Developmental and spatial variation in the microbial communities associated newly hatched juveniles and adults of the marine snail, *Nucella ostrina*

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ABSTRACT

Marine invertebrates host diverse microbial communities on their surfaces and within their digestive systems, which can aid digestion, support immune defence, or increase susceptibility to pathogens. Despite their importance, little is known about how these communities assemble and function during early life stages, when invertebrates are particularly vulnerable to environmental stressors. While previous studies have examined the entire microbiome of adult marine invertebrates in laboratory settings, no study to date has examined the external microbiome of juvenile invertebrates in their natural habitat. To address this knowledge gap, we developed a method to separately characterize exterior-associated and gut-associated microbial communities of both juvenile and adult *Nucella ostrina* life stages and applied this method to investigate developmental and environmental variation in the host-associated microbiome across two distinct habitats on Vancouver Island. This method successfully recovered distinct microbial communities associated with the exterior and interior tissues of both juvenile and adult snails. The microbiome associated with juvenile snails included high abundances of bacteria that have been previously determined to aid in immune defence or be pathogenic, whereas the microbiome associated with adult snails was dominated by a range of species, with organic matter degradation, secondary metabolite production, and other functions that can benefit host metabolism. Microbial communities associated with *N. ostrina* differed significantly between the two field sites, with minimal overlap in microbial taxa between locations. This study demonstrates that microbial community composition varies with development and location in *N. ostrina*, and establishes a foundation for future research on host-associated microbiomes in marine invertebrates.

INTRODUCTION

Marine invertebrates harbour a diverse range of bacteria within their digestive systems and on the external surfaces of their bodies. In some species, microbial symbionts can account for a substantial portion of host biomass, up to 40% in the sponge *Crambe crambe*, and provide compounds essential for development and growth (Wilkins et al. 2019; Turon et al. 2024). In the digestive system, bacteria are involved in nutrient processing and host immune defence (Khan et al., 2018). On the body surface, microbial associations are more variable: some bacteria appear to have no functional benefit to the animal at all, while others may be beneficial to the host (e.g., reducing vulnerability to pathogens and predators) or harmful (e.g., pathogens) (Khan et al., 2018; Menabit et al. 2024). It is becoming increasingly clear that the microbiome is critically important for an individual's survival and growth (Apprill 2017; Gonzalez et al. 2024).

Microbiome composition can vary across host developmental stages, with both vertical and horizontal transmission shaping microbial acquisition (Wilkins et al. 2019). For example, vertically transmitted symbionts have been observed across all life stages of the sponge *Crambe crambe*, suggesting evolutionary selection for beneficial microbes during early development (Turon et al. 2024). However, not all systems show such continuity. In the freshwater snail *Cipangopaludina chinensis*, total microbiota composition was found to be relatively stable between juveniles and adults, with similar diversity and functional profiles, despite environmental exposure and differences in feeding (Zhou et al. 2022). In general, microbiome composition reflects a combination of inherited and environmentally acquired taxa (Almeida et al. 2023; Mintin et al. 2024). Environmental exposure becomes particularly relevant during transitions such as metamorphosis or the onset of feeding, which can trigger a restructuring of the microbial community (Turon et al. 2024). While some studies report stable microbiomes across life stages (Zhou et al. 2022), others show that adults tend to harbour more diverse microbial communities (Mintin et al. 2024), indicating ontogenetic changes in microbiome structure and function are often species- and tissue-specific.

The early juvenile stage is often the most vulnerable in the life cycle of marine invertebrates (Gosselin & Qian 1997), as individuals face high mortality risk due to underdeveloped immune systems and increased exposure to pathogens in benthic habitats (Sandee et al. 2016). While immunocompetence develops near the end of the larval phase or at metamorphosis (Balseiro et al. 2013; Song et al. 2016), juveniles initially rely on maternally derived immune factors such as proteins and mRNA (Yue et al. 2013; Wang et al. 2015). However, microbial pathogens alone do not appear to drive early mortality. Laboratory experiments determining the effects of pathogen exposure reported low mortality rates (0–3%) in early juveniles of *Nucella ostrina*, suggesting that developing immune function or early microbiome establishment provides effective protection (Sandee et al. 2016).

There is also growing concern that climate change, which is causing a rise in seawater temperature, could disrupt the natural microbial communities associated with marine invertebrates (Iwabuchi & Gosselin 2019). Rising seawater temperatures alter microbial community composition, often increasing the abundance of opportunistic or pathogenic taxa (Iwabuchi & Gosselin 2019; Apprill 2017). In the oyster *Crassostrea virginica,* when stressed by increased temperatures, microbial diversity was significantly lower than that of oysters which did not experience the same temperature stresses (Khan et al. 2018). Similarly, temperature stress in *Crassostrea gigas* led to shifts in gut microbiota and suppressed immune function, highlighting the compounding effects of microbial associations and host vulnerability under warming conditions (Zheng et al. 2024). Sponges exposed to heat stress also experienced colonization by transient pathogens and elevated mortality (De Castro-Fernández et al. 2024). Laboratory experiments investigating the role of temperature in shaping symbiotic microbiota on oysters showed that microbial diversity and abundance increase under warming temperatures, particularly for disease-associated taxa (Liu et al. 2023). These studies collectively indicate that microbiome composition plays a central role in host resilience to climate-driven stress and highlight a need to establish baseline information on typical host-microbiome associations before they are influenced by environmental change.

Though several studies have examined the diversity and activity of the microbial communities living on marine invertebrates, these studies have focused on the gut microbiome or entire microbiome of individuals from homogenized samples; to our knowledge, no study to date has examined variation in the microbiome by body site. In addition, most previous studies have focused on adult invertebrates and no study to date has investigated the external microbiome of early juveniles. Furthermore, the majority of microbial studies have focused on invertebrates reared in aquaculture facilities, with few studies examining the microbial communities associated with wild invertebrates in natural habitats.

To address these knowledge gaps, the present study sought to characterize the microbiome of early juveniles of a marine invertebrate living in the natural habitats of Barkley Sound on the west coast of Vancouver Island, Canada. The animal selected for this study, the intertidal gastropod *N. ostrina,* has been extensively studied, and the early juvenile phase of this species has been found to be ecologically distinct from the adult phase (Gosselin 1997, de Bruyn & Gosselin 2014, Hamilton & Gosselin 2020). The specific goals of this work were to 1) determine an effective methodology for characterizing the bacterial communities from external and internal body sites of both the juvenile and adult stages of *N. ostrina*, 2) determine if the associated microbiomes change between the juvenile and adult stages of an invertebrate species, and 3) determine if the associated microbiomes vary between animals living in different habitats. This work improves understanding of the microbial association's characteristic of *N. ostrina*, and provides a foundation for future studies investigating host–microbe interactions in marine invertebrates across life stages and environments.

METHODS

Study sites and field collection

This study was conducted in Barkley Sound on the west coast of Vancouver Island, near the Bamfield Marine Sciences Center (BMSC). Fieldwork took place in May and June 2024 at two intertidal field sites: the Ross Islets (48.869˚N, -125.160˚W) and Prasiola Point (48.817˚N, -125.169˚W). These sites were selected for their abundant populations of *N. ostrina* and accessibility during low tide.

Adult *N. ostrina* and egg capsules were collected by hand from the intertidal zone, using gloves and sterilized tweezers to minimize the potential for microbial contamination. Egg capsules were placed in sterile 1.5 ml microcentrifuge tubes (FroggaBio Inc., Canada), and adults were transported in sterile 50 mL conical tubes (FroggaBio Inc., Canada). All samples were returned to the BMSC laboratory and transferred immediately into holding tanks filled with seawater from their respective collection sites. A total of 40 L of seawater was collected from the sites in clean plastic water jugs for transport to the laboratory.

Hatching and preservation of snails for microbial DNA extraction

Newly hatched snails were obtained from the egg capsules collected from the field. At BMSC, the egg capsules and adults were placed together in cleaned tanks, ensuring the hatchlings and adult snails were exposed to the same seawater and microbial community. In addition, the tanks were filled with seawater collected from the field site where the snails and capsules had been collected, to ensure the snails remained exposed to the same microbes as were present at their source location. Hatchlings emerged from the egg capsules over the following 36 h, at which time both adults and juveniles were moved to a different cleaned tank containing seawater from the field site for an additional 48 h to allow a microbiome to develop on the surfaces of newly hatched juvenile snails. Snails were then placed in sterile tubes (50 ml conical tubes for adults, 1.5 ml microcentrifuge tubes for juveniles) in groups of either five adults or twenty juveniles and frozen at -20 °C. A total of 12 mL of seawater was also collected from the tank, divided into 1.5 mL microcentrifuge tubes, and frozen for subsequent microbial community analysis.

Sample Preparation

DNA extraction was performed on 12 replicates of 20 juvenile snails and 12 replicates of 5 adult snails in three consecutive steps. In the first step, sterile artificial seawater was used to extract the surface microbial communities. Artificial seawater was prepared using Instant Ocean Sea Salt (Instant Ocean, USA), following the manufacturer's instructions and sterilized by filtration through 0.22 micron filter towers (Vavantor TM., USA) and 15 min treatment under UV light. To confirm the effectiveness of the sterilization procedure, 1 ml subsamples of sterilized seawater were set aside for extraction and subsequent PCR. Artificial seawater was added to each sample—

1 mL for juvenile replicates and 5 mL for adult replicates, and samples were then vortexed three times at maximum speed for 60 seconds to remove the external microbiome present on the snails. One ml from each tube was collected and placed in a separate sterile 1.5 mL microcentrifuge tube, hereafter referred to as the *artificial seawater wash* sample. The second step involved a second wash to remove microbes that might have been attached to the external surfaces of the snail strongly enough to avoid being dislodged by the artificial seawater wash. ATL Lysis Buffer (Qiagen, USA) was added to each tube, 1 mL for juvenile samples and 5 mL for adult samples. Samples were incubated at 60˚C for 30 min, then vortexed for 30 sec, after which 1 mL of the ATL buffer mixture from each sample was collected and placed in a separate sterile 1.5 ml microcentrifuge tube, hereafter referred to as the *ATL buffer wash* samples. In the third step, the same snails were homogenized by sonication and incubated in ML1 buffer and Proteinase K (Omega Bio-tek, USA) at 60 °C for 30 minutes to break down the tissues and extract the remaining microbiome from internal tissues, including the gut. For adult snails, the soft body was first removed from the shell using sterile tweezers prior to sonication to ensure thorough homogenization of tissues otherwise protected by the shell; these are hereafter referred to as *homogenized samples*. Additionally, twelve 1 mL subsamples of seawater were collected from the field sites for microbiome extraction and are hereafter referred to as *field seawater* samples.

DNA Extraction

Each 1 mL subsample (artificial seawater wash, ATL buffer wash, homogenized samples) from juvenile and adult snails, twelve 1 mL field sea water subsamples from each field location, and 1 ml of artificial seawater were processed for DNA extraction. These samples were centrifuged at 14000 RCF for 10 min to pellet cells and to separate cells from mucopolysaccharides in the snail mucus (Adema 2021). The supernatant was discarded, and DNA extraction of the pellet was carried out using the E.Z.N.A Mollusc & Insect DNA Kit (Omega Bio-Tek) following the manufacturer's protocol. This kit was chosen for its ability to break down complex mucopolysaccharides that may be carried over in snail mucus, which is known to inhibit polymerases in PCR (Belouhova et al., 2022, Casale & Crane, 2019, Sidstedt et al. 2020). DNA extracts were quantified using a Qubit fluorometric assay (Thermo Fisher Scientific, USA) prior to PCR.

Amplicon preparation and sequencing

Prior to PCR, DNA extracts containing the entire microbiome from the adult snails were diluted with sterile water to adjust concentrations to be within the range of 1 – 100 ng/μL (Adema 2021). The V4 hypervariable region of the 16S rRNA gene was amplified for all samples in 20 μL reactions containing 1 X GoTaq Green Master Mix (Promega, USA), 0.5 μM each of forward primer 341F (5'-TACGGGAGGCAGCAG-3') and reverse primer 806R (5'-GGACTACVSGGGTATCTAAT-3'), and 2 μl of template DNA or PCR water for no-template controls. Thermocycling conditions consisted of initial denaturation at 94 °C for 120 sec, 30 cycles of 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 30 sec, and a final extension of 72 °C for 5 min.

The PCR products were resolved on a 1% agarose gel to confirm successful PCR amplification, and confirmed there was no contamination in the artificial seawater extraction control and no-template PCR control. PCR products were purified using Agencourt AMPpure XP beads (Beckman-Coulter, USA) following manufacturer instructions. Cleaned products were barcoded in a second-round PCR using Ion Xpress modified forward primers and P1 adaptor modified reverse primers. PCR reactions were prepared as described for first-round PCR and thermocycling conditions consisted of initial denaturation at 94 °C for 240 sec, 25 cycles of 94 °C for 30 sec, 65 °C for 45 sec, and 72 °C for 120 sec, and a final extension of 72 °C for 5 min. Second-round PCR products were again resolved on a 1% agarose gel and cleaned using Agencourt Ampure XP beads as described above. Amplicons were sequenced at the TRUGen sequencing facility on an Ion Genestudio S5 platform (Thermo Fisher Scientific).

Statistical analysis

A total of 120 samples were evaluated by 16s rRNA gene amplicon sequencing. Of these 120 samples, ten were removed from the dataset due to low sequencing read depth (<10,000 reads): the samples removed were distributed across sample types and included two Prasiola adults ATL wash, three Ross Juveniles homogenized, three Ross Juveniles ATL wash, and three Ross seawater. After removing samples with low sequencing read depth, samples were rarefied to an even sequencing depth of 10,991 reads per sample to allow for standardized comparisons of microbial diversity. Bray–Curtis dissimilarity matrices were calculated using the v*egan* package (Oksanen et al. 2025) in R. Non-metric multidimensional scaling (NMDS) ordinations based on Bray-Curtis dissimilarity were used to visualize community differences across samples. The multivariate homogeneity of groups was assessed using the betadisp function in *vegan*, to determine if within-group dispersion was significantly different between groups. In cases where the assumption of multivariate homogeneity between groups was met, significant differences in community structure were assessed using PERMANOVA (Permutational Multivariate Analysis of Variance) via the adonis function in *vegan*, which tests for multivariate differences in community composition based on dissimilarity (Oksanen et al. 2025, McMurdie & Holmes 2013, Wickham, 2016). To determine which taxa contributed most to differences between groups, SIMPER (Similarity Percentage) analysis was performed in *vegan*. The top 10 taxa accounting for the greatest cumulative contribution to Bray-Curtis dissimilarity between groups (typically ≥40% contribution) were visualized using whisker plots of OTU abundance. Species-level identification was conducted by comparing representative 16S rRNA gene sequences against the NCBI nucleotide database using BLAST.

Alpha diversity (Shannon index) and within group dispersion of beta diversity (based on Bray-Curtis dissimilarity) were calculated separately for juvenile and adult snail samples to compare microbial diversity and dispersion of beta diversity across juvenile and adult external and internal microbiomes. The dispersion across microbial communities of juvenile internal, adult internal, juvenile external and adult external was assessed by applying the Kruskal–Wallis rank sum test to Shannon index values, and applying ANOVA followed by Tukey's Honest Significant Difference (HSD) multiple comparisons test on Bray-Curtis dissimilarity comparisons.

RESULTS

Development of a DNA extraction method targeting different body sites of N. ostrina

To assess the success of the extraction protocol, microbial communities from seawater, artificial seawater wash, ATL buffer, and homogenized samples were compared. NMDS plots revealed distinct clustering of microbial communities by sample type for both juvenile and adult snails, and consistent relationships between sample types for both juveniles and adults (Fig. 1). In both juvenile and adult snails, seawater communities were more similar to artificial seawater wash communities than to the ATL buffer and homogenized sample communities. ATL buffer and homogenized sample communities were most similar to each other and were clearly distinct from the artificial seawater wash and seawater communities. For adult snails, PERMANOVA revealed statistically significant differences between the ATL buffer and homogenized samples (R2 = 0.257, F1, 17 = 7.61, $p < 0.003$) and the artificial seawater wash and homogenized samples (R2 = 0.703, F1, 23 = 52.2, $p < 0.001$). PERMANOVA comparisons for juveniles were not carried out as within-group dispersion was not homogeneous between groups. However, NMDS plots revealed clear differences in compositions between sample types, supporting the interpretation that the artificial seawater wash captured a distinct microbial community associated with the external body sites of juvenile and adult snails. Based on these findings, the artificial seawater wash represents the external microbiome, and the homogenized samples represent the internal microbiome for the remaining analyses. The ATL buffer wash was left out of further analysis.

Microbiome comparison between juvenile and adult snails – external

External microbial community composition varied across life stages (Fig. 2A). A comparison between external microbiomes of juvenile and adult *N. ostrina* revealed significant differences between these groups (PERMANOVA, $R^2 = 0.44$, $F_{1,22} = 14.99$, $p < 0.001$), indicating a clear shift in microbial community structure with host development. The relative abundance of several microbial taxa also varied between juvenile and adult external microbiomes (Fig. 2B). *Alkaliphilus metalliredigens*, and *Aestuariivita boseongensis* were more abundant in the juvenile external microbiome with a mean of 17.5 ± 7.0% and 7.04 ± 5.16% respectively, compared to only 0.83 ± 0.49% and 0.079 ± 0.070% in adults. *Phaeobacter porticola* follows a similar trend of 5.42 ± 3.02% in juveniles and only 0.707 ± 0.239% in adults. In contrast, taxa such as *Flavimaricola marinus* (5.20 ± 0.77%), and *Alteromonas genovensis* (6.31 ± 2.01%), were highly abundant compared to the juveniles with 0.823 ± 0.362% and 1.73 ± 0.68% respectively.

Microbiome comparison between juvenile and adult snails – internal

Internal microbial community composition varied between life stages, with adult and juvenile communities showing distinct compositions (Fig. 3A). The relative abundance of several microbial taxa also differed between juvenile and adult internal microbiomes (Fig. 3B). Juvenile samples had higher abundances of *Alkaliphilus metalliredigens* (8.04 ± 5.58%), *Aestuariivita boseongensis* (4.92 ± 5.80%), Halarcobacter ebronensis (2.32 ± 1.54%), *Haliangium tepidum (4.61 ± 3.30%), Rubritalea marina (*__9.01 ± 2.97%)__ and *Balneicella halophila* (2.54 ± 5.59%), while these taxa were nearly all less than 0.1% in adults with the exception of *Rubritalea marina* at 3.88 ± 1.95%. Conversely, *Shimia aestuarii* (21.00 ± 13.44%), *Hyella patelloides* (17.53 ± 7.85%), and *Flavimarcola marinus* (3.41 ± 1.03%) were more abundant in adults with less than 1.0% in juveniles.

Diversity Metric's

Alpha diversity was assessed using the Shannon diversity index, where higher values indicate a more diverse community (Fig. 4). A Kruskal-Wallis rank-sum test revealed a significant overall difference in Shannon diversity between the four groups. Adult external communities had significantly higher Shannon diversity than adult internal communities ($p = 4.2\times10^{-5}$). Adult external communities were also more diverse than juvenile external communities ($p = 1.8\times10^{-5}$). Juvenile internal communities showed higher diversity than adult internal communities ($p = 0.011$). No other pairwise comparisons were statistically significant.

Differences in within-group variation in beta diversity, as quantified by betadisper, were assessed with ANOVA and Post hoc Tukey's HSD tests, which showed that juvenile external communities were significantly more variable than adult external (p = 0.030), adult internal (p = 0.00094), and juvenile internal (p = 0.0017) communities (Fig. 5). No other pairwise comparisons were statistically significant.

Spatial variation in snail-associated microbiome – juveniles

The internal microbial communities of juvenile *N. ostrina* from the Ross and Prasiola field sites were distinct from each other (Fig. 6A). Differences in composition were reflected in variation of several taxa between these sites (Fig. 6B). *Rubritalea marina* (9.01 ± 2.97% at Ross; 0.009 ± 0.019% at Prasiola), *Haliangium tepidum* (4.61 ± 3.30% at Ross; 0.0819 ± 0.034% at Prasiola) and *Alkaliphus metalliredigens* (8.04 ± 5.58%at Ross; 0.317 ± 0.284% at Prasiola) were dominant in Ross juveniles. Whereas, G*ranulosicoccus marinus* (17.29 ± 6.87% at Prasiola; 0% at Ross), *Fusobacterium perfoetens* (4.11 ± 2.20% at Prasiola; 0.031 ± 0.094% at Ross), *Marinicaulis aureus* (4.88 ± 4.14% at Prasiola; 0% at Ross) and *Algoriphagus chordae* (4.32 ± 2.00% at Prasiola; 0.056 ± 0.074% at Ross) were more abundant in Prasiola juveniles.

Spatial variation in snail-associated microbiome – adults

The microbial communities associated with adult *N. ostrina* cluster separately at the Ross and Prasiola field sites (Fig. 7A). Differences in composition were also reflected in the abundance of several taxa (Fig. 7B). Ross adult microbiomes contained higher abundances of *Shimiae aestuarii* (20.86 ± 13.29% at Ross; 0.0637 ± 0.057% at Prasiola), *Hyella patelloides* (17.48 ± 8.05% at Ross; 0.0034 ± 0.0068% at Prasiola), and *Flavimaricola marinus* (3.50 ± 1.03% at Ross; 0.621 ± 0.251% at Prasiola). In contrast, *Falsigemmobacter intermedius* (11.19 ± 2.25% at Prasiola; 3.52 ± 0.79% at Ross), *Pontimicrobium aquaticum* (8.37 ± 1.31% at Prasiola; 1.26 ±

0.96% at Ross), and *Aurantibacter aestuarii* (2.85 ± 0.89% at Prasiola; 1.26 ± 0.96% at Ross), were more abundant in Prasiola adults.

DISCUSSION

Development of a DNA extraction method targeting different body sites of N. ostrina

This study successfully validated a method for extracting microbial DNA from both the external and internal microbiomes of juvenile and adult *N. ostrina*. Microbial community composition was clearly distinct among seawater, artificial seawater wash, ATL buffer, and homogenized samples, indicating that the method is effective in isolating microbial DNA from spatially distinct regions of the same host.

The external microbiome was best represented by the artificial seawater wash. The artificial seawater wash samples formed a distinct cluster in NMDS space, positioned between the environmental (seawater) and internal host-associated (ATL and homogenized) communities. This intermediate but non-overlapping positioning suggests that the microbial community recovered from the artificial seawater wash is ecologically and compositionally distinct from both the surrounding seawater and the internal microbiome. The artificial seawater wash was highly dissimilar from environmental seawater, indicating that it does not simply capture planktonic environmental microbes. At the same time, the artificial seawater wash was also different from both the ATL buffer and homogenized tissue, with similar levels of dissimilarity to each. This consistent pattern across life stages supports the idea that the artificial seawater wash captures a distinct community localized to the external surfaces of the snail, rather than being dominated by either environmental carryover or contamination from the internal microbiome. In contrast, ATL buffer samples showed greater similarity to homogenized tissue, suggesting that ATL buffer may partially lyse or extract microbes from internal tissues, making it less suitable as a stand-alone representation of the external microbiome.

Together, these findings demonstrate that the artificial seawater wash method provides a reliable and conservative approach for sampling the external microbiome of both juvenile and adult *N. ostrina*. By minimizing overlap with both environmental and internal microbial communities, this method can help resolve location-specific host–microbe interactions, which are critical for understanding microbiome function and structure in marine invertebrates.

Shifts in microbial community composition across life stages

The associated microbial communities shifted significantly across developmental stages of *N. ostrina*, suggesting ontogenetic changes in host–microbe interactions. The external microbiome of juvenile *N. ostrina* is enriched in *Phaeobacter porticola*, a bacterium that has been isolated from barnacles and aquaculture organisms and is known to produce antimicrobial metabolites such as tropodithietic acid, which inhibits the growth of pathogens like *Vibrio* spp. (Breider et al. 2017). Adult *N. ostrina,* in contrast, harbour external microbiomes enriched in *F. marinus*, and *A. genovensis* all of which have been isolated from marine biofilms and known to colonize marine surfaces (Vandecandelaere et al. 2008; Schober et al. 2024). The increase of antimicrobial-producing taxa in juveniles' external microbiomes may indicate that structural protection provided by the shell is insufficient as a primary barrier to infection in early life stages. The external microbiome of juveniles was also the most variable in community composition, suggesting that although juveniles have symbiotic antimicrobial bacterium present, they may also very susceptible to random colonization from environmental microbes on the exterior of their bodies.

The internal microbiome of juvenile *N. ostrina* exhibited the least variability across all samples and a higher overall diversity to the internal microbiome of adults. While the internal tissues of adult *N. ostrina* were primarily dominated by three microbial taxa (*Shimia aestuarii*, *Hyella patelloides*, and *Flavimaricola marinus),* juvenile snails displayed a broader diversity of abundant species, including *Rubritalea marina*, *Aestuariivita boseongensis*, *Haliangium tepidum*, *Balneicella halophila*, *Halarcobacter ebronensis, Alkaliphilus metallirdigens*. These taxa span a range of ecological roles: *H. patelloides* has capabilities for nitrogen fixation and extracellular polymer secretion that support biofilm stability (Becerra-Absalón et al. 2019); *H. tepidum* lyses other bacteria, thus helping to regulate microbial communities (Iizuka et al. 2003); *B. halophila* is a fermentative anaerobe associated with organic matter degradation in anoxic microenvironments (Fadhlaoui et al. 2016); and *A. boseongensis* is a bacterium linked to carbon cycling of coastal sediments (Park et al. 2014). In contrast, the internal microbiome of adults included *S. aestuarii*, *H. patelloides,* both mentioned previously, which have known associations with the gut of invertebrate hosts, and *F. marinus*, a bacterium known to be associated with kelp, which could be present due to the diet of the prey consumed by *N. ostrina* (Weigel et al. 2022). This taxonomic richness in the juvenile internal microbiome compared to the adults suggests juveniles host a diverse microbiome strongly shaped by environmental exposure and random colonization, whereas adults have an established gut microbiome.

Diversity Metrics Across Host Microbiomes

The patterns observed in alpha diversity help explain likely modes of colonization in early juvenile life. Adult snails exhibited significantly higher alpha diversity on their external surfaces compared to internal tissues, suggesting a more complex community associated with the shell or outer epithelium. The adult external microbiome was also more diverse than that of juveniles, indicating that microbial richness and evenness on the body surface may increase with age, exposure, and surface area. In contrast, the external microbiome of juveniles only had days to be colonized, resulting in reduced species diversity.

The internal communities of both adults and juveniles had lower Shannon diversity compared to external communities. Adults, presumably with established diets, may support more specialized gut microbiomes shaped by long-term host–microbe and diet–microbe interactions, which explains the reduced diversity (Russel et al. 2024). In juveniles, the internal microbiome may not reflect stable or specialized communities, as juveniles in this study had no access to food, and their gut microbiota had no opportunity to develop niche associations tailored to digestive function; therefore, the internal microbiome of juveniles reflects microbes that colonize the gut in the capsule or in the first few days since hatching.

Beta diversity analyses further underscore these distinctions in colonization patterns between juveniles and adults. Juvenile external communities were significantly more variable in composition than any other group, indicating a higher degree of heterogeneity in the juvenile external microbiome. The internal microbiome reflects greater stochasticity in early colonization events due to lower selective pressure in the juvenile gut. In contrast, the adult microbial communities were less variable, indicating host-regulated microbiomes in older individuals. The reduced variability in the juvenile internal microbiome lends support to the possibility that juveniles develop a predictable gut microbiome in the egg capsule.

Microbial variation across sites

Microbial communities associated with *N. ostrina* differed significantly between snails collected from Ross Islets and Prasiola Point. While Ross juveniles hosted microbiomes enriched in *A. metalliredigens, R. marina* and *H. tepidum*, those from Prasiola were enriched in *G. marinus* and *M. aureus*. *G. marinus* has been identified in Arctic seawater and plays roles in nutrient cycling (Bae et al. 2013), whereas *M. aureus* produces bioactive secondary metabolites and may confer chemical protection during early stages of colonization (Feng et al. 2024).

Adult *N. ostrina* from Ross Islets similarly displayed high abundance in *H. patelloides*, and *S. aestuarii,* both of which were isolated from ocean sediments found in sheltered inlets (Schober et al. 2024; Brito et al. 2020). Prasiola Point adults were dominated by a different set of taxa, including *F. intermedius*, *P. aqaticum*, and *A. aestuarii*, none of which were abundant in adult snails from Ross Islets. *F. intermedium* is an Alphaproteobacterium associated with biofilms and mucosal colonization (Fadhlaoui et al. 2016). *P. aquaticum*, originally isolated from seawater, is thought to be an opportunistic colonizer of marine invertebrates (Jang et al. 2011), while *R. marina* is commonly associated with sponge and coral microbiomes and may support antimicrobial defence (Yoon et al. 2007). These site-specific differences suggest local environmental conditions play a strong role in shaping microbial community assembly, even within the same host life stage. Variation in surrounding flora, salinity, wave exposure, temperature, tidal dynamics, and composition of the local algal and animal community likely drives the observed taxonomic differences between sites.

CONCLUSION

The significant differences between the microbiomes associated with wild juvenile and adult *N. ostrina* suggest microbial communities undergo substantial changes with host development. Juveniles are colonized by transient environmental taxa found in seawater samples. The prevalence of antimicrobial-producing bacteria such as *P. porticola* and *M. aureus* in the exterior microbiome of juveniles suggests a potential role in microbial defence against pathogenic threats when physical barriers like the shell are underdeveloped (Breider et al. 2017; Feng et al. 2024). In contrast, when looking at the whole adult microbiome, it appears to host a less variable range of microbial communities with some noted functions related to surface adhesion and host–microbe symbiosis. These findings emphasize that microbial acquisition in early life stages is shaped both by environmental influence and host life stage.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS

L.A. Gosselin and E.M Bottos devised the conceptual ideas of the study, helped develop methodology and experimental design, assisted with field and laboratory work, and contributed to writing the manuscript. J.D. Van Hamme assisted with laboratory work and sequence data analysis. T.L. Dixon was involved in all aspects of the study.

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FIGURE LEGENDS

Figure 1. NMDS plot based on Bray-Curtis dissimilarity in microbial community compositions associated with *N. ostrina.* (A) juveniles, and (B) adults; demonstrating that distinct communities were extracted from external (Artificial Seawater Wash) and internal

(ATL Buffer Wash and Homogenized) body sites and that these communities were distinct from environmental samples (Seawater).

Figure 2. NMDS plot based on Bray-Curtis dissimilarity in external microbial community compositions associated with *N. ostrina* adults and juveniles from the Ross Islets (A), and box and whisker plot representing OTUs of each of the top 10 species accounting for 43.6% of the observed variation between the external microbiomes of juveniles and adults (B).

Figure 3. NMDS plot based on Bray-Curtis dissimilarity in internal microbial community compositions associated with *N. ostrina* adults and juveniles from the Ross Islets (A), and box and whisker plot representing OTUs of each of the top 10 species accounting 50.8% of the observed variation between the internal microbiomes of juveniles and adults (B).

Figure 4. Box and whisker plot representing alpha diversity of the microbial communities associated with *N. ostrina*. Error bars represent 1.5 times the interquartile range, indicating the spread of dispersion values within each group.

Figure 5. Box and whisker plot representing within-group dispersion of beta diversity of microbial communities associated with *N. ostrina*. Error bars represent 1.5 times the interquartile range, indicating the spread of dispersion values within each group.

Figure 6. NMDS plot based on Bray-Curtis dissimilarity in internal microbial community compositions associated with *N. ostrina* juveniles from Ross Islets and Prasiola Point (A), and box and whisker plot representing OTUs of each of the top 10 species accounting for 38.0% of the observed variation between the internal microbiomes of juveniles and adults.

Figure 7. NMDS plot based on Bray-Curtis dissimilarity in internal microbial community compositions of juveniles from Ross Islet and Prasiola Point (A), and box and whisker plot representing OTUs of each of the top 10 species accounting for 52.4% of the observed variation between the internal microbiomes of juveniles and adults.

FIGURES

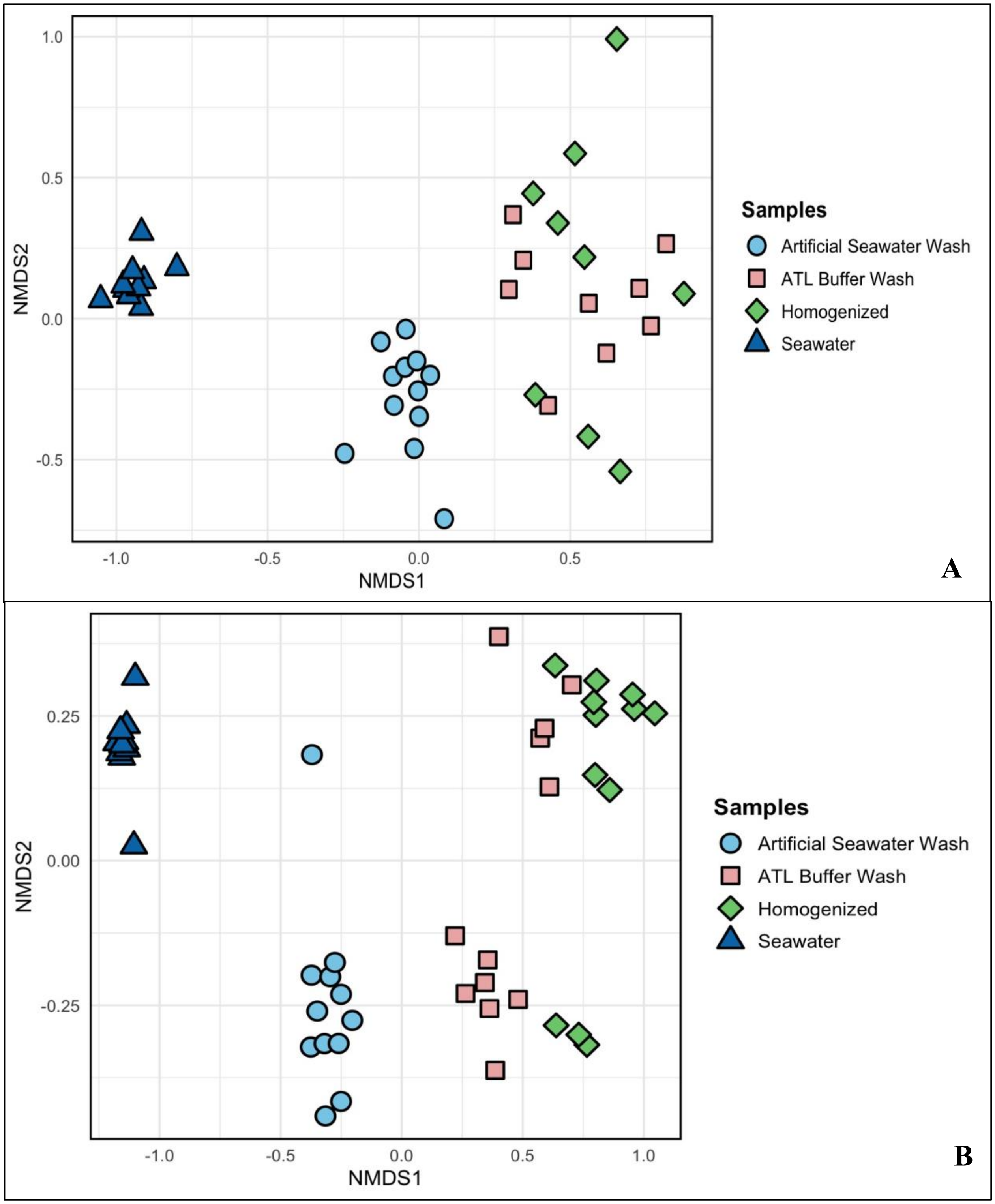


Figure 1.

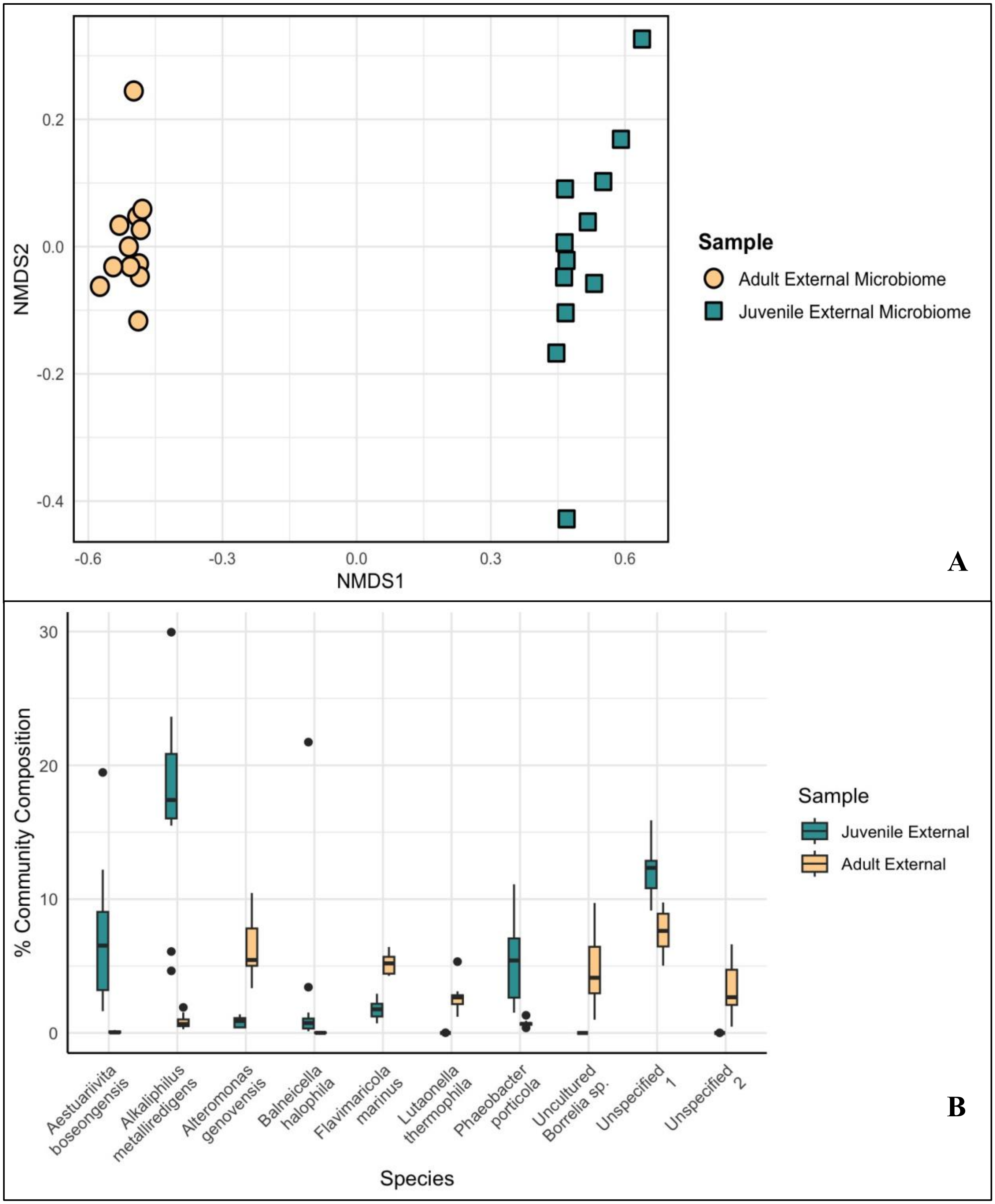

0.2
0.0
-0.2
-0.4
NMDS2
-0.6
-0.3
0.0
0.3
0.6
NMDS1
Sample
Adult External Microbiome
Juvenile External Microbiome
A
30
20
10
0
% Community Composition
Aestuariivita boseongensis
Alkaliphilus metalliredigens
Alteromonas genovensis
Balneicella halophila
Flavimaricola marinus
Lutaonella thermophila
Phaeobacter porticola
Uncultured Borrelia sp.
Unspecified 1
Unspecified 2
Species
Sample
Juvenile External
Adult External
B

Figure 2.

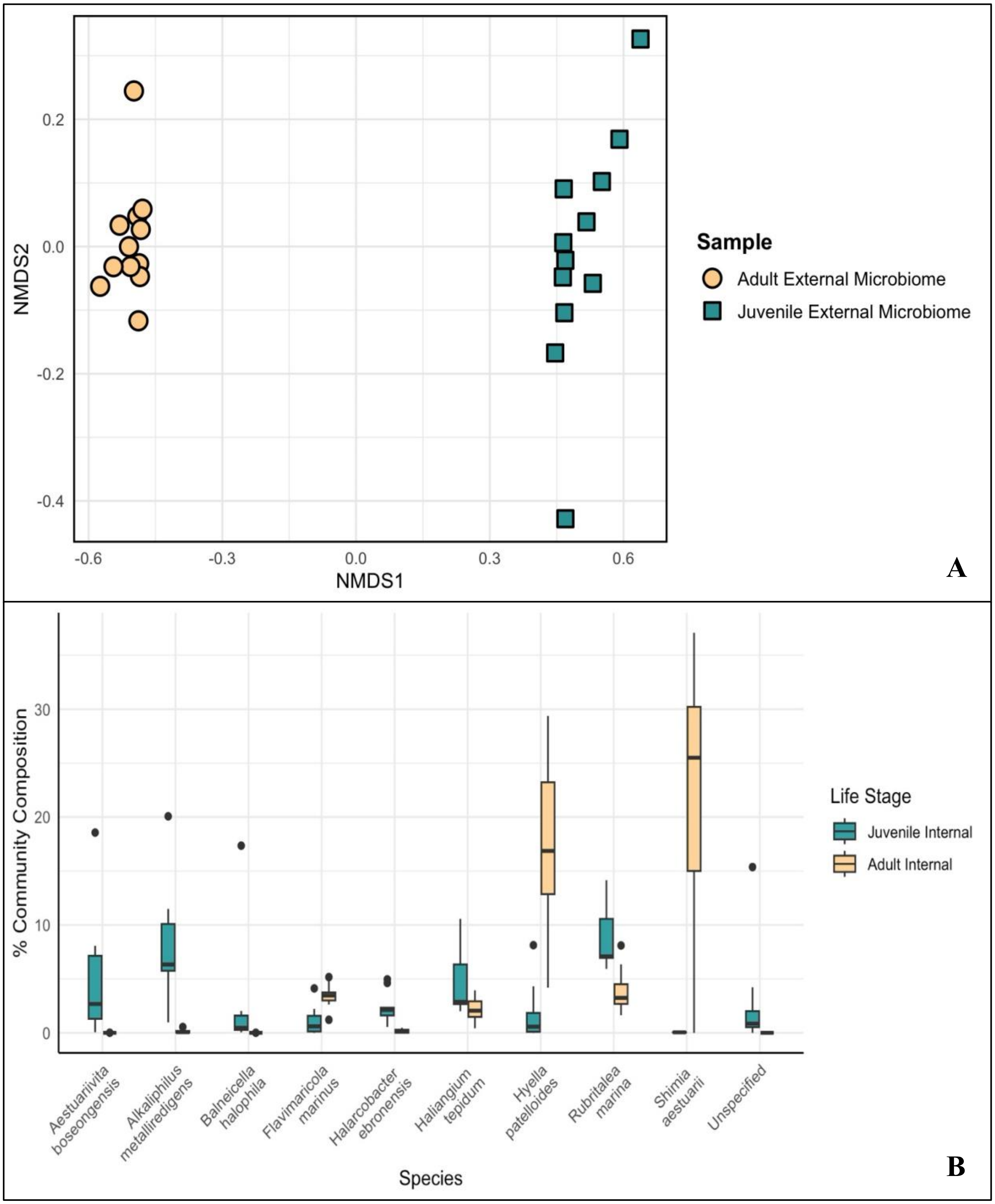

NMDS2
0.2
0.0
-0.2
-0.4
-0.6
-0.3
0.0
0.3
0.6
NMDS1
Sample
Adult External Microbiome
Juvenile External Microbiome
A
% Community Composition
30
20
10
0
Life Stage
Juvenile Internal
Adult Internal
Aestuariivita boseongensis
Alkaliphilus metalliredigens
Balneicella halophila
Flavimaricola marinus
Halarcobacter ebronensis
Haliangium tepidum
Hyella patelloides
Rubritalea marina
Shimia aestuarii
Unspecified
Species
B

Figure 3.

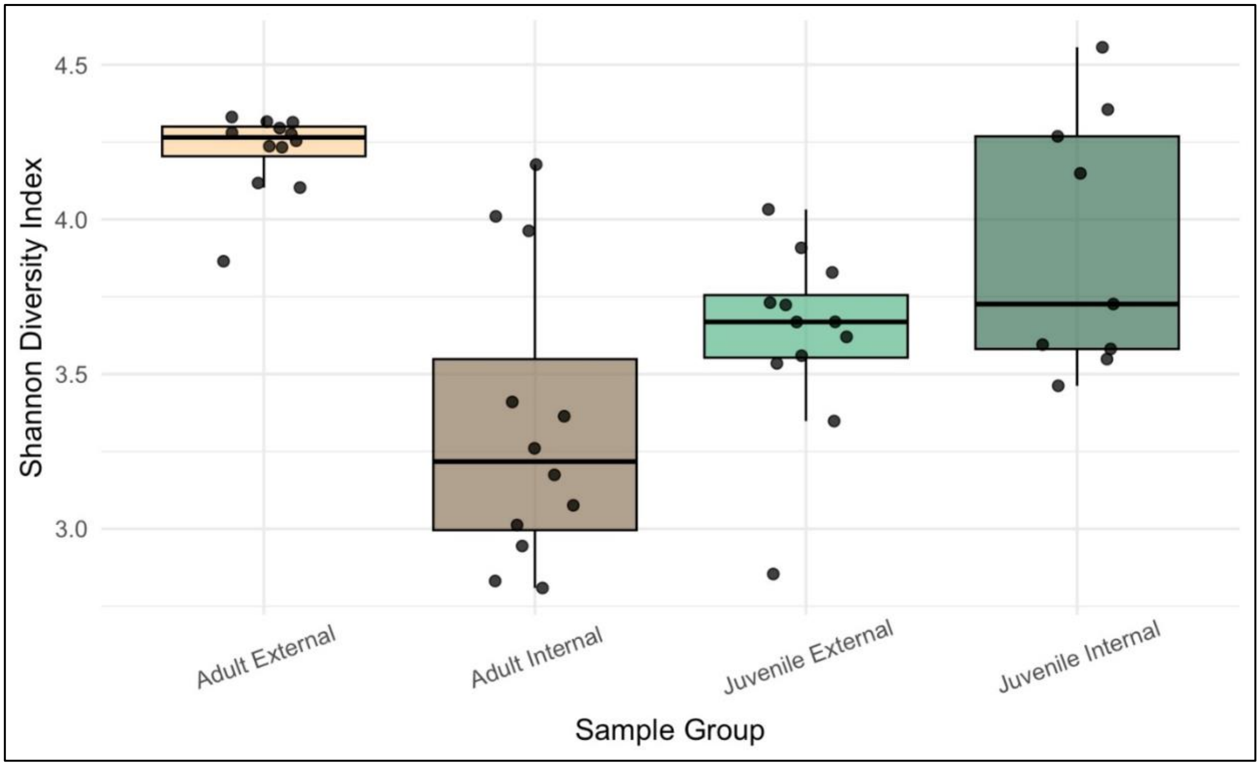
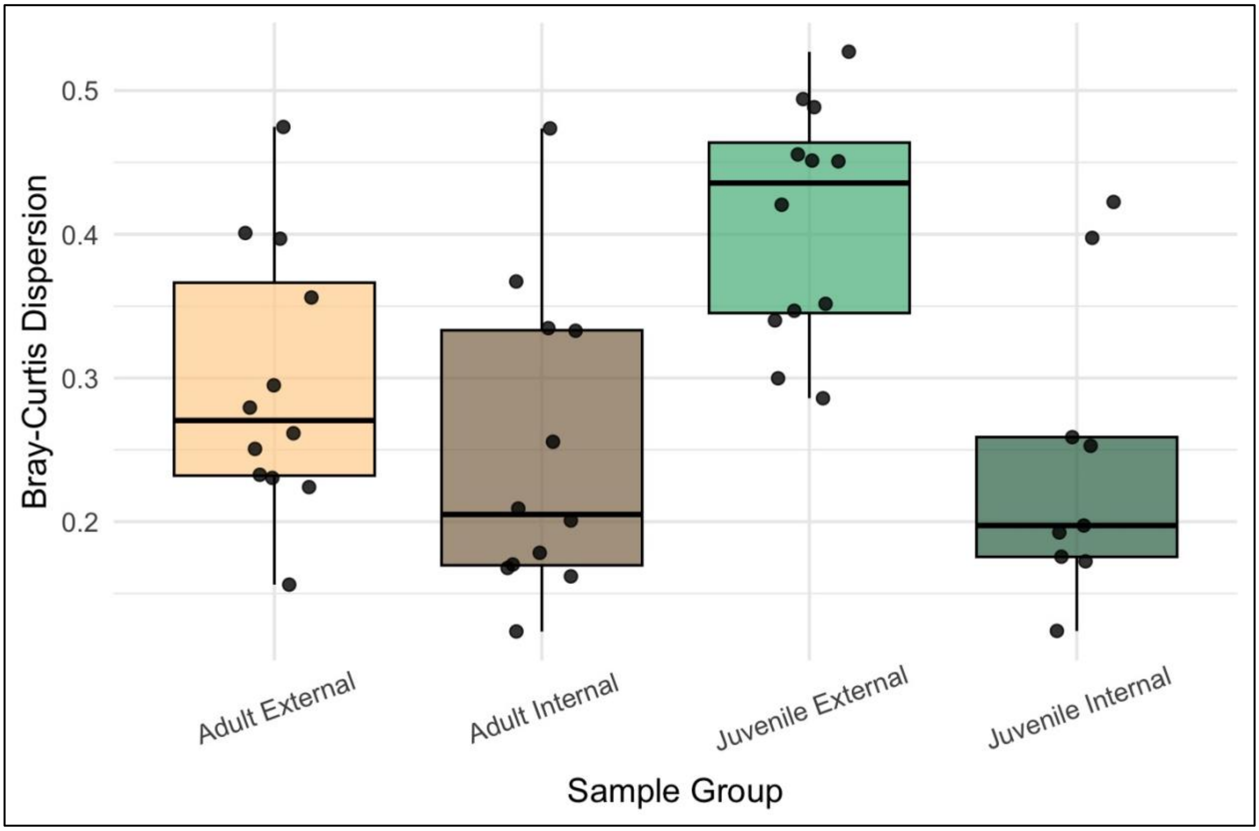


Figure 4.



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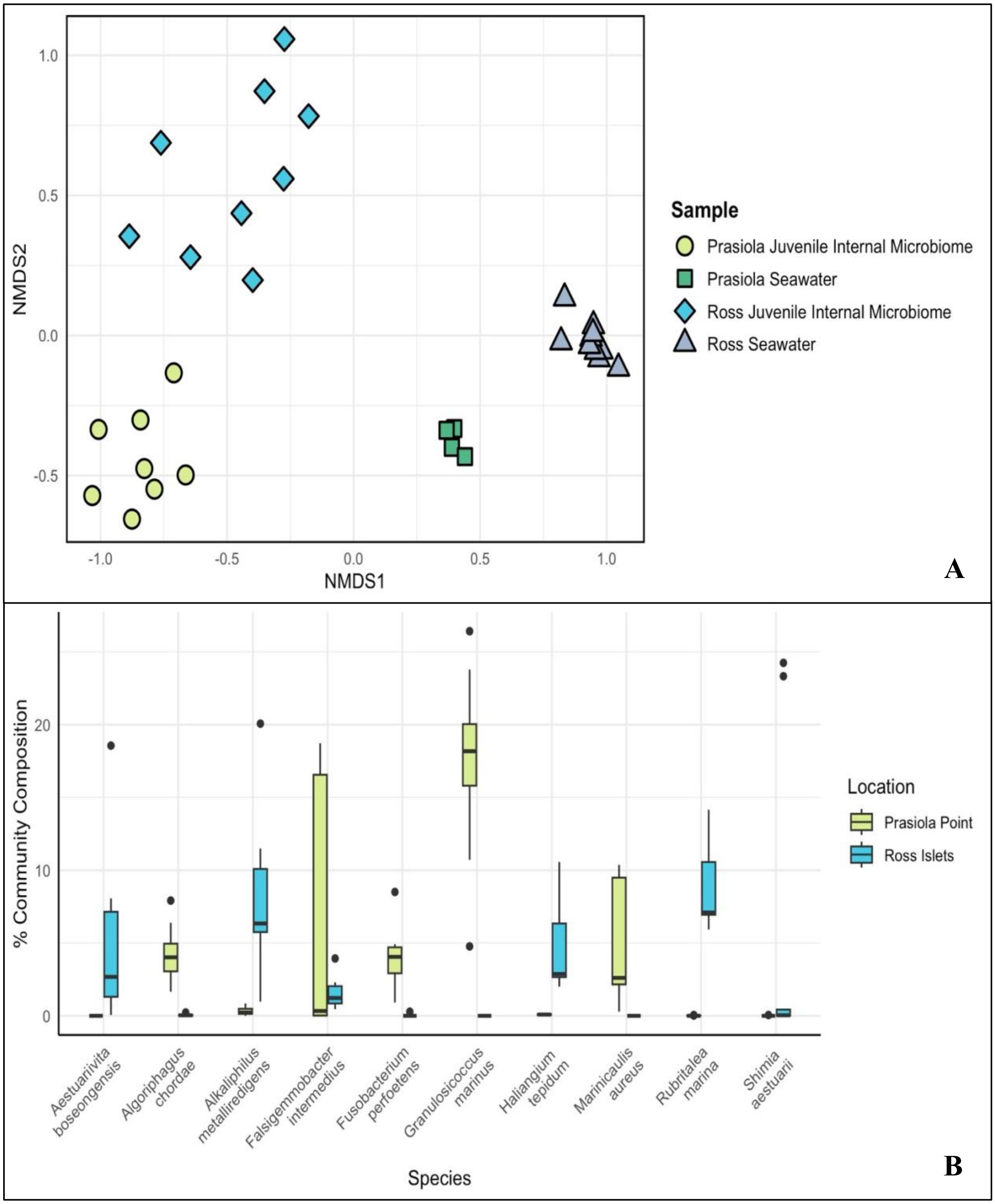
1.0
0.5
0.0
-0.5
NMDS2
-1.0
-0.5
0.0
0.5
1.0
NMDS1
Sample
Prasiola Juvenile Internal Microbiome
Prasiola Seawater
Ross Juvenile Internal Microbiome
Ross Seawater
A
% Community Composition
20
10
0
Aestuariivita boseongensis
Algoriphagus chordae
Alkaliphilus metalliredigens
Falsigemmobacter intermedius
Fusobacterium perfoetens
Granulosicoccus marinus
Haliangium tepidum
Marinicaulis aureus
Rubritalea marina
Shimia aestuarii
Species
Location
Prasiola Point
Ross Islets
B

Figure 6.

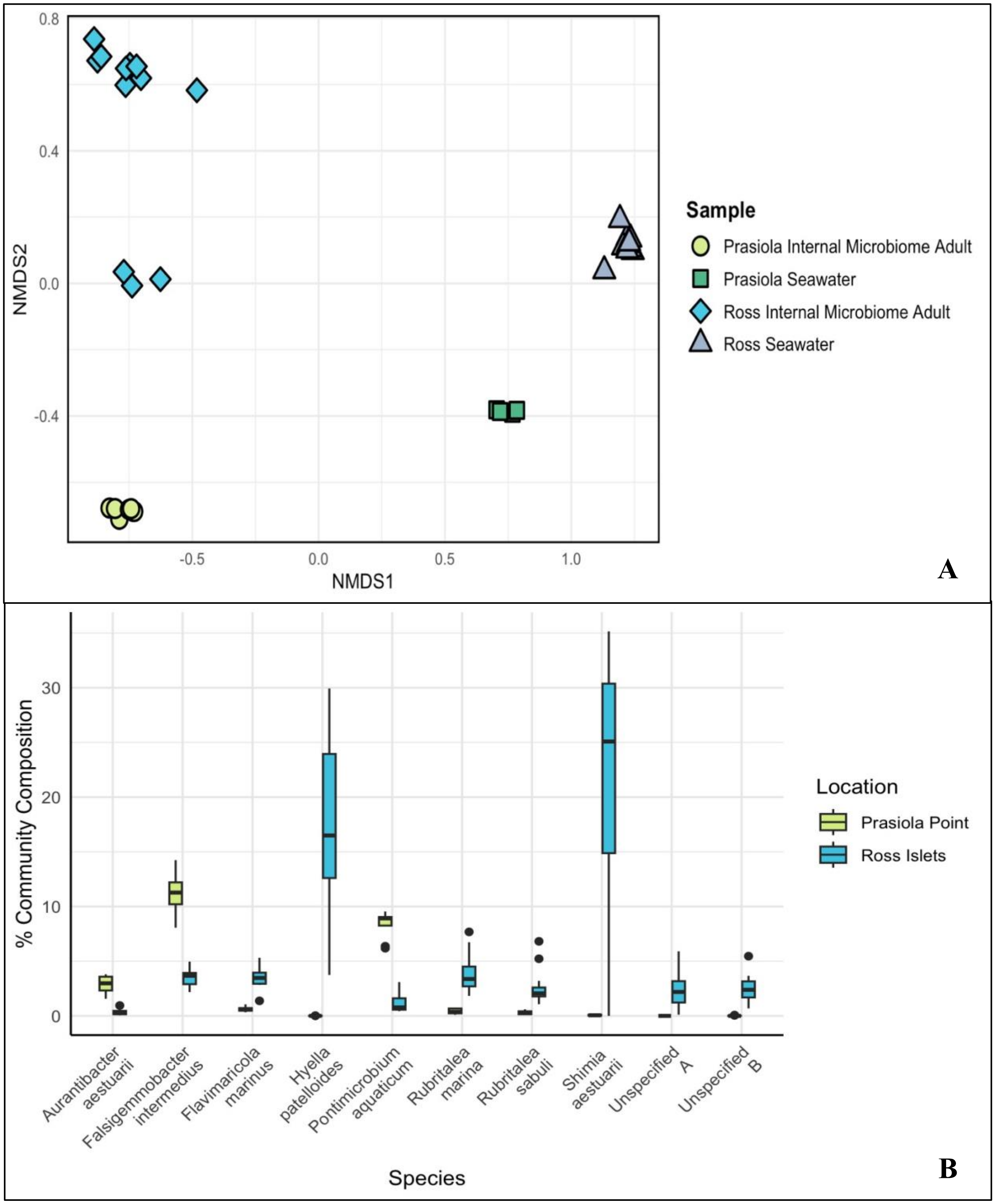
0.8
0.4
0.0
-0.4
NMDS2
-0.5
0.0
0.5
1.0
NMDS1
Sample
Prasiola Internal Microbiome Adult
Prasiola Seawater
Ross Internal Microbiome Adult
Ross Seawater
A
30
20
10
0
% Community Composition
Location
Prasiola Point
Ross Islets
Aurantibacter aestuarii
Falsigemmobacter intermedius
Flavimaricola marinus
Hyella patelloides
Pontimicrobium aquaticum
Rubritalea marina
Rubritalea sabuli
Shimia aestuarii
Unspecified A
Unspecified B
Species
B

Figure 7.