfer to the offspring (Diaz & Restif, 2014), uptake from the surrounding environment, and direct feeding (Samuel *et al.*, 2016). Similar processes have been observed in a vast array of animals including anemones (Baldassarre *et al.*, 2021), corals (Sharp *et al.*, 2012), and fish (Sullam *et al.*, 2012). The quantity of bacterial cells introduced into *C. elegans* is important for successful colonization into the intestine (Vega & Gore, 2017). A similar experimental system in a model aquatic invertebrate would increase our knowledge on the mechanisms of bacterial colonization for these organisms living in environments surrounded by water.

Artemia salina and *Brachionus plicatilis* are small planktonic animals used as prey in aquaculture for carnivorous aquatic animals (Lubzens *et al*., 1989; Bengtson *et al*., 1991). *Artemia salina* are utilized in the laboratory setting for their ease of culture and stable long-term storage of cysts (Clegg, 1967). Juvenile *A. salina* are approximately 1mm long whereas adult *B. plicatilis* reach 500 μm. This size difference as well as their respective swimming strengths may influence a predator's ability to catch prey, particularly for small juveniles. Both *A. salina* and *B. plicatilis* have been shown to transmit bacteria through feeding mechanisms to invertebrates including corals (Assis *et al*., 2020; Galand *et al*., 2020). *Artemia salina* harbors less bacteria compared to *B. plicatilis* in short-term feeding experiments (Makridis *et al.*, 2000). With respect to the bacterial load of each individual prey item, it is unknown how efficiently the bacteria are transferred and maintained in predatory organisms after consumption. While *B. plicatilis* are easily caught, *A. salina* are larger and have a higher nutritional content (Haché & Plante, 2011), which may allow for extended retention of bacteria. These differences could influence the successful inoculation of newly introduced bacteria into a predatory organism. Assessing the sustainability of introduced probiotic microorganisms could be essential for effective protection or treatment of diseased animals.

Here, we utilize the sea anemone *Nematostella vectensis* as an experimental organism for quantification of bacterial transplantation in aquatic invertebrates. In recent years, *N. vectensis* has become a model organism for cnidarians and other invertebrates due to their ease of culture in a laboratory setting, the ability to recapitulate the life cycle, an extensive set of molecular tools, and their broad distribution in coastal habitats (Darling *et al.*, 2004; Darling *et al.*, 2005; Al-Shaer *et al.*, 2021). *Nematostella vectensis*

has also grown into a model for microbiome studies where previous studies have described the shifts in bacterial communities in the lab and field (Har *et al.*, 2015), identified robust microbial communities in particular life cycle stages or abiotic conditions (Mortzfeld *et al.*, 2016; Leach *et al.*, 2019), and determined the contribution of vertical and horizontal transmission (Baldassarre *et al.*, 2021). Because many organisms are predatory in nature, describing the uptake efficiency of bacteria is a key component to determine how the bacterial community changes over time, especially with feeding organisms.

In this study we quantify the colonization and establishment of bacteria to this cnidarian host. We utilize two different prey species (*Artemia salina* and *Brachionus plicatilis*) to better understand the impact of organismal level interactions with respect to the delivery of bacteria. For this study we selected three bacterial species, *Bacillus velezensis,* a documented probiotic (Yi *et al.,* 2018, Thurlow *et al.,* 2019), *Pseudoalteromonas spiralis,* an antagonist to other bacteria (Holmström & Kjelleberg, 1999), and *Vibrio alginolyticus*, a known marine pathogen (Zhenyu *et al.,* 2013, Yang *et al.,* 2021). This approach is compared to the transmission of bacteria through solution to assess how contrasting inoculation approaches impact colonization of these environmental isolates. This method can be extended to other aquatic organisms, particularly predatory species capable of acquiring microbes through their prey.

Materials and Methods

Bacterial cultivation

We selected three environmental isolates originally collected from Georgetown, South Carolina, *Bacillus velezensis*, *Pseudoalteromonas spiralis*, and *Vibrio alginolyticus*, for the colonization and growth efficacy trials. *Bacillus velezensis* and *P. spiralis* were cultivated on Estuarine Broth agar (EB) (Table S2.1 for recipe), while *V. alginolyticus* was cultivated on Heart Infusion (HI) broth agar plates (Criterion Cat. no.: C5831) in preparation for seeding in 15 ppt Artificial Seawater (ASW). Before transfer to the specific seeding condition, the Optical Density (OD) of the bacterial cultures were determined for a cell concentration of 10^8 cells mL^{-1}. Growth curves were measured in the BioTek LogPhase 600 for all bacteria (Figure S2.1). Samples were serially diluted in 90 µL Phosphate Buffered Saline (PBS), and 10 µL of liquid were drop spotted on the appropriate media. The plates were grown at 25°C overnight, and Colony Forming Units (CFUs) were counted the following day.

Prey cultivation

Artemia salina cultivation

Artemia salina cysts were purchased from Artemia International. One gram of *A. salina* cysts were cultivated in 250 mL of 30 ppt ASW with bubbling to maintain cysts in suspension while under a light source (NT). For axenic culturing (AB), *A. salina* were exposed to an antibiotic cocktail (50 µg mL^{-1} Ampicillin (Acros Organics Cat. No.: 61177), Chloramphenicol (VWR, Cat. No.: 0230), Kanamycin (Fisher BioReagents, Cat. No.: BP906), and Neomycin (Alfa Aesar, Cat. No.: J61499) each) for 24 hours under

identical cultivation conditions as NT. After 24 hours hatched *A. salina* were subsequently washed three times in sterile 15 ppt ASW two hours before bacterial inoculation. Both 'No Treatment' (NT) and 'Antibiotic' (AB) conditions utilized this wash step to eliminate either antibiotics or bacteria in solution.

Brachionus plicatilis cultivation

Brachionus plicatilis were purchased from LiveAquaria (#BEH-75641) that were pre-fed with algae. This stock culture was maintained by feeding with *Storeatula major* cultivated in 15 ppt F/2 media with continuous vigorous aeration. One-quarter volume of the culture was cycled weekly to reduce the accumulation of waste products in solution. Prior to experimentation, *B. plicatilis* were reared in 15 ppt ASW for 24 hours to allow clearing of algae from the digestive system. Antibiotic treated *B. plicatilis* were exposed to the same antibiotic cocktail as *A. salina* over 24 hours to remove resident bacteria, as described for *A. salina*. *B. plicatilis* were rinsed 2 hours before application of bacteria used for experiments.

Determination of *Artemia salina* and *Brachionus plicatilis* short-term feeding capacity

Two groups of both *A. salina* and *B. plicatilis* were grown, one axenically (AB), and the other with no treatment (NT). After the wash step, the prey were exposed to individual or combinations of bacteria. For all conditions, the prey items were exposed to bacteria in a 1:1 v/v ratio, at a concentration of 10^8 CFUs mL^{-1}. After 10 minutes of exposure, the prey items were rinsed with 30 mL sterile 15 ppt ASW, and groups of 10 were dissociated with sterile pestles in 1.5 mL microcentrifuge tubes, then serial diluted

as previously described and drop spotted for CFU counts. Four types of media were utilized in all experiments for CFU calculations, Heart Infusion (HI) agar plates (Criterion Cat. No.: C5831), CHROMagar Vibrio plates (CAV) (CHROMagar Ref. No.: VB912), Phenylethyl Alcohol (PEA) agar (BD Ref. No.: 211539), and Marine Broth (MB) agar plates (Table S2.1). All media were made using instructions provided by the respective manufacturer.

Nematostella vectensis cultivation

*Nematostella vectensi*s adults were cultured in 15 ppt ASW and spawned weekly, following previously established methods that include feeding adults with mussel tissue once and *A. salina* three times per week, with weekly water changes (Hand & Uhlinger, 1992; Fritzenwanker & Technau, 2002; Stefanik *et al.*, 2013). Fertilized eggs were separated into new bowls of 15 ppt ASW for development. Recently metamorphosed juveniles were fed *Brachionus plicatilis* several times until the approximate size reached (>3 mm). To create axenic individuals, the polyps were exposed to the antibiotic cocktail for 24 hours. Two hours before treatment, organisms were washed with and transferred to sterile 15 ppt ASW.

Seeding *Nematostella vectensis* with bacteria

Direct application of bacteria

The three bacterial species were grown to a concentration of 10^8 cells mL^{-1} in EB. *Nematostella vectensis* larvae and young juveniles were exposed to approximately 10^8 cells in 3 mL of sterile ASW. After 10 minutes the animals were removed from the

bacterial solution, washed over a 40 µm Nylon filter with 30 mL sterile ASW, and aliquoted into flat bottom 96-well plates with 100 µL sterile 15 ppt ASW. At each time point animals (NT, AB, and SUM treated) were dissociated using a sterile pestle in a 1.5 mL microcentrifuge tube, then serially diluted and plated on the applicable media for CFU counts (n=5). We refer to this approach as the solution uptake method (SUM).

Feeding *Nematostella vectensis* seeded prey

Artemia salina (PFM-A) and *B. plicatilis* (PFM-B) were exposed to individual bacterial cultures of approximately 10^8 cells in 15 ppt ASW. For the combination of *B. velezensis* and *V. alginolyticus,* a 50:50 mix of both bacteria for a total of 10^8 cells was exposed to the prey items. After 10 minutes of exposure prey were washed with 30 mL of sterile ASW and placed into a 6-well plate with *N. vectensis*. To increase the efficiency of the animals' ability to catch prey, the plates were placed over a light source. As the anemones caught single prey, they were isolated to a single well in a 96 well plate containing with 100 µL of sterile ASW. At 6 hours, 48 hours, 7 days, and 14 days entire anemones were washed over a 40 µm Nylon Mesh Filter with 30 mL sterile ASW and dissociated in sterile 1.5 mL tubes filled with 100 µL 15 ppt ASW (n=5). The resulting slurry was serially diluted and 10 µL drop spots were plated on the appropriate media for each individual animal. The total number of CFUs were counted the subsequent day. We refer to this approach as the prey feeding method (PFM). To determine the number of bacteria prey items shed into solution, we serially diluted the previously sterile ASW the prey resided in for 10 minutes after bacterial exposure. Additionally, to determine the relative transfer of non-treated prey to *N. vectensis*, juveniles were antibiotically treated

and exposed to NT prey and plated on MB, HI, PEA, and CAV, as described above over the time course.

Saturated feeding of adult *Nematostella vectensis* with *Bacillus velezensis*

Using fully grown axenic adult anemones, we fed animals approximately 50 *A. salina* each. Following methods described above (section 2.3), *A. salina* were exposed to *B. velezensis*. Adult anemones were starved for 5 days prior to the exposure of the prey items. Over the same time course as juveniles, adult animals were dissociated, serially diluted, and plated on PEA as previously described.

Results

Prey seeding

Recently hatched *A. salina* harbored similar bacterial concentrations (Figure 2.1A) to those previously reported (Makridis *et al.*, 2000). Our laboratory acclimated *B. plicatilis* had lower quantities of *Vibrio* spp. and gram-positive bacteria than has been shown in previous studies (Martínez-Díaz, 2003), but this did not appear to influence their ability to uptake additional bacteria (Figure 2.1A-B). To test the variance in the differing methods we used two-way ANOVA to determine the difference in associated bacterial concentrations between treatments. When exposing the No Treatment (NT) prey to the bacteria, there was significantly less bacteria when adding *V. alginolyticus* than in the axenic (AB treated) organisms (two-way ANOVA, $p = 0.0067$, $p < 0.0001$, PFM-A, PFM-B, respectively). No statistical difference was detected between the NT and AB treated organisms for *B. velezensis* and *P. spiralis* in both prey items (all p-values above

0.977, see Table S2.2). Additionally, when comparing the bacterial isolates, *V. alginolyticus* accumulated in untreated organisms with significantly higher numbers than *B. velezensis* in *A. salina* ($p = 0.0142$), and both *B. velezensis* and *P. spiralis* in the *B. plicatilis* ($p < 0.0001$, both comparisons). In axenic prey, *V. alginolyticus* seeded with higher numbers than the two other bacteria in both *A. salina* ($p < 0.0001$, both comparisons) and *B. plicatilis* (*B. velezensis* $p = 0.0027$, *P. spiralis* $p = 0.0025$). We found that short term exposure to dense bacterial culture results in high concentrations of seeded bacteria, which in some cases exceeded normal bacterial concentrations in the prey items (Figure 2.1B). When *A. salina* (Figure 2.2A) was exposed to combinations of *B. velezensis* and *V. alginolyticus*, *B. velezensis* seeded similarly to individual inoculations ($p > 0.9673$), and *V. alginolyticus* decreased ($p < 0.0001$) from the concentrations they were exposed to individually (Figure 2.2A). For *B. plicatilis* combination exposures, *B. velezensis* remained close to individual exposures, while *V. alginolyticus* also decreased ($p < 0.0001$) relative to individual exposures (Figure 2.2B). Thus, short term exposures with combinations of microbes at equal concentrations in the surrounding media resulted in differential concentrations in the prey item.

Predator seeding

Seeding *Nematostella vectensis* larvae

Under NT conditions, 2-day old larvae harbor between 10^1 and 10^3 CFUs per individual, depending on media type. While CAV and PEA selected microbes did not increase in CFUs over the time course, both MB and HI had a 1-fold increase after 2 days (Figure 2.3A, all statistical values for *Nematostella vectensis* time courses can be found

in Table S2.2). For the larval stage of *N. vectensis*, SUM had the greatest retention of microbes (Figure 2.3B), as the organisms pre-settlement stages are not capable of predatory feeding (Figure 2.3C-D) and are not known to actively consume bacteria (Kehr & Jaeckle, 2013). The colonization of *B. velezensis, P. spiralis,* and *V. alginolyticus* through the SUM was significantly higher in the short term (2D SUM vs. PFM-A and PFM-B, $p < 0.0001$, all comparisons). Additionally, there was a significant difference between prey feeding methods for *V. alginolyticus* (2D PFM-A vs. PFM-B, $p = 0.0040$). While direct uptake of bacteria from PFM is unlikely in this non-feeding life stage (no prey consumption was observed), bacteria that were present in solution seeded these developmental stages. The concentration of bacteria increases from 6 hours to 2 days under all conditions (Figure 2.3). *Vibrio alginolyticus* increased 2-fold over this time course. Both *B. velezensis* and *P. spiralis* also increased over this early development phase. We found concentrations ranging from 7.0×10^3 - 3.6×10^5 bacterial CFUs in solution after washing *A. salina* and *B. plicatilis*, indicating association of bacteria with the larvae was likely through this indirect mechanism (Figure S2.3). The concentration of bacteria shed into solution was similar to the SUM concentrations of larvae. At 48 hours both SUM and Prey Feeding Methods resulted in similar concentrations that exceed the initial 6-hour larvae measurements.

Feeding vs. solution uptake by juvenile *Nematostella vectensis*

When juvenile sea anemones were fed NT prey items, the relative uptake of bacteria from *A. salina* (Figure 2.4A) and *B. plicatilis* (Figure 2.4B) were similar, while trending downward through the 7-day cycle. Juvenile animals over 7 days did not

increase in measurable CFUs over the time course (Figure 2.5A). SUM transferred microbes resulted in less associated bacteria when compared to the PFM at 2 days and retained less CFUs over time in juvenile organisms. The SUM transplanted microbes increased at 2 days, with general decrease in CFUs at and after 7 days (Figure 2.5B). Additionally, both prey vectored methods resulted in a higher concentration of bacteria in the organisms at 2 days than at 6 hours, except for *P. spiralis* in *A. salina*. When *B. velezensis* and *P. spiralis* colonized juveniles through the SUM, the number of CFUs transferred were significantly lower and higher, respectively, at 2 days (2D PFM-A, $p < 0.0001$, $p = 0.0018$, and 2D PFM-B, $p > 0.9999$, $p = 0.0010$, respectively). Additionally, the SUM resulted in significantly higher accumulation of CFUs at 2 days than both PFMs for *V. alginolyticus* (6H PFM-A, $p < 0.0001$, 6H PFM-B, $p < 0.0001$). Bacteria vectored with *A. salina* increased up to 2-fold than initial concentration (Figure 2.5C), while the *B. plicatilis* vectored bacteria were up to 1-fold greater than initial concentrations at 2 days (Figure 2.5D). We further observed that the highest concentration of vectored bacteria was *V. alginolyticus* within *B. plicatilis*, potentially indicating a preference of microbial uptake, or differential microbial retention at the genus level. At 7-days the number of vectored microbes from both prey decreased to the 6-hour concentrations and further decreased at 14 days for most conditions (Figure 2.5C-D). For *B. velezensis* and *P. spiralis* transferred to *N. vectensis*, both *A. salina* and *B. plicatilis* shared similar short-term capacities (Figure 2.5C-D). In a satiated feeding experiment, we observed that adult *N. vectensis* can consume large volumes of prey and still retain the bacteria through the digestion of the prey species. At 2 days the concentration of bacteria increased 2-fold

(Figure 2.6), likely due to the availability of freshly digested *A. salina*. At 7 days the concentration of *B. velezensis* decreased from the 2-day concentration.

Discussion

With the pace of climate change likely exceeding the ability for many animals to adapt genetically, the introduction and retention of beneficial microbes represents a promising approach for future conservation efforts (Bourne *et al.*, 2016; Peixoto *et al.*, 2017; Rosado *et al.*, 2019). Methodology for controlled manipulation of the microbiome has extensive application for modifying the health and resilience of aquatic hosts. Approaches for successful, efficient introduction of microbes to aquatic animals remains understudied to inform these interventions (Welsh *et al.*, 2017; Damjanovic *et al.*, 2019; Rosado *et al.*, 2019; Doering *et al.*, 2021; Santoro *et al.*, 2021; Silva *et al.*, 2021; Zhang *et al.*, 2021). The use of axenic prey that can be quickly reseeded with probiotic isolates at or above standard bacterial concentrations could be an efficient alternative to introduction of these bacteria in solution. *Brachionus plicatilis* has been utilized as a vehicle to potentially transmit probiotic bacteria to cnidarians (Assis *et al.*, 2020). In our study, we build upon this research by showing that multiple species of bacteria are concentrated in *B. plicatilis* and *A. salina*, and that bacteria seeded into each prey item are successfully transferred to a predatory cnidarian. The utility of small planktonic organisms for delivery of selected bacteria from different phyla has the potential for wide application for conservation of species under threat. Additionally these methods will allow us to better understand the interactions of cnidarians and their associated microbial communities (Peixoto *et al.,* 2021).

Controlled introduction of specific bacteria can be informative for discerning microbe-microbe interactions and the mechanisms of host-microbe interactions. For example, application of *Phaeobacter inhibens* in non-axenic *A. salina* exhibits antagonistic properties against *Vibrio anguillarum* (Grotkjær et al., 2016). Interactions such as these can be investigated within the context of the host organism. To understand the different interactions between individual bacteria, it is important to determine the stoichiometric ratio present in the host system. Here, we quantified the aggregation of both *V. alginolyticus* and *B. velezensis* concurrently and found that both prey items accumulate less *V. alginolyticus* when both bacteria are presented in equal concentration. When constructing engineered microbiomes, such as for probiotic applications, it is important to consider the ratio of seeded bacteria that aggregate in the terminal animal.

The host microbiome can be a protective mechanism that interferes with the colonization of new bacteria, a factor to be considered when investigating host-bacteria interactions. While this mechanism of exclusion is likely dependent on the bacterial interactions, our results suggest that removal of the native microbial community had limited impact on the ability to successfully seed new bacteria. When the native bacteria are removed, *V. alginolyticus* had increased aggregation in *A. salina*, while *B. plicatilis* had less than control animals. Alternatively, *B. velezensis* and *P. spiralis* did not differentially seed either prey, indicating this may be species dependent. Additionally, we found *V. alginolyticus* seeded *B. plicatilis* with a 2-fold higher CFUs when compared with *B. velezensis* and *P. spiralis*. To effectively study changes in individual or combinations of bacterial interactions with the host it may be beneficial to eliminate the

pre-existing microbiome to control the composition for some bacteria. For example, to study the direct interactions between a single bacterial species and its host, it is imperative to eliminate other bacteria to reduce unnecessary interactions. We anticipate that there is a species dependent uptake according to what other microbes are present in solution, and in the host. This result suggests that it may be difficult to predict the microbiome of prey items based on the composition of microbes in solution (Carrier & Reitzel, 2019).

Nematostella vectensis larvae acquire their microbiomes at early developmental stages which differs from the microbiome of later life stages (Mortzfeld *et al.,* 2016). For the larval stage, short term uptake of all bacteria at 6 hours was equitable across all treatment types. At 48 hours, SUM treated larvae had increased acquisition for all bacterial species against both PFMs. While active bactivory has not been documented in *N. vectensis*, there were still associations of bacteria in the larval stages (Mortzfeld *et al.*, 2016), even when exposed to bacteria shed from seeded prey items. After prey were seeded, we measured different shedding between bacterial species. Both *B. velezensis* and *P. spiralis* shed 1-fold more CFUs into solution when associated with *A. salina*, while *V. alginolyticus* shed 1-fold more with *B. plicatilis* (Figure S2.3). Despite this differential shedding of bacteria into solution, the number of bacteria measured with the larvae remained similar, indicating there may be other factors such as surface area and binding affinities influencing the quantity of associated bacteria.

Juvenile anemones that consumed seeded prey had an increase in CFUs compared with SUM, likely due to additional nutrients from the metabolism of the prey organisms. With *B. plicatilis*, the CFUs increased 2-fold at 48 hours, decreased 2-fold at 7 days, and

maintained these concentrations over 14 days. With *A. salina* anemones had a greater increase in CFUs but these were also lost through 7 days, returning to the 6-hour concentrations. The retention of high concentrations of introduced bacteria for less than one-week indicates that continuous feeding would likely be required to maintain the seeded bacteria, between 2 and 7 days. The tradeoff of size and nutrient composition between *A. salina* and *B. plicatilis* is important for applications comparing species or life stages. Young polyps and other small planktivorous organisms may require the smaller prey for ease of capture and consumption. Adults may benefit from larger nutrient-rich prey to both seed and maintain the transplanted microbes. In adults, a higher initial number of seeded prey does not appear to influence bacterial retention in *N. vectensis*, and resulted in a decrease to the original CFU load for *B. velezensis* at 7 days. This indicates repeated inoculations is necessary to maintain high concentrations of probiotic organisms.

Our work targeted the quantification of bacterial inoculation of a model cnidarian species through two approaches: solution and prey items. To determine the impact of probiotics or any bacterial species on stressed or diseased organisms, it is important to understand how bacteria colonize hosts, including how different methods of introduction influence these symbioses. Together, these combinations of methods and comparisons result in a quantitative assessment for how particular microbes can be successfully seeded into focal species to permit introduction of bacteria for future applications. Future studies will help determine the longevity of transplanted bacterial species under different abiotic conditions that more closely resembles natural environments to help inform the field-based application of probiotic supplements.

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Table 2.1: Estuarine Broth Recipe.

1L Estuarine Broth	
NaCl	12.36g
KCl	0.34g
$CaCl_2 \cdot 2\ H_2O$	0.68g
$MgCl_2 \cdot 6\ H_2O$	2.33g
$MgSO_4 \cdot 7\ H_2O$	3.15g
$NaHCO_3$	0.18g
Peptone	10g
Yeast Extract	3g
Agar (Plates only)	15g

S2.2 : Statistical results (p-values) of all experiments.

	Artemia salina	*Brachionus plicatilis*
	NT - AB	NT - AB
V. alginolyticus	0.0067	<0.0001
P. spiralis	0.977	0.9998
B. velezensis	>0.9999	>0.9999
	Artemia salina	*Brachionus plicatilis*
	Single vs. Combo	Single vs. Combo
V. alginolyticus	<0.0001	<0.0001
B. velezensis	0.9673	0.9948
Nematostella vectensis	NT	
	Larvae 6H - 2D	
NT - MB	<0.0001	
NT - HI	0.0216	
NT - CAV	0.9928	
NT - PEA	0.1171	
	Larvae 6H SUM, PFM-A, PFM-B	Larvae 2D SUM, PFM-A, PFM-B
Bacillus velezensis		
SUM 6H vs. PFM-A 6H	0.9903	<0.0001
SUM 6H vs. PFM-B 6H	0.9902	<0.0001
PFM-A 6H vs. PFM-B 6H	>0.9999	>0.9999
Pseudoalteromonas spiralis		
SUM 6H vs. PFM-A 6H	>0.9999	<0.0001
SUM 6H vs. PFM-B 6H	>0.9999	<0.0001
PFM-A 6H vs. PFM-B 6H	>0.9999	>0.9999

Vibrio alginolyticus				
SUM 6H vs. PFM-A 6H	>0.9999	<0.0001		
SUM 6H vs. PFM-B 6H	>0.9999	<0.0001		
PFM-A 6H vs. PFM-B 6H	>0.9999	0.0040		
Nematostella vectensis	SUM	PFM-A		PFM-B
	Larvae 6H - 2D	Larvae 6H - 2D		Larvae 6H - 2D
B. velezensis	0.2217	>0.9999		0.9961
P. spiralis	<0.0001	0.986		0.9376
V. alginolyticus	0.0026	0.1266		0.0033
Nematostella vectensis	*Artemia salina*			
AB - NT Prey	MB	HI	CAV	PEA
6H vs. 2D	0.1231	0.0015	<0.0001	0.0063
6H vs. 7D	0.0819	0.0008	<0.0001	0.0064
2D vs. 7D	0.0109	0.0034	0.3156	0.0239
Nematostella vectensis	*Brachionus plicatilis*			
AB - NT Prey	MB	HI	CAV	PEA
6H vs. 2D	0.1559	0.1295	<0.0001	0.015
6H vs. 7D	0.0185	0.2536	<0.0001	0.0004
2D vs. 7D	0.1121	0.0328	<0.0001	0.0351
Nematostella vectensis	SUM			
	Bacillus velezensis	*Pseudoalteromonas spiralis*		*Vibrio alginolyticus*
6H vs. 2D	0.0947	0.0772		0.0291
6H vs. 7D	0.0035	0.259		0.0061
6H vs. 14D	0.5145	0.0016		0.0115
2D vs. 7D	0.7075	0.0868		0.0298
2D vs. 14D	0.0102	0.0695		0.029
7D vs. 14D	0.0036	0.0017		0.0059

Nematostella vectensis	PFM-A		
	Bacillus velezensis	*Pseudoalteromonas spiralis*	*Vibrio alginolyticus*
6H vs. 2D	0.0013	0.992	0.4974
6H vs. 7D	0.6321	0.9854	<0.0001
6H vs. 14D	>0.9999	0.0435	<0.0001
2D vs. 7D	0.0489	0.9538	0.7972
2D vs. 14D	0.0057	0.2197	0.5148
7D vs. 14D	0.3802	0.1768	<0.0001
Nematostella vectensis	PFM-B		
	Bacillus velezensis	*Pseudoalteromonas spiralis*	*Vibrio alginolyticus*
6H vs. 2D	0.5807	0.0508	<0.0001
6H vs. 7D	0.9982	0.1291	0.8093
6H vs. 14D	0.9997	0.1226	<0.0001
2D vs. 7D	0.628	0.0512	0.677
2D vs. 14D	0.5725	0.0507	<0.0001
7D vs. 14D	0.994	0.0006	0.5382

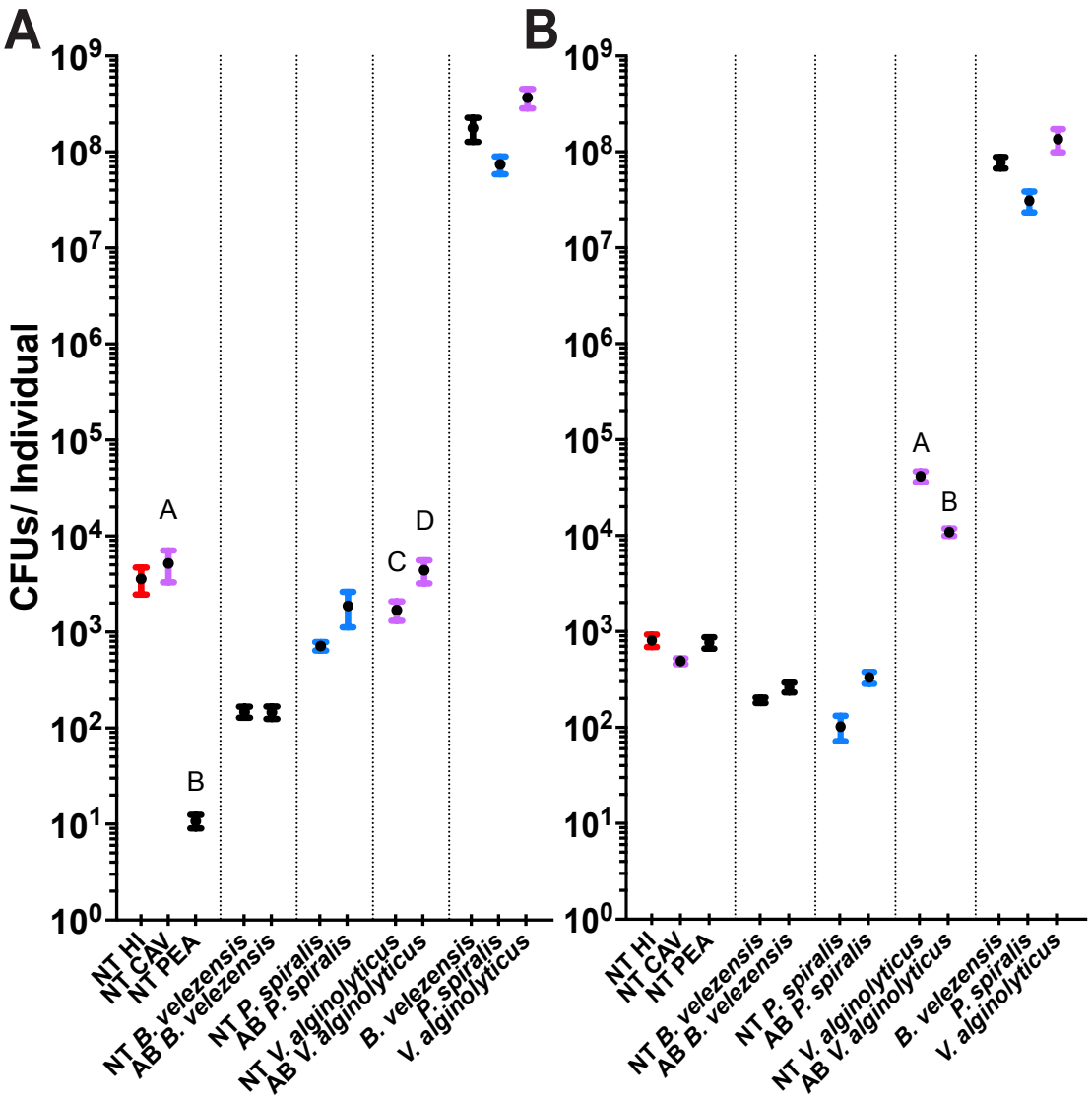

Figure 2.1: Prey individual CFU counts. (A) Artemia salina individual bacterial inoculation plate counts 10-minute after introduction to each bacterial species. Dissociated groups of 10 prey (n = 15) were serially diluted on non-selective (MB, HI) and selective media (CAV, PEA) where appropriate. A-B ($p = 0.0168$) C-D ($p = 0.0067$). (B) Brachionus plicatilis individual 10-minute bacterial inoculation plate counts. A-B ($p < 0.0001$). Technical replicates across media types were averaged. Antibiotic treated control animals yielded no culturable growth under the same plating conditions. Separate values are graphed in Figure S2.1. Points represent the mean, and the bars indicate the Standard Error of the Mean (SEM).

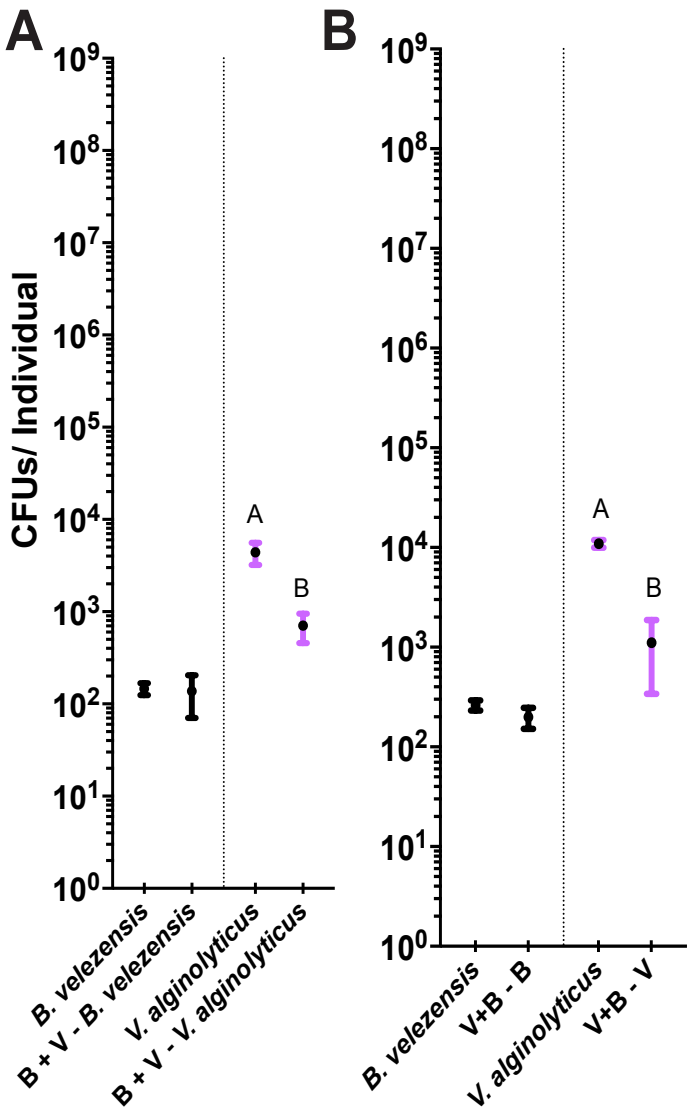

Figure 2.2: Counts of bacteria resulting from prey combination exposures. Groups of 10 prey (n = 15) were dissociated and serially diluted on appropriate media types. (A) *Artemia salina* combination 10-minute bacterial inoculation plate counts. A-B ($p < 0.0001$). Combinations containing *P. spiralis* were uncountable due to the inability to effectively select against the other cohorts. (B) *Brachionus plicatilis* combination bacterial exposure plate counts. A-B ($p < 0.0001$).

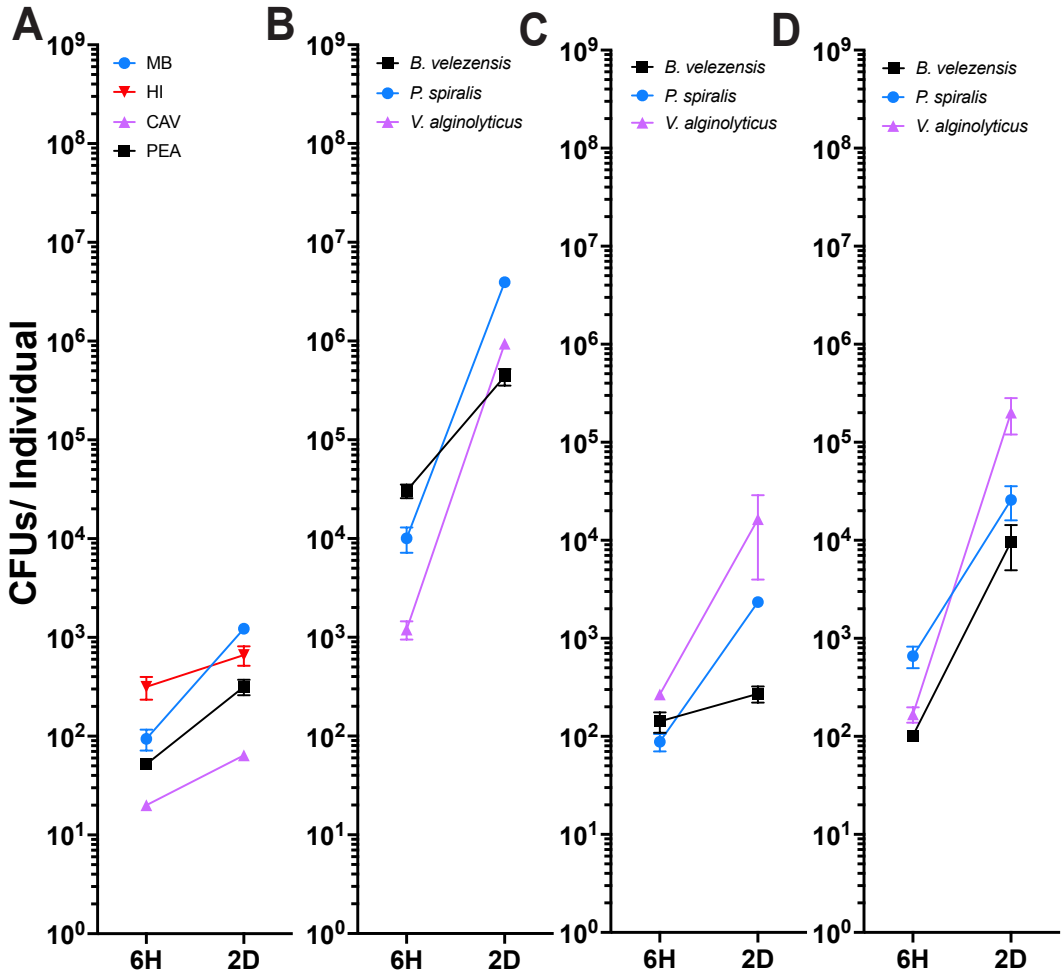

Figure 2.3: *Nematostella vectensis* larvae CFU counts over 2 days (n = 5). Points represent the mean and bars represent the SEM. (A) Untreated control larvae. The number of CFUs associated significantly increased from 6 hours to 2 days. (B) SUM treated larvae. All bacterial species increased from 6 hours to 2 days. (C) *Artemia salina* PFM treated larvae. CFUs for all bacterial species trended upward from 6 hours to 2 days. (D) *Brachionus plicatilis* PFM treated larvae. *Vibrio alginolyticus* significantly increased over the time course ($p < 0.0033$). All statistical values can be found in Table S2.2.

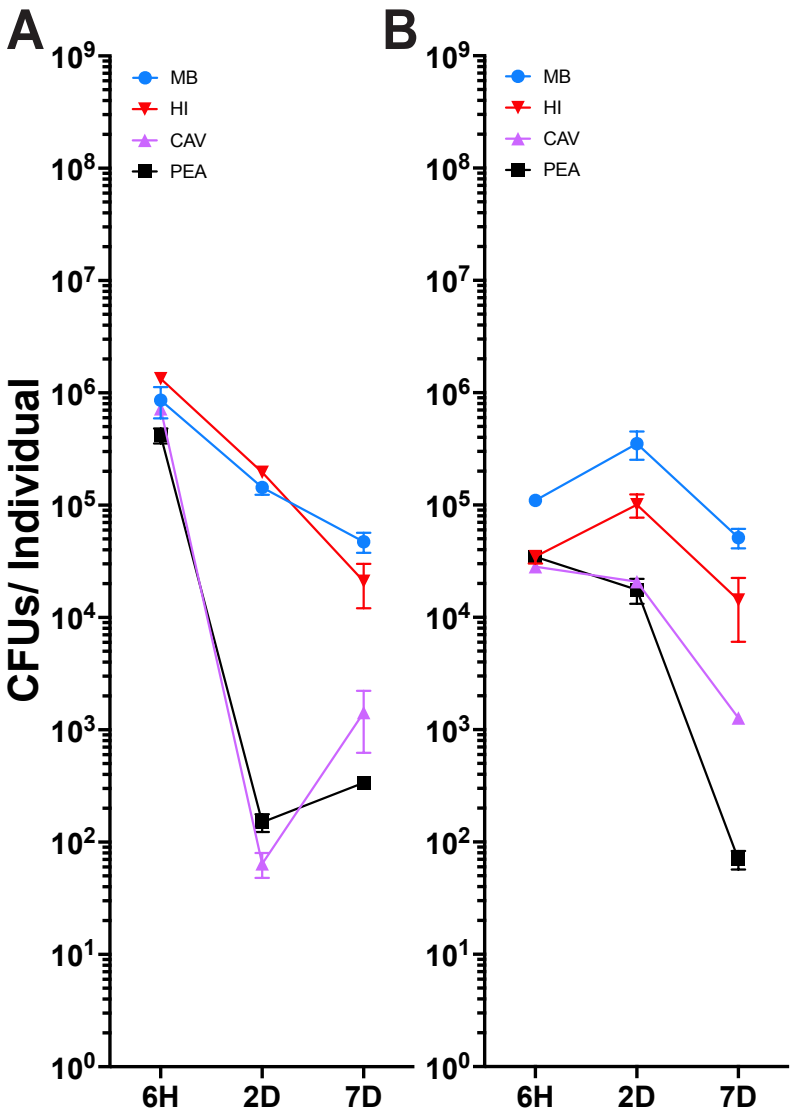

Figure 2.4: *Nematostella vectensis* juvenile CFU counts over 7 days. (A) Antibiotic treated juveniles fed Non-Treated *Artemia salina*. (B) Antibiotic treated juveniles fed Non-Treated *Brachionus plicatilis*. All statistical values can be found in Table S2.2.

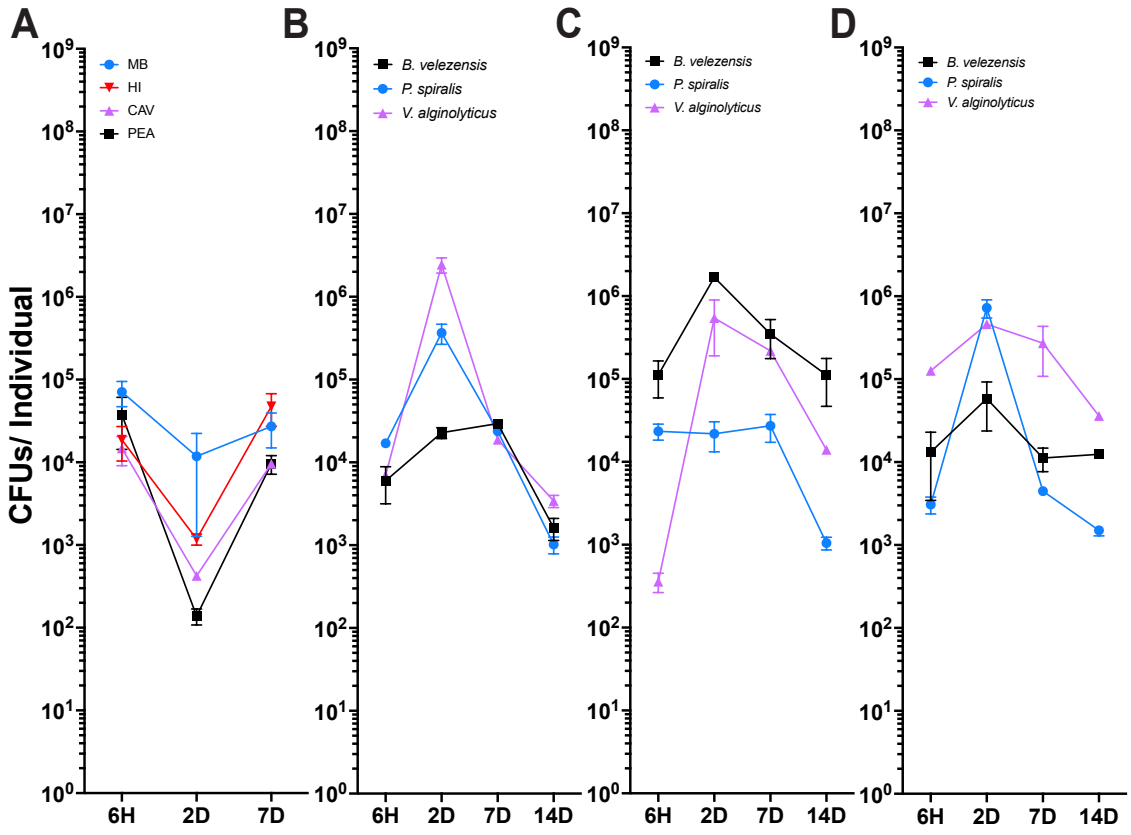

Figure 2.5: Juvenile *Nematostella vectensis* CFU counts. (A) Non-treated juveniles over 7 days. (B) SUM treated juveniles over 14 days. (C) *Artemia salina* PFM fed juvenile *N. vectensis*. (D) *Brachionus plicatilis* PFM fed juveniles.

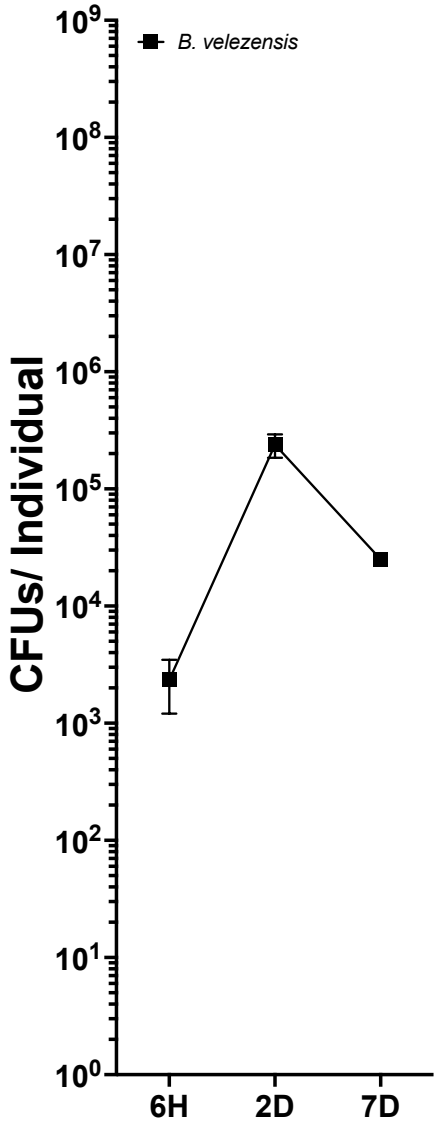

Figure 2.6: Adult *Nematostella vectensis* fed *Artemia salina* seeded with *Bacillus velezensis* to satiation, CFUs measured over a 7-day time course.

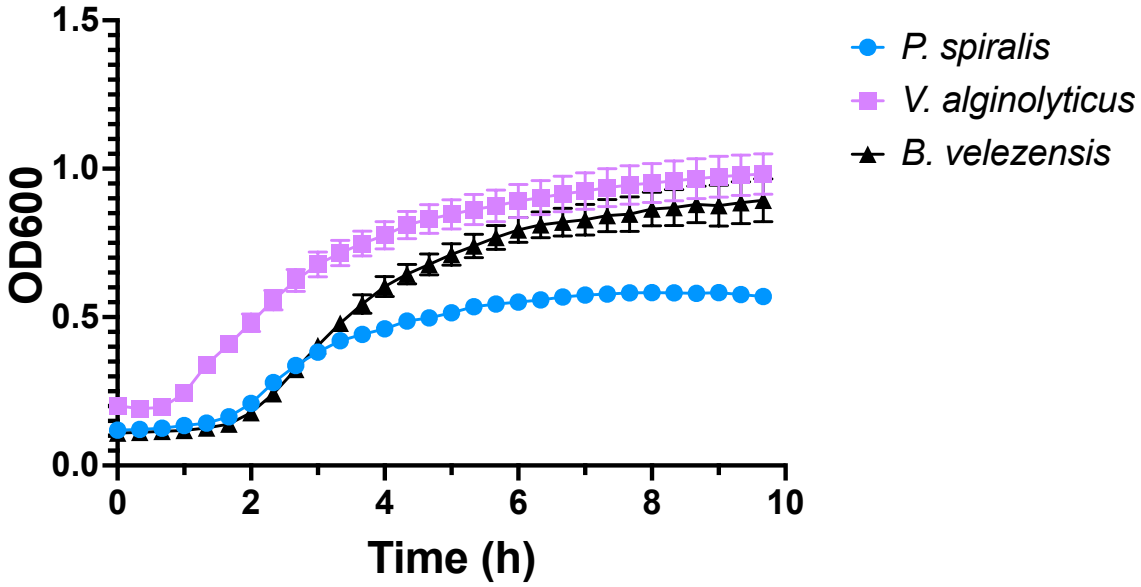

Figure S2.1: Growth curves of *Bacillus velezensis*, *Pseudoalteromonas spiralis*, and *Vibrio alginolyticus* recorded with the BioTek Logphase600. Optical Density (OD600) was measured in 20-minute intervals in Estuarine Broth at 30°C for 10 hours.

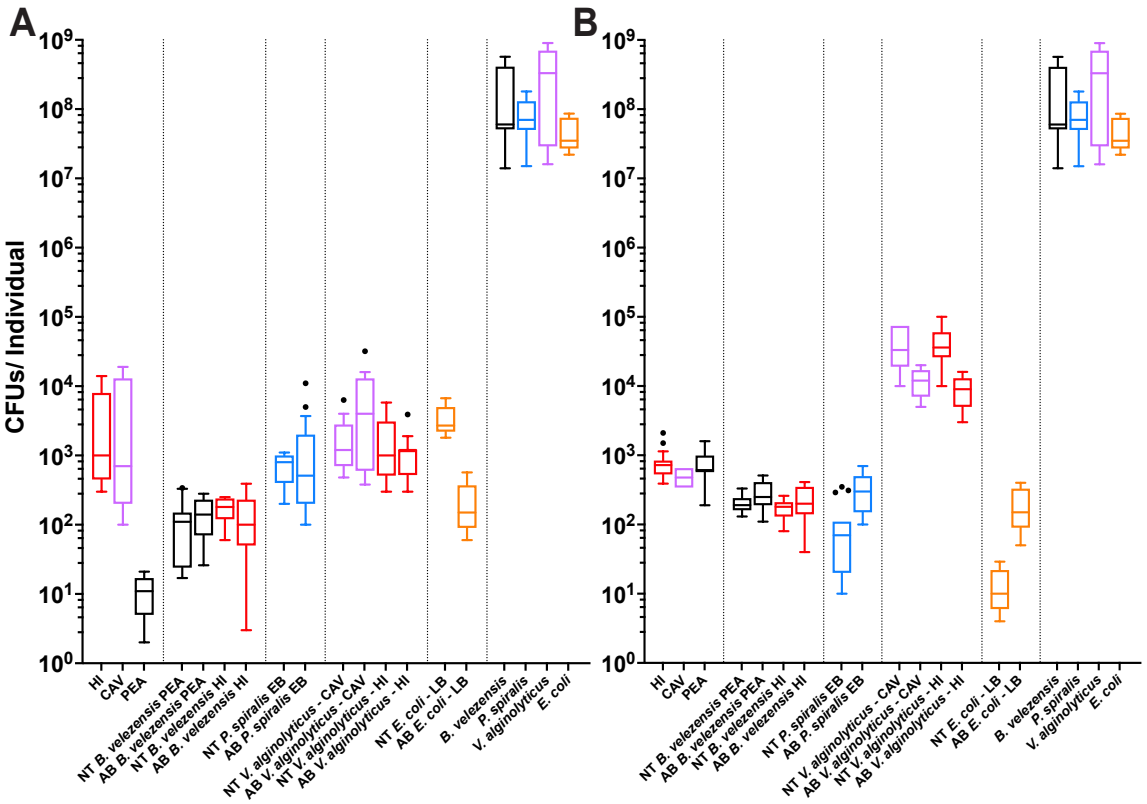

Figure S2.2: Prey item exposures with separated technical replicates across media types. See Figure 2.1 for a detailed explanation. *Escherichia coli* S-17 lambda pir inoculated into both prey types, and plated on Lysogeny Broth Agar plates (Fisher Scientific, BP-1425). (A) *Artemia salina* exposures. (B) *Brachionus plicatilis* exposures.

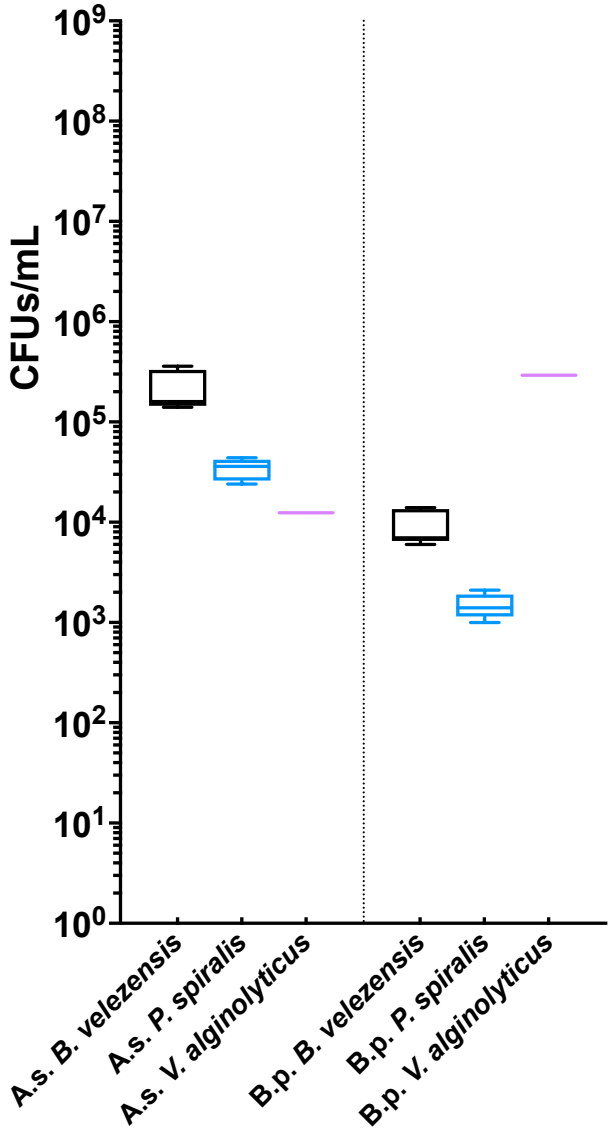

Figure S2.3: Concentration of bacterial CFUs shed from washed *A. salina* and *B. plicatilis*. Prey washed after a 10-minute exposure to bacteria. Prey were resuspended in 15ppt ASW for 10 minutes, and the solution was sampled for resultant bacteria shed into solution.

CHAPTER 3

IMPACTS OF THERMAL AND SALINE STRESSORS ON THE ASSOCIATED BACTERIA OF THE CNIDARIAN HOST NEMATOSTELLA VECTENSIS

Quinton A. Krueger, Madisun H. Shore, Adam M. Reitzel

Abstract

The associated microorganisms ('microbiome') of multicellular individuals ('host') are important for the physiology and survival of the host. Individual bacterial species vary in environmental tolerances that may limit their associations with hosts, especially when their range of survivable conditions is narrower. To elucidate the roles for different niche spaces of the microbiome, we evaluated the performance of individual and combinations of bacteria with and without an animal host, *Nematostella vectensis* (Cnidaria, Anthozoa). We assessed 62 environmental isolates (*Alteromonas*, *Bacillus*, *Grimontia*, *Photobacterium*, *Pseudoalteromonas*, *Shewanella*, and *Vibrio* genera) from six geographically distinct estuaries and the host to determine their tolerance across a gradient of temperatures (30° - 40°C) and salinities (5 ppt - 30 ppt). Growth rates and plate counts revealed members of the *Vibrio* genus had the fastest growth rate at higher salinities (15 and 30 ppt), while *Bacillus* and *Alteromonas* spp. exhibited the growth over a broader scope of all tested conditions, indicating these genera could be present in the host at extreme conditions of these estuarine environments. Interestingly, only 15% of isolates were capable of growth at the highest temperature and lowest salinity (40°C 5 ppt). We further assessed three isolates (*Bacillus velezensis*, *Pseudoalteromonas spiralis*, and *Vibrio alginolyticus*) for how bacterial survival changed *in vivo* with *Nematostella*

vectensis. When anemones were exposed to heat stress over three days, quantities of each tested bacteria varied across conditions. *Pseudoalteromonas spiralis* had higher growth at lower salinities and maintained stable concentrations. Conversely, *V. alginolyticus* grew at higher salinities, and maintained higher concentrations in most conditions. Temperature poses a serious threat to the individual bacteria, where concentrations diminished following thermal spikes inside the host. Together, these results support a hypothesis that environmental conditions drive the microbial community and relative abundance of certain isolates.

Introduction

The composition of microbes (microbiome) of an environment is dependent on a variety of biotic and abiotic factors, e.g., temperature (Lokmer & Mathias Wegner 2015; Ramsby et al. 2018), salinity (Dulski et al. 2020; Röthig et al. 2016), and the host organism (Ahern et al. 2021). These factors can influence the diversity and abundance of microbes present in the environment. A core microbiome, which is composed of bacteria over a broad range of environmental conditions, has been described in many animals (Dirksen et al. 2016; Douglas 2018; Dunphy et al. 2019). While the mechanisms for the formation and modification of a host's microbiome are difficult to elucidate, several host-centric hypotheses have been proposed.

Current research describes the role of host genomics in microbial selection (Ahern et al. 2021). Microbes associated with host diet have been shown to influence the microbiome structure (Macke et al. 2020; Roura et al. 2017; Samuel et al. 2016). Additionally, environmental conditions (McDevitt-Irwin et al. 2017; Sepulveda &

Moeller 2020; Woodhams et al. 2020), tissue specificity (Marchioro et al. 2020; Van Oppen & Blackall 2019), and competition between individual bacteria can shape these communities (Prigot-Maurice et al. 2022). Invertebrates can acquire microbes vertically from the maternal organism (Giraud et al. 2022; Unzueta-Martínez et al. 2022), and this composition can change over the various life stages (Lee et al. 2018; Vijayan et al. 2019; Wu et al. 2018; Zhou et al. 2017). Additionally, bacteria such as some described within the *Pseudoalteromonas* genus, have been found to provide settlement cues in a variety of marine invertebrates (Huggett et al. 2006b; Sneed et al. 2014; Tebben et al. 2011; Unabia & Hadfield 1999). These bacteria can play a vital role in mechanisms through the life stages of the host, which necessitates further investigation of these interactions.

Bacteria that are capable of survival in highly dynamic and extreme environments may be better equipped for growth and survival in a host under stressful conditions. These bacteria in conjunction with the host may assist in the holobionts' survival while under stress (Fallet et al. 2022; Marangon et al. 2021; Reina et al. 2022). The synergisms of the host and microbiome may expand the environments in which both organisms can survive (Williams & Carrier 2018; Yang et al. 2022). In addition, bacteria can modulate immune responses in invertebrates, and protect against pathogens (Giarma et al. 2017; Li et al. 2019; Sampath 2018; Wang et al. 2021b). It is important to identify these individual bacteria to determine which are capable of surviving or thriving in the environment. The physiological tolerance of each bacterial species is a critical factor that determines how microbiomes assemble and how they may vary in different environments.

In aquatic ecosystems, host-microbe interactions have been studied in both the natural environment and in aquaculture. For aquatic invertebrates, cnidarians, and

particularly corals, have well characterized microbiomes (Bourne et al. 2016; Pollock et al. 2018; Tran 2022b), where the bacterial communities are distinct across geographically distinct populations (Botté et al. 2022; Morelan et al. 2019). Variance in the microbiome can also be attributed to distinct species of coral (Price et al. 2021; Ricci et al. 2022) and host genome (O'Brien et al. 2020). Corals have limited environmental ranges and when they develop to the adult stage are incapable of movement. Individual corals must acclimate to the changing environment, a process which has been shown to include the fluctuations of associated prokaryotic and eukaryotic microbes (Ahmed et al. 2019; Dungan et al. 2022; Rosado et al. 2019; Santoro et al. 2021). At extreme temperatures algal symbionts from the family Symbiodiniaceae can be purged from the host eukaryote (e.g., coral, anemone, mollusk), resulting in bleaching and potentially lead to death (Strychar et al. 2005). White Band Disease, a common lethal affliction in corals, is attributed to the increased presence of *Vibrio carchariae*, indicating changes in the microbiome can lead to physiological duress of the host (Sweet et al. 2014). Microbes localized in the mucus layer of corals assist in maintaining the overall health of the holobiont (Huntley et al. 2022; Hussien et al. 2019; Vanwonterghem & Webster 2020). Bacteria have been isolated from marine invertebrate such as corals, though the implementation for probiotic applications is poorly characterized (Keller et al. 2021; Pereira et al. 2017; Sweet et al. 2021). To better understand the impacts on host survival, assessment of the bacteria is required to understand these interactions.

Nematostella vectensis has been used as an experimental system for characterization of variation and potential function of the microbiome in cnidarians. This species is easily cultured in a laboratory setting and inhabits estuarine environments that

vary in abiotic and biotic factors. These estuaries experience extreme water temperature fluctuations daily up to 20ºC (Reitzel et al. 2013). *Nematostella vectensis* can survive at 40ºC for several hours, but prolonged exposure will result in mortality (Reitzel et al. 2013). The microbiome of *N. vectensis* can vary due to seasonal shifts (Har et al. 2015), daily variability in light (Leach et al. 2019), and by geographic location (Mortzfeld et al. 2016). *Nematostella vectensis*, like other marine organisms, have several life stages, each of which has significant variation in the microbiome (Mortzfeld et al. 2016). These differences in the associated bacteria can be exacerbated with incubation in differing temperatures and salinities (Mortzfeld et al. 2016). Long term thermal exposures can select for a microbiome that can confer thermal tolerance to acclimated juvenile *N. vectensis*, and their acclimated offspring (Baldassarre et al. 2021). Few studies have described the effects of adding ecologically relevant bacteria to *N. vectensis* and characterized the response (Domin et al. 2018). *Vibrio coralliilyticus* has pathogenicity against *N. vectensis* at temperature conditions above 30ºC (Brennan et al. 2017). Because microbes can be important facilitators for the survival of cnidarians, assessing how the individual microbes survive with and without the host will provide fundamental knowledge regarding conditions that may favor and limit assembly of microbial communities in different environmental conditions.

Here we identified bacterial isolates that survive beyond the host's physiological range, utilizing a variety of techniques. First, we analyzed the survival and growth of 62 bacterial isolates from the host anemone and its natural environment across relevant stressors characteristic of estuarine environments. Second, we co-cultured several bacteria to determine if there is a synergistic effect while in proximity with other species.

Third, we determined if these bacterial species survive within the host at extreme temperatures and salinities. Last, we determined bacterial isolate stability in *N. vectensis* through extended thermal exposures with life/death assays and turbidity tests.

Methods and Materials

Animal and Sediment Collection

Anemones, water, and sediment were collected from six locations along the coast of the United States: Georgetown, South Carolina, Great Sippewissett Marsh, Massachusetts, Odiorne, New Hampshire, Saco, Maine, Crescent Beach, Nova Scotia, Chezzetcook, Nova Scotia. Animals were collected from a 1 mm mesh filter and transferred into 50 mL conical tubes for transport back to the lab. These animals were used for isolations of bacteria. Separate water and sediment samples were collected and used for environmental isolates.

Microbial Isolations

Animals and water/sediment collections were serial diluted in sterile Phosphate Buffered Saline (PBS) and spread on either Marine Broth Agar or Heart Infusion Agar (Criterion, C5831). The plates were incubated overnight, and distinct morphological colonies were selected for isolation. A total of 196 bacterial isolates were taxonomically identified via 16S rRNA sequencing (Table S3.1). DNA was extracted with Promega Wizard® Genomic DNA Purification kit and a majority of the 16S rRNA gene was amplified using Q5 Mastermix (New England Biolabs) with the 27F forward and 1492R reverse primers. The amplicons were directly sequenced with Sanger sequencing

(Eurofins). The sequences were imported into RDP for identification in the SILVA database. Resulting sequences were aligned and matched with the highest similarity to 16S rRNA sequences in SILVA database with a threshold of 97% similarity (Quast et al. 2012). The sequence taxonomies were identified to the genus level.

Growth Curve of Isolates

Isolated bacteria from diverse taxonomic groups were selected for growth analysis under distinct extreme conditions. Of 196 isolates, 62 were selected for growth assays (Table S3.2). This diverse group was selected from a variety of locations and genera for a wide representation of the isolated bacteria. We tested six environmental conditions, three salinities and three temperatures, in a full factorial design. For salinity, we used: ⅓ Estuarine Broth (EB), Estuarine Broth, Marine Broth (MB). The calculated salinities of ⅓ EB, EB, and MB are 5, 15, and 30 parts per thousand (ppt), respectively. For temperature, we used three high but environmentally relevant temperatures, 30°C, 35°C, and 40°C. The BioTek LogPhase 600 (Agilent Technologies, LP600) was used for the growth assays. We measured six replicates per condition per bacteria with optical density (OD) measured every 20 minutes for 48 hours. Cultures were acclimated to the specific growth conditions overnight before inoculations for the growth curves. These cultures were run in an incubator at the specific temperature and appropriate media in 96 well plates with 149 µL media and 1 µL inoculum. After overnight acclimations 1 µL was transferred to 149 µL of fresh media, and the OD at 600 nm was measured through the time course in the LogPhase 600. The growth rate of each isolate was calculated using the average of the six replicates followed by a log transformation and linear regression of the resulting data.

AMiGA Analysis

The Analysis of Microbial Growth Assays (AMiGA) pipeline was utilized for the analysis of the growth curves generated from the BioTek LogPhase 600 (Midani et al. 2021). First, individual runs were concatenated to a single file for batch analysis. Growth curves were exported from the LogPhase 600 app and imported into the AMiGA pipeline. In AMiGA, the area under the curve (AUC) was calculated for all growth curves. This, in combination of the growth rates, was used for PCA analysis in R. The PCA was visualized with the package ggplot2. To determine statistical significance, the function adonis from the vegan package was used for a PERMANOVA.

Culture of Select Isolates

Two isolates of distinct genera were chosen for co-culture experiments, *Bacillus velezensis* and *Vibrio alginolyticus*. The goal for these experiments was to determine potential evidence for bacteria-bacteria competition. Cultures were acclimated overnight to the respective growth condition. To accommodate for *V. alginolyticus* limited ability to survive in 40°C ⅓ EB (see Results), the overnight culture was set to 35°C to maintain a viable culture for the co-culture experiment. To determine the proportions of cells in the culture, plate counts were conducted to determine the Colony Forming Units (CFUs) of each isolate in solution. Absorbance (OD600) was recorded every 20 minutes with the BioTek LogPhase 600, and CFUs were measured at 0, 8, 32, and 56 hours. Using a 96 well plate each replicate was diluted using 10 µL in 90 µL sterile Phosphate Buffered Saline (PBS), respective to the growth condition, and repeated depending on current cell

density. These diluted cultures were drop spotted with 10 µL of inoculum on Phenyl-Ethyl Alcohol Agar (Becton, Dickinson and Company, 211539) for selection of *B. velezensis*, and ChromAgar Vibrio (CHROMagar, VB912) for selection *V. alginolyticus*. These were grown at 30°C, 40°C, in ⅓ EB and MB (5ppt and 30ppt, respectively). Additionally, these isolates were exposed to three thermal spikes at maximums of 30°C and 40°C at salinities of 5ppt and 30ppt. Bacteria were cultured in EB Broth at 25°C until a mid-log concertation of 10^8 CFUs/mL. A volume of 100 µL culture was aliquoted into 900 µL of ASW respective to condition for 10 minutes, then 10 µL was aliquoted into a 96-well plate (n = 5), and covered with a gas exchange membrane and placed into the thermocycler (BioRad T100). After each 8-hour thermal spike, the 10 µL was removed and serial diluted and plated on appropriate media to approximate culturable CFUs.

Nematostella vectensis cultivation

Nematostella vectensis adults were cultured in 15 ppt ASW and spawned weekly, following previously established methods that include feeding adults with mussel tissue once and *Artemia salina* three times per week, with weekly water changes (Fritzenwanker & Technau 2002; Hand & Uhlinger 1992; Stefanik et al. 2013). Fertilized eggs were separated into new bowls of 15 ppt ASW for development. Recently metamorphosed juveniles were fed *Brachionus plicatilis* several times until animals were approximately 3 mm in length. Adults were collected from the general population in the Reitzel Lab (University of North Carolina at Charlotte). To create axenic individuals, the polyps were exposed to the antibiotic cocktail (50 µg/mL Ampicillin (Acros Organics Cat. No.: 61177), Chloramphenicol (VWR, Cat. No.: 0230), Kanamycin (Fisher

BioReagents, Cat. No.: BP906), and Neomycin (Alfa Aesar, Cat. No.: J61499) each) for 24 hours. Two hours before treatment, organisms were washed with and transferred to sterile 15 ppt ASW.

Host Inoculation with Isolates

Bacillus velezensis, P. spiralis, and *V. alginolyticus* were quantified in adult *N. vectensis* to determine how these species interact within the animal model across a range of temperatures and salinities. Five days before the thermal exposures *N. vectensis* juveniles were transferred to either 5ppt ASW or 30ppt ASW and exposed to the antibiotic cocktail. Two hours before bacterial exposures all organisms were washed with 30 mL of either 5 ppt or 30 ppt sterile ASW. Bacteria were cultured as previously described. Each day after the initial inoculation, five organisms were sampled using previous methods, to determine the relative Colony Forming Units (CFUs).

Isolate Survival in Host's Experiencing Abiotic Stressors

Adult *N. vectensis* were treated with the antibiotic cocktail for 24 hours. *Nematostella vectensis* adults were washed with 30 mL of sterile ASW to remove residual antibiotics. Two hours after the wash, anemones were exposed to 10^8 CFUs of bacteria in sterile 15 ppt ASW. After 10 minutes of exposure, animals were washed with 30 mL sterile ASW animals and then isolated into to 96-well plates (VWR, 10861-562). Over 56 hours, *N. vectensis* was exposed to three 8-hour thermal spikes at 30°C or 40°C using a thermocycler (BioRad T100). The details of the temperature profiles are provided in Table S3.3. At the end of each cycle five animals were removed and dissociated by 1.7

mL Graduated Microcentrifuge Tubes (VWR, 87003-294) and pestle (Axygen, PES-15-B-SI). The resulting homogenate was serially diluted in PBS, and 10 µL aliquots were drop spotted on appropriate agar media. To identify isolate stability in ASW, individual bacteria were grown to log phase and subsequently diluted to 10^4 CFUs/mL into either 5 ppt or 30 ppt sterile ASW. Over the previously described thermal spikes, 10 µL aliquots were serially diluted in PBS drop spotted on appropriate media.

Results

Growth Rates

Sixty-two isolates from five geographically distinct estuaries were assessed for growth at three saline conditions and three temperatures (Table S3.2). The calculated growth rate (hr^{-1}) ranged from 0 (no detectable growth) to a maximum of 36.13 hr^{-1}. Members of the *Photobacterium* (0/3), *Vibrio* (0/15), and *Grimontia* (0/1) phyla were unable to grow at 40°C 5 ppt. South Carolina (3/27), Massachusetts (4/12), New Hampshire (1/5), Maine (1/7), Nova Scotia (0/3) bacterial isolates were unable to grow at 40°C 5ppt. The fastest change in OD occurred with *Vibrio* sp. (isolate ID 46) isolated from Maine, under the 30°C 15ppt condition (Table 3.1). This was followed by *Alteromonas* spp. (W15) from South Carolina, at 35°C MB. The third fastest organism was *Grimontia* spp. (W7) isolated from South Carolina. At each condition, there is no clear isolate that is capable of fast growth under all conditions. When comparing the area under the curve and the growth rates of all organisms tested, the PCA reveals a strong effect due to conditions (PERMANOVA, $R^2 = 0.336$, $p = 0.001$) (Figure 3.1). Most organisms grouped tightly in the 40°C 5ppt condition, as many organisms failed to grow

in this condition. Decrease in temperature resulted in a greater dispersion in the data, and when salinity increases most bacteria were able to grow in these conditions.

Bacterial plate counts in broth

Individual bacteria (*B. velezensis*, *P. spiralis*, and *V. alginolyticus*) began at 10^5 CFUs/mL (Figure 2). Through 8 hours, *V. alginolyticus* increases in concentration that exceeds the relative concentrations of both *B. velezensis* and *P. spiralis* under all tested conditions. Furthermore, *V. alginolyticus* maintains a higher concentration through 32 hours when compared to *B. velezensis* and *P. spiralis* at all salinities and temperatures. Through 56 hours, *V. alginolyticus* continues to maintain higher concentrations than the other bacteria under all conditions. When *B. velezensis* and *V. alginolyticus* are co-cultured at 5ppt, at 8 hours *V. alginolyticus* maintains the initial CFU concentration at 30°C, while losing CFUs at 40°C (Figure 3). After 32 hours, *V. alginolyticus* in 30°C increases to 10^9 CFUs/mL at both 15 and 30 ppt, while at 5 ppt the concentration was 1-fold lower. At 40°C, *V. alginolyticus* under 5 ppt salinity increases 2-fold from the lowest concentration. *Vibrio alginolyticus* reaches its maximum at 32 hours under 30 ppt, while it took 56 hours to reach its maximum in 5 ppt broth. Alternatively, at 5 ppt, *B. velezensis* does not increase in culturable CFUs until 32 hours and reaches its peak at 56 hours.

Bacterial plate counts in Artificial Seawater

Individual isolates began at 10^5 CFUs/mL (Figure 3.4). At 30°C, both *B. velezensis* and *P. spiralis* decrease from the initial concentrations at 8 hours but recover at 32 hours to the original concentrations and maintain through 56 hours. Alternatively,

V. alginolyticus maintained the original concentration throughout the time course at both temperatures. At 40°C, both *B. velezensis* and *P. spiralis* decrease at 8 hours, while *V. alginolyticus* maintained its concentration through the elevated thermal spike. At 32 hours, both *B. velezensis* and *P. spiralis* maintain their respective concentrations relative to 8 hours, and *V. alginolyticus* maintains its elevated concentration. At 56 hours, *P. spiralis* maintains its concentration from 32 hours, while *B. velezensis* has an increase in CFUs, and *V. alginolyticus* sees a slight decrease.

In vivo Nematostella vectensis

Plate counts

Axenic organisms yielded no colony growth, and all animals survived the 30°C spikes. While most anemones survived the 40°C thermal spikes, all individuals exposed to *V. alginolyticus* at 5 ppt appeared to have died following the second thermal exposure, but the tissues remained intact. Remaining individuals survived the 40°C condition but were visibly stressed, which was observed by limited response to tactile stimulation. Non-treated individuals also survived these stressors and CFU counts at each timepoint are recorded in Figure 3.2. For NT anemones, the number of cultivated bacteria remained similar at 30°C (Figure 3.5A) and 40°C (Figure 3.5B) with most conditions. At 15 ppt with MB counted bacteria, the two temperatures were statistically different, where 30°C was significantly higher (two-way ANOVA, $p = 0.0053$). Additionally, anemones exposed to 15 ppt and 30 ppt MB organisms at 40°C were statistically different, where the increase in temperature increased CFU concentration ($p = 0002$). The remaining treatments were not statistically different from controls. *Vibrio alginolyticus* maintained

higher concentrations than the other two transplanted bacteria. At 30°C, *V. alginolyticus* is significantly higher in concentration in the anemone with the host only at 30 ppt, when compared against both *B. velezensis* and *P. spiralis* in the same conditions (two-way ANOVA, $p = 0.0074$, $p = 0.0112$, respectively). At 40°C, *B. velezensis* and *P. spiralis* were lower relative to *V. alginolyticus*. At 5 ppt *Vibrio alginolyticus* associates with the animal at higher concentrations than both *B. velezensis* and *P. spiralis* ($p = 0.006$, $p = 0.0123$, respectively). Alternatively, *V. alginolyticus* concentrations through the time course at 40°C and 30ppt were not statistically different when compared against the other bacteria across any treatment.

Discussion

Salinity in combination with temperature drives growth rate

After testing a broad array of conditions and environmental isolates, salinity in combination with temperature are the driving factors for growth performance for most bacteria isolated. These different bacteria were isolated from a broad range of estuaries along the eastern coast of the United States. Temperature of all these locations can fluctuate upwards of 20°C daily, and the minimum and maximums can be from -5°C and 42°C, respectively, depending on the location (Darling et al. 2005; Reitzel et al. 2013). While these temperatures are permissive for growth and sustainability of their respective microbiomes (Mortzfeld et al. 2016), the combination of high temperature and low salinity can be difficult for isolate growth on their own. Here, we tested extreme temperatures and salinities, which differs from those tested in long term studies at

moderate temperatures (18°C and 25°C) and salinities (16 and 25 ppt). These elevated conditions may result in differential communities under control conditions.

As *Pseudoalteromonas spiralis* (W20) is one of the fastest growing microbes tested at 5ppt, it is possible that this isolate could outcompete pathogenic *Vibrio* species in similar low saline conditions. *Pseudoalteromonas* sp. have shown antagonistic properties against pathogens isolated from marine systems (Holmström & Kjelleberg 1999; Nichols et al. 2005), sponges (Hamid 2020), and corals (Tang et al. 2020). Alternatively, when *P. spiralis* is inoculated into sterile 5ppt sea water, it is outperformed by *V. alginolyticus*. This measurement in realistic conditions helps elucidate the performance of these bacteria in relevant conditions, where broth culturing may limit interpretability. In *N. vectensis*, a low salinity of 5 ppt alone does not induce mortality. For many bacterial isolates, growth conditions in 5 ppt Marine Broth resulted in lower growth rates during log phase growth. Additionally, the static temperature of 40°C does not reflect the conditions of the field but does provide insight to the capabilities of the individuals of the microbiome. The log phase of a bacterial growth curve is indicative of favorable conditions and evaluating the capabilities of these isolates will help us determine if individual isolates may be present in the host.

When comparing the growth of *V. alginolyticus* and its co-inoculation with a potential probiotic species (*B. velezensis*), we found that under certain conditions, there may be active antagonism. This antagonistic effect is exacerbated by both temperature and salinity, as the relative concentration of *V. alginolyticus* drops several-fold under high temperature and low salinity conditions likely due to *B. velezensis*. This result suggests that antagonisms may be dependent on the conditions of the system, and the

abiotic preferences of each organism. *Bacillus velezensis* has been documented to be an antagonistic probiotic in a variety of systems (Emam & Dunlap 2020; Jamali et al. 2015; Thurlow et al. 2019; Yi et al. 2018). More specifically in *Artemia naupulii*, another probiotic organism, *Bacillus subtilis,* has been shown to be a direct antagonist against *Vibrio anguillarum* by controlling the oxidative stress in the host. This indicates that this strain of *B. velezensis* may also be a beneficial probiotic, which necessitates future investigation into this bacterial species.

Bacterial concentration fluctuates over time

Non-treated *N. vectensis* resulted in minimal shifts in culturable CFUs over the time course. In the treated conditions, the bacterial CFU's present inside of the host fluctuated daily. Additionally, the relative number of bacteria within *N. vectensis* can fluctuate over the hours of the day (Leach et al. 2019). While the normal conditions resulted in low fluctuations of culturable CFUs throughout the time course, this number remains consistent at both temperatures, which is a potential indicator for microbiome stability. Alternatively, when single isolates are introduced into the host, elevated temperatures create instability for the individual bacteria. This supports the idea of engineering microbiomes, as opposed to the addition of individual isolates (Domin et al. 2018). Few studies have cultured bacteria from cnidarian hosts, where most interactions are hypothesized through genome sequencing (Huggett & Apprill 2019). Determination of isolate stability is vital for proper implementation of probiotic methodologies in the field.

When bacteria are added to sterile ASW, temperatures up to 30°C does impact the culturable CFUs in solution, after initial stabilization. Alternatively, 40°C appears to be more stressful for these isolates, resulting in a significant decrease in CFUs for both of the "probiotic" organisms *B. velezensis* and *P. spiralis*. Alternatively, *V. alginolyticus* maintained CFUs at these elevated temperatures under both salinities. Some methods suggest direct application of bacteria to diseased organisms. In some systems such as *C. elegans* there is a stoichiometric dependence for the host to obtain bacteria (Vega & Gore 2017). This result supports the idea that solution mediated methods may not be effective for probiotics. If bacteria are not taken up by the target host before high thermal fluctuations, the chance of success may be reduced significantly. Prey-mediated methods may prove to be a better method, as this provides a direct route of transmission into the terminal organism (Assis et al. 2020; Jamali et al. 2015; Peixoto et al. 2017a). Prey vectoring methods have proven to be effective at transmitting bacteria to predatory hosts, while reducing the number of CFUs that are shed into solution (unpublished data). Considering this dependence of quantity of cells, if the number of cells that remain in the environment that are pathogenic, these methods may produce less than ideal results.

Bacteria are sustained with the host under stressful conditions

Vibrio alginolyticus, *B. velezensis*, and *P. spiralis* were all successfully reinoculated into the host organism. Here were found that the individual isolates in the host fluctuated over short thermal spikes. Additionally, we found that when these bacteria are not introduced into the native microbiome, they readily associated with the animal. Over a longer time course, if the native microbiome is present in the host, specific

isolates that are transferred into the organism are lost (Domin et al. 2018). We found that these three bacteria can be differentially retained in axenic organisms over the time course. The dominate bacteria in our trials was *V. alginolyticus*, a documented pathogen (Castillo et al. 2015; Lajnef et al. 2012; Yang et al. 2021; Zhenyu et al. 2013). At elevated temperatures we found an increased concentration of *V. alginolyticus* at both salinities. This result indicates the importance of microbiome maintenance, as pathogens may associate with the host before the application of probiotics.

Alternatively, the number of CFUs in the organism after host mortality remained elevated, indicating the bacteria continue to grow following host death. The sustained elevated temperature of 48°C is approximately 8°C above the previously recorded maximum of *N. vectensis* (Reitzel et al. 2013). While this thermal stress is beyond the capabilities of the host, it is not beyond the tolerance range of the bacteria in some conditions. We observed turbidity in many conditions at both high and low salinities. Additionally, we found the number of CFUs of a monoculture of either *B. velezensis* or *P. spiralis* resulted in lower concentrations than the non-treated organisms. While total concentrations do not necessarily determine microbiome health, the change in relative abundance may be an indicator of heat stress (Li et al. 2019). Additionally, when *V. alginolyticus* is the only introduced bacteria, the concentration of CFUs increases through heat stress. This is consistent with former studies of marine invertebrates where the prevalence of *Vibrio* spp. increases with temperature increases (Ketchum et al. 2021; Tout et al. 2015).

Conclusion

This work sought to assess the capabilities of bacterial isolates across a broad range of conditions, both in growth media, and within the host. This study characterized the stoichiometric dependencies of condition on *Bacillus velezensis*, *Pseudoalteromonas spiralis*, and *Vibrio alginolyticus*. A variety of bacteria, including isolates from the genera *Vibrio, Bacillus, Alteromonas,* and *Pseudoalteromonas*, exhibited growth across a broad range of temperatures and salinities. When select bacteria were grown in broth media, most increased in CFUs through a short time course. In sterile artificial seawater, *V. alginolyticus* retains the highest CFUs through high successive thermal spikes. At moderate 30°C thermal spikes, all organisms trend in similar CFUs in *N. vectensis*, except for *V. alginolyticus* at 30 ppt, which exceeds all other bacteria. At 40°C , bacteria have less predictable concentrations, but *V. alginolyticus* exceeds the concentrations of both *B. velezensis* and *P. spiralis* in as little as 32 hours. Lastly, we found bacteria persist through extended elevated temperatures that *N. vectensis* cannot survive, indicating the potential for bacterial influence on host survival.

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Table 3.1: Top three bacteria that grew the fastest (hr^{-1}) in each condition.

1				
Condition	Isolate ID	Genus	Location	hr^{-1}
40°C MB	W7	*Grimontia*	South Carolina	34.27
35°C MB	W15	*Alteromonas*	South Carolina	35.46
30°C MB	46	*Vibrio*	Maine	30.73
40°C EB	W7	*Grimontia*	South Carolina	33.76
35°C EB	W22	*Pseudoalteromonas*	South Carolina	26.55
30°C EB	46	*Vibrio*	Maine	36.13
40°C 1/3EB	98	*Bacillus*	Maine	18.65
35°C 1/3EB	W20	*Pseudoalteromonas*	South Carolina	22.66
30°C 1/3EB	A6	*Vibrio*	South Carolina	20.22
2				
Condition	Isolate ID	Genus	Location	hr^{-1}
40°C MB	86	*Shewanella*	Massachusetts	33.43
35°C MB	98	*Bacillus*	Maine	31.66
30°C MB	W12	*Alteromonas*	South Carolina	28.85
40°C EB	49	*Vibrio*	Maine	32.54
35°C EB	82	*Vibrio*	New Hampshire	26.03
30°C EB	W6	*Vibrio*	South Carolina	29.79
40°C 1/3EB	W20	*Pseudoalteromonas*	South Carolina	18.22
35°C 1/3EB	A6	*Vibrio*	South Carolina	20.90
30°C 1/3EB	W20	*Pseudoalteromonas*	South Carolina	19.27
3				
Condition	Isolate ID	Genus	Location	hr^{-1}
40°C MB	56	*Vibrio*	Maine	32.01
35°C MB	W1	*Vibrio*	South Carolina	28.94
30°C MB	47	*Pseudoalteromonas*	Maine	28.51
40°C EB	W18	*Vibrio*	South Carolina	31.55
35°C EB	60	*Vibrio*	Nova Scotia	24.84
30°C EB	58	*Vibrio*	Maine	27.10
40°C 1/3EB	1	*Vibrio*	Massachusetts	17.74
35°C 1/3EB	98	*Bacillus*	Maine	18.21
30°C 1/3EB	33	*Shewanella*	Massachusetts	14.71

Table 3.2: Highest growth rates across all conditions.

Highest Overall	Isolate ID	Genus	Location	hr^{-1}	Condition
1	46	*Vibrio*	Maine	36.134	30°C EB
2	W15	*Alteromonas*	South Carolina	35.461	35°C MB
3	W7	*Grimontia*	South Carolina	34.274	40°C MB
4	W7	*Grimontia*	South Carolina	33.756	40°C EB
5	86	*Shewanella*	Massachusetts	33.426	40°C MB

Table S3.1: Distribution of isolates collected from estuaries along the eastern coast of the United States. Isolates were clustered by genus, and by northern sites (ME, NH, NS, and MA) and southern site SC. ME = Maine, NH = New Hampshire, NS = Nova Scotia, MA = Massachusetts, SC = South Carolina.

	ME NH NS MA		SC	
Genus	Identified	Selected	Identified	Selected
Agarivorans	0	0	2	1
Shewanella	3	3	2	2
Vibrio	119	14	24	13
Alteromonas	0	0	4	3
Pseudoalteromonas	7	7	10	7
Bacillus	4	4	4	3
Grimontia	0	0	1	1
Photobacterium	0	0	1	1
Exiguobacterium	1	0	0	0
Psychrobacter	1	0	0	0
Total	148	31	48	31

Table S3.2: Distribution of microbial isolates selected for growth assays.

Isolate composition	Nova Scotia	Maine	New Hampshire	Massachusetts	South Carolina
Agarivorans					1
Alteromonas					3
Bacillus		2	1		3
Grimontia					1
Photobacterium		1			1
Pseudoalteromonas	1	1		3	6
Shewanella				3	2
Vibrio	3	9	2	7	13

Table S3.3: Temperature profiles for thermocycler experiments at 30°C and 40°C.

40°C	25°C	30°C	35°C	40°C	35°C	30°C	25°C	20°C
30°C	22.5°C	25°C	27.5°C	30°C	27.5°C	25°C	22.5°C	20°C
Step Duration (h)	1	1	1	1	1	1	1	17
Total Duration (h)	1	2	3	4	5	6	7	24

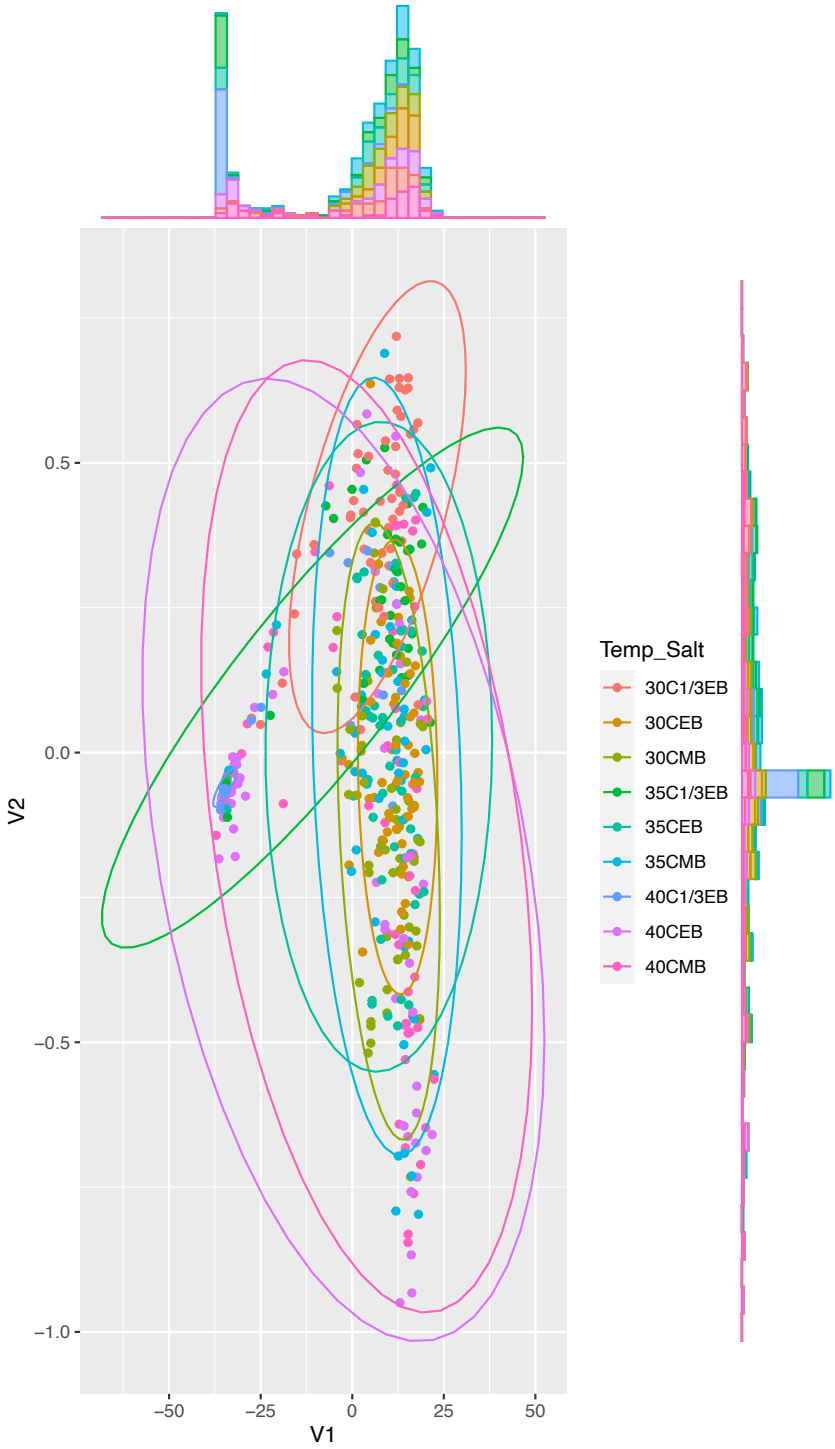

Figure 3.1: Principle component analysis of the 62 isolates growth capabilities across thermal and saline conditions. Ellipses represent 95% confidence intervals.

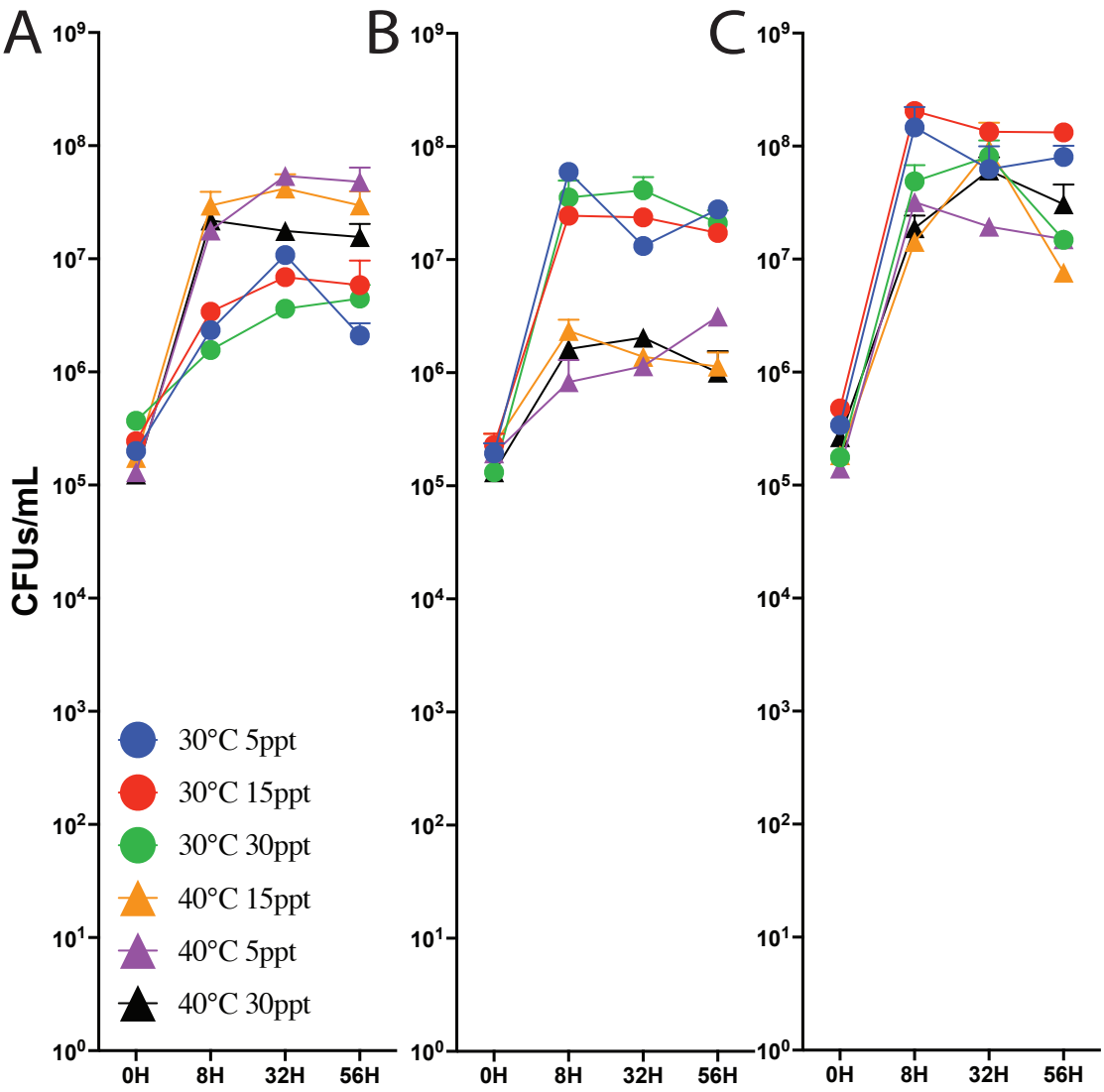

Figure 3.2: Plate counts of the three individual bacterial isolates exposed to extended thermal exposure across saline gradients over three days. (A) *Bacillus velezensis* (B) *Pseudoalteromonas spiralis* (C) *Vibrio alginolyticus*.

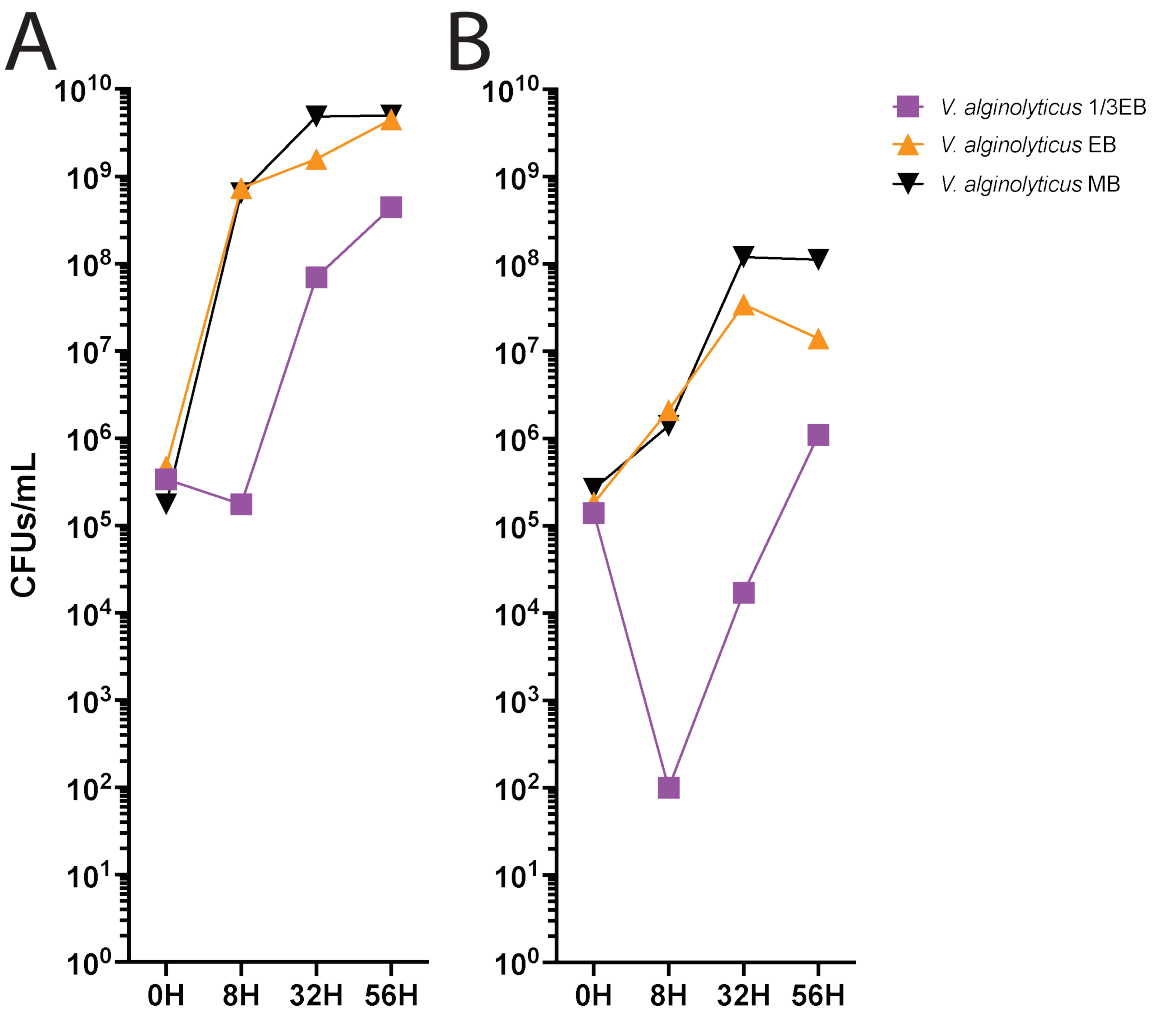

Figure 3.3: Co-culture of *B. velezensis and V. alginolyticus*. Plate counts for the number of culturable CFUs through thermal and saline gradients. (A) 30°C *V. alginolyticus* counts for co-culture in broth media (B) 40°C *V. alginolyticus* counts for co-culture in broth media.

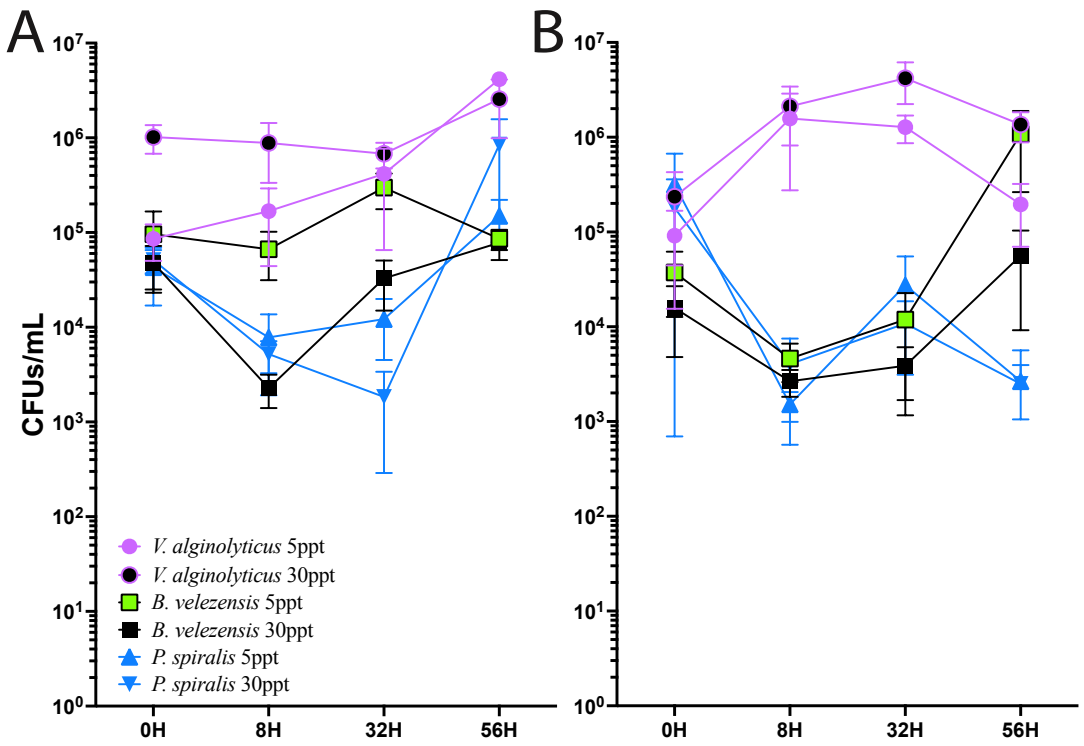

Figure 3.4: Colony Forming Units for *B. velezensis*, *P. spiralis*, and *V. alginolyticus* in 5 ppt and 30 ppt ASW from (A) 30°C and (B) 40°C thermal spikes.

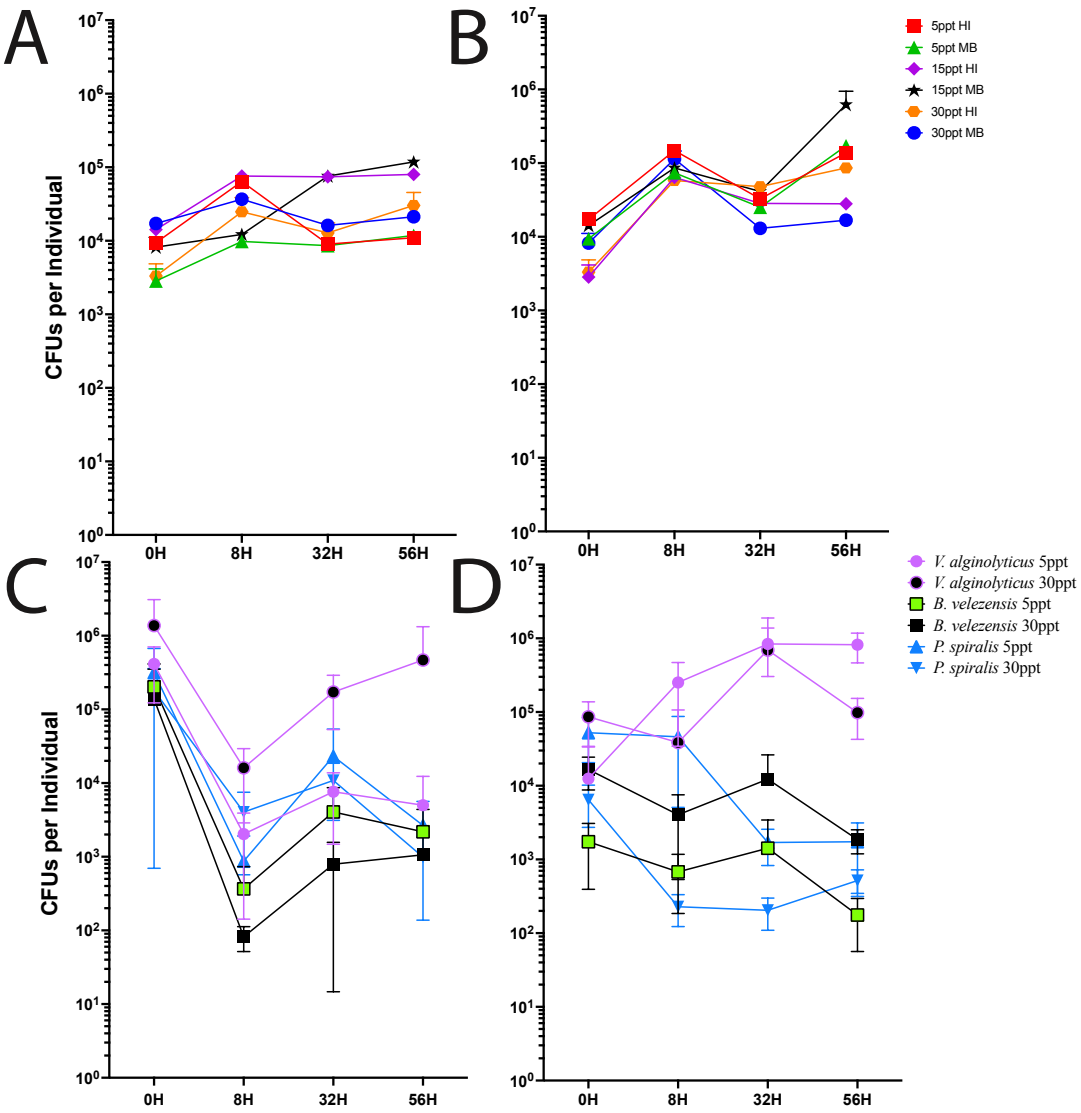

Figure 3.5: Colony Forming Units of three environmental isolates: *B. velezensis*, *P. spiralis,* and *V. alginolyticus.* (A) NT conditions, CFUs were measured with MB and HI agar media. 30°C treatment (B) NT conditions. 40°C treatment (C) Thermal spikes increased to 30°C and organisms were inoculated with either *B. velezensis*, *P. spiralis*, or *V. alginolyticus* (D) Thermal spikes increased to 40°C and organisms were inoculated with either *B. velezensis*, *P. spiralis*, or *V. alginolyticus.*

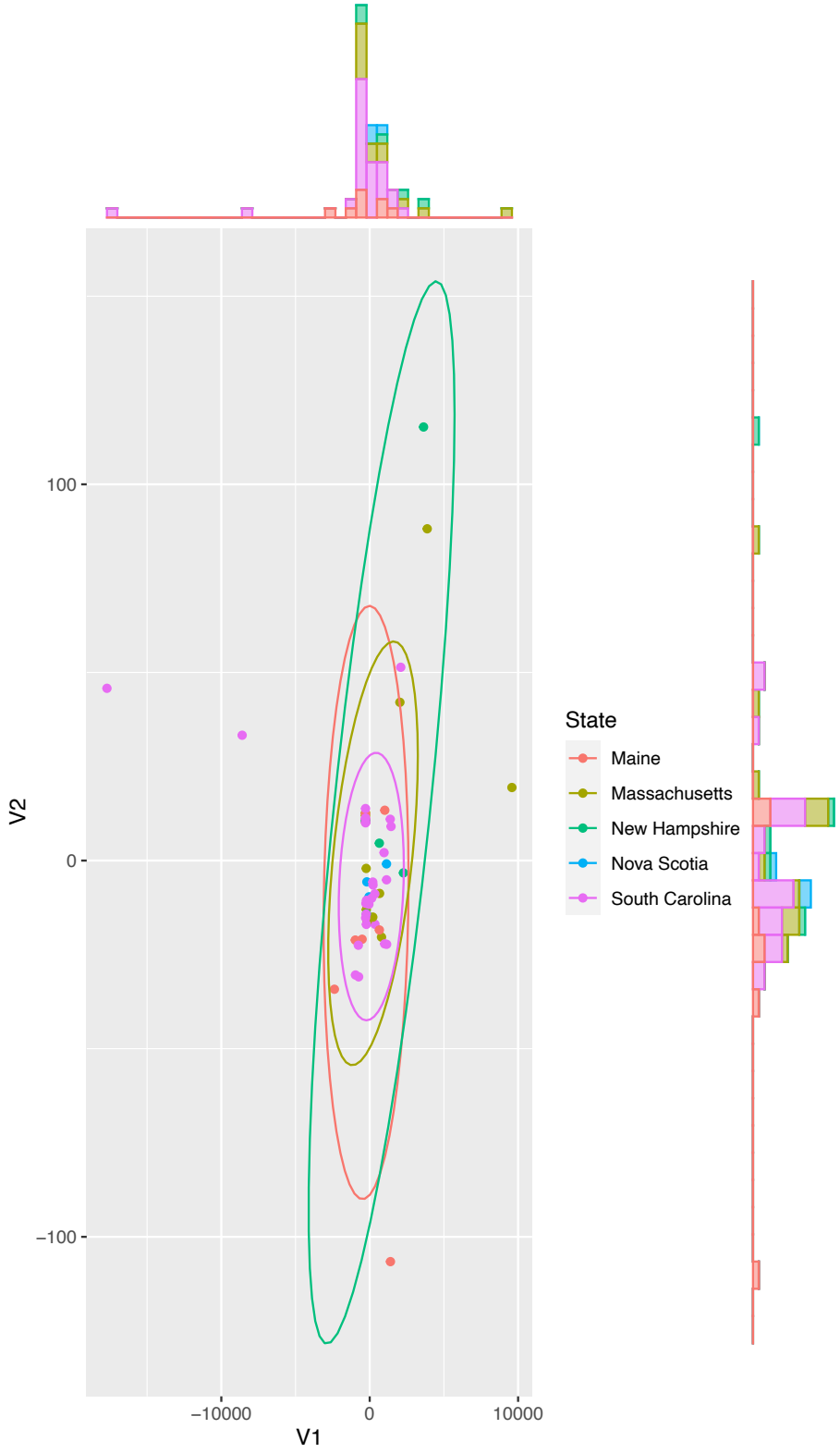

Figure S3.1: PCA of only 40°C and 5 ppt growth curves, separated location (state) for each isolate.

OVERALL CONCLUSIONS

This dissertation sought to experimentally test factors that influence the interaction of bacteria and the sea anemone *Nematostella vectensis*. The starlet sea anemone, *N. vectensis* is a cnidarian model for marine invertebrates that extends to microbiome and microbe-host interactions. Chapter 1 explored the sublethal effects of antibiotics on *N. vectensis* and the culturable portion of the microbial community. The second chapter described quantification methods to transfer individual bacteria into the predator *N. vectensis* through the surrounding water and prey items. The third chapter implemented methods described in Chapter 2 to understand the growth dynamics of environmental bacterial isolates in specific temperature and salinity conditions, and within the host anemone. The combination of insights from this research have resulted in an understanding of methods that are commonly implemented and can be widely adopted across experimental studies of the microbiome of animals, particularly marine invertebrates to complement approaches primarily based on sequence-only methods.

In Chapter 1, antibiotics significantly increased the time for larval settlement. Additionally, bacteria were readily culturable after removal of antibiotics in the anemone, including a variety of members that belong to gammaproteobacteria. Lastly, anemones that were acutely treated with antibiotics, and subsequently removed, showed lasting effects on the host with differential expression of genes. The anemones that received constant antibiotic treatment were transcriptionally impacted across a broad range of processes including metabolism, development, and neuron function. This work contributed to the characterization of antibiotic treatments in cnidarians. Additional assessment of the sublethal effects is needed to determine the total impact on the host

organism. Future research will help elucidate the role of bacteria have in organisms under antibiotic stress, and discover additional targets antibiotics have in *N. vectensis*.

In Chapter 2, two methods to transplant associated bacteria into *N. vectensis* are development and compared quantitatively. Here, the number of culturable bacteria was determined in two prey types, and the addition of two different bacterial species (probiotic and pathogen) resulted in a reduced uptake of the pathogen. The Prey Feeding Method, in comparison to Solution Uptake Method, resulted in higher associations of bacteria in the terminal host. Both prey items tested are capable of transmitting bacteria through the feeding process, indicating it is a viable method for introducing bacteria into a terminal organism. This method described in Chapter 2 can be utilized in a vast array of organisms that are predatory in their environment. Future research could implement these methods in other model organisms, in combination with other bacterial assessment tools such as differential gene expression and interaction networks.

In Chapter 3, a variety of culturable isolates were tested for grow efficacy in specific temperature and salinity conditions that represent the estuarine environment. The combination of higher temperatures and low salinity resulted in poor growth across most bacteria. Additionally, the method described in Chapter 2 was utilized to transfer bacteria into *N. vectensis*. Here, a single bacterial species present in the host fluctuated significantly under heat stress relative to the control microbiome. This work contributed to the characterization of the individuals that compose the microbiome of *N. vectensis*, both outside and inside of the host organism. Future research could investigate additional bacteria isolated from *N. vectensis* and determine if other bacteria have the potential to

extend the hosts survivable range. Antagonism between isolates should also be characterized further, and their direct interactions inside of the host.

Nematostella vectensis is an informative model for understanding and identifying interactions between the host and its microbiome. The ease of culture, regular spawning intervals, and diversity of microbial species (microbiome) make it a unique experimental organism for extrapolating interactions to other cnidarians. The development of quantification methods is applicable across a broad range of hosts, and can provide insight to the interactions bacteria may have inside of the host. Cumulatively, this dissertation provides knowledge to assist in understanding and implementing microbiological practices to marine invertebrates.