Thompson Rivers University

Developmental and environmental variation in the microbial communities of the newly hatched juvenile marine snail, Nucella ostrina.

UREAP Project

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INTRODUCTION

The microbiome plays an important role in maintaining the health and fitness of marine invertebrates by facilitating immune defence, aiding in digestion, and enabling adaptation to environmental conditions (Khan et al. 2018). Marine invertebrates harbour a diverse variety of bacterial communities, predominantly within their digestive systems as well as on the external surfaces of the body. Some bacteria found on the body surface of marine invertebrates have beneficial properties, others can be harmful pathogens, and some may have no functional benefit to the animal at all. The bacteria associated with marine invertebrates have been found to alter the animal's vulnerability to pathogens and predators (Menabit et al. 2024). In addition, studies using PCR (polymerase chain reaction) sequencing and synthesis techniques have identified bioactive metabolites with antimicrobial properties in marine invertebrates (El Samak et al. 2018), an important consideration given the current increase in antibiotic resistance among medically important pathogens. There is also a growing concern that climate change, which is causing a rise in seawater temperature (Iwabuchi & Gosselin 2019), could disrupt the natural microbial communities associated with marine invertebrates. For instance, Khan et al. (2018) discovered that bacteria in the genera *Clostridiales* and *Verrucomicrobiales* were present in high densities on the gills and in the gut of heat-resistant oysters, and those bacteria improved the oyster's ability to respond to heat; however, *Acidobacteriales* and *Betaproteobacteriales* were potential pathogens and were found more frequently on heat-susceptible oysters. Additionally, PCR analysis of sponge microbiomes before and after heat stress events revealed the presence of transient bacteria on vulnerable

sponges, resulting in fatalities of some of those sponges (De Castro-Fernández et al. 2024). Warming oceans can also affect the gut microbiome of marine invertebrates, particularly the abundance of disease-causing bacteria (Liu et al. 2023); Liu et al. (2023) found microbial diversity and abundance increased under warming temperatures in the laboratory, and to a lesser extent in the field. Evidence to date therefore indicates that the microbial community associated with marine invertebrates plays an important role in the survival and growth of these animals.

Though several studies have examined microbial diversity and activity on marine invertebrates, most have focussed on the gut microbiome, and all studies have focussed on adult invertebrates. No study to date has investigated the outer microbiome of early juveniles, which is known to be the most vulnerable phase of the life cycle of marine invertebrates (Gosselin & Qian 1997). In addition, a majority of microbial studies have focussed on invertebrates reared in aquaculture facilities; far fewer studies have examined the microbial community associated with wild invertebrates in natural habitats. This project, carried out in part at the Bamfield Marine Sciences Center, seeks to characterize the microbiome of early juvenile marine invertebrates living in natural habitats. The specific goal of the project was to determine an effective methodology for the extraction and amplification of bacterial DNA from both the juvenile and adult stages of the snail, *Nucella ostrina*.

METHODS

Field collection at BMSC

The first part of the study aimed to collect juvenile and adult *N. ostrina* from their natural habitats and preserve them for subsequent analysis of their microbiome. Field collection was carried out from May 15th to June 30th 2024 in Barkley Sound, near the Bamfield Marine Sciences Center (BMSC) on the west coast of Vancouver Island. Adult snails and egg capsules of *N. ostrina* were collected by hand, using sterilized gloves and tweezers, from the intertidal zone of an island within the Ross Islets (48° 52′ 18.8″ N, 125° 09′ 43.7″ W) and brought back to the BMSC laboratory, where they were placed immediately into holding tanks containing water collected from their field site.

Hatching and preservation of snails for DNA extraction

The next step was to obtain newly hatched snails from the egg capsules collected from the field and to preserve snails for eventual microbiome analysis. The egg capsules and adults were placed together in cleaned tanks; these tanks contained seawater collected from their field site to ensure the snails remained exposed to the same microbes and conditions they were exposed to in the field. Hatchlings emerged from the egg capsules over the following 36 h into the same water as the adults, ensuring consistency across the microbes the snails were exposed to. After a sufficient number of juveniles hatched, both adults and juveniles were then placed for 48h into a different clean tank containing seawater from the field site to allow a microbiome to develop on the surfaces of the juvenile snails. Snails were then placed in sterilized centrifuge tubes containing either 5 adults or 20 juveniles, and frozen at -20˚C for 24 h to euthanize. Seawater from the tanks was also collected and frozen for subsequent

DNA analysis to determine whether the seawater microbiome is significantly different from the snail microbiome.

DNA extraction

The second part of this study aimed to develop a method to extract DNA from the outside of both the juvenile and adult snails. DNA extraction and laboratory work was conducted at the Microbial Ecology and TRUGen sequencing laboratory at Thompson Rivers University from July to September 2024. Juveniles and adult snails were thawed and washed three times in sterile seawater by vortexing, and then the seawater was removed via pipet and placed in separate collection tubes. This was performed to remove transient bacteria in the surroundings that are not part of the snail's microbiome. ATL buffer was used to remove the outer microbiome; snails were placed in the buffer for 30-40 minutes at 60 °C, then the surrounding buffer containing the outer microbiome was removed into a separate 2mL microcentrifuge tube. Sterile seawater was used to wash the remaining ATL buffer off the snails, then using sterilized tweezers the body of each adult snail was removed from its shell to allow the following buffers to reach body tissues normally located under the shell. The samples of ATL buffer containing DNA from wash water, seawater, or the outer body surface were all centrifuged at 14,000 rpm for 10 min into a pellet, and the supernatant ATL buffer was then poured off. The pellet samples were also rinsed with sterile seawater to remove all ATL buffer for future steps.

DNA purification

Amplification of DNA from the pellet samples was carried out using the E.Z.N.A Mollusc & Insect DNA Kit® (Omega Bio-Tek), which is designed for the efficient

recovery of genomic DNA from molluscs and was chosen for its ability to break down complex mucopolysaccharides that are present within the mucus of snails (Belouhova et al., 2022). Mucopolysaccharides are negatively charged polysaccharide compounds that are believed to function in cell signalling and other biochemical processes (Casale & Crane, 2019). Their negative charge allows them to interact with a variety of proteins and ions as well as interacting with DNA polymerases (Casale & Crane, 2019, Sidstedt et al. 2020). The binding of mucopolysaccharides could impede the activity of the polymerase stopping effective transcription (Sidstedt et al. 2020). The following steps were applied as described by the manufacturer of the E.Z.N.A Mollusc & Insect DNA Kit. The procedure relies on the properties of the cationic detergent, cetyltrimethyl ammonium bromide (CTAB), present in the ML1 buffer to break down and dissolve membrane lipids. Proteinase K digests proteins, histones and nucleases. Together, these compounds lyse the cell and release the DNA. Samples were incubated at 60˚C for 30 min or until soluble, as proteinase K is the most effective at this temperature, maximizing its ability to degrade proteins surrounding the DNA. Once soluble, a solution of chloroform:isoamyl alcohol (24:1) was added to separate the DNA and RNA from the mucopolysaccharides that inhibit transcription. This resulted in two distinct layers: a top layer containing RNA and DNA (which was removed into separate tubes), and a lower layer (which was discarded) containing proteins, lipids, other organic molecules; in between these layers was a central milky interface containing denatured proteins and debris. BL buffer and RNase A were then added to the tube containing the RNA and DNA. BL buffer helps promote the binding of the DNA to the silica column, as it contains chaotropic salts that disrupt hydrogen bonds allowing the DNA

to bind more easily. RNase A is an enzyme that cleaves the phosphodiester bonds between ribonucleotides, selectively destroying all the RNA in the sample. Following this, ethanol was then added to precipitate DNA and help it bind to the silica; DNA is soluble in water because of its negatively charged backbone, but in ethanol the hydration shell is disrupted allowing the DNA to precipitate out of the solution. This allows the DNA to bind onto the silica spin column, to isolate and discard other liquids. HBC buffer is then added to disrupt hydrogen bonds after the DNA has bound, aiding to denature any remaining proteins and increasing the affinity of DNA for the column. A DNA wash buffer was then used to remove residual proteins, polysaccharides, lipids, salts and organic solvents, and an elution buffer heated to 70˚C rehydrated the DNA, releasing it from the silica into the collection tube below.

Analysis of extracted DNA

This step served to isolate the 16S rRNA bacterial gene sequence by thermal cycle amplification to visualize the presence of bacterial DNA in the samples. This gene was targeted in sequencing due to being highly conserved across bacterial phyla allowing for comparThons. The samples containing the outer microbiome and the samples containing the entire microbiome from the adult snails were diluted to reduce the amount of DNA in the sample, as high concentrations inhibit binding of primers during PCR. The outer samples were diluted in sterile PCR water to a 1:5 ratio, and samples of entire homogenized snails were diluted to a 1:10 ratio.

Each purified DNA sample was processed using PCR to amplify the 16S rRNA gene. The DNA along with Taq polymerase (a type of polymerase that can withstand high temperatures), the 341F primer, and the 806R primer were run through 30 cycles

in the thermocycler. These samples were then run on a 1% agarose gel to confirm the amplification of this gene region and presence of bacterial DNA.

RESULTS

The initial PCR trials using the DNeasy PowerBiofilm Kit resulted in the majority of samples not producing visible DNA bands (Figures 1, 2). This was likely due to the kit not containing chloroform:isoamyl alcohol (24:1), a critical step in the removal of the

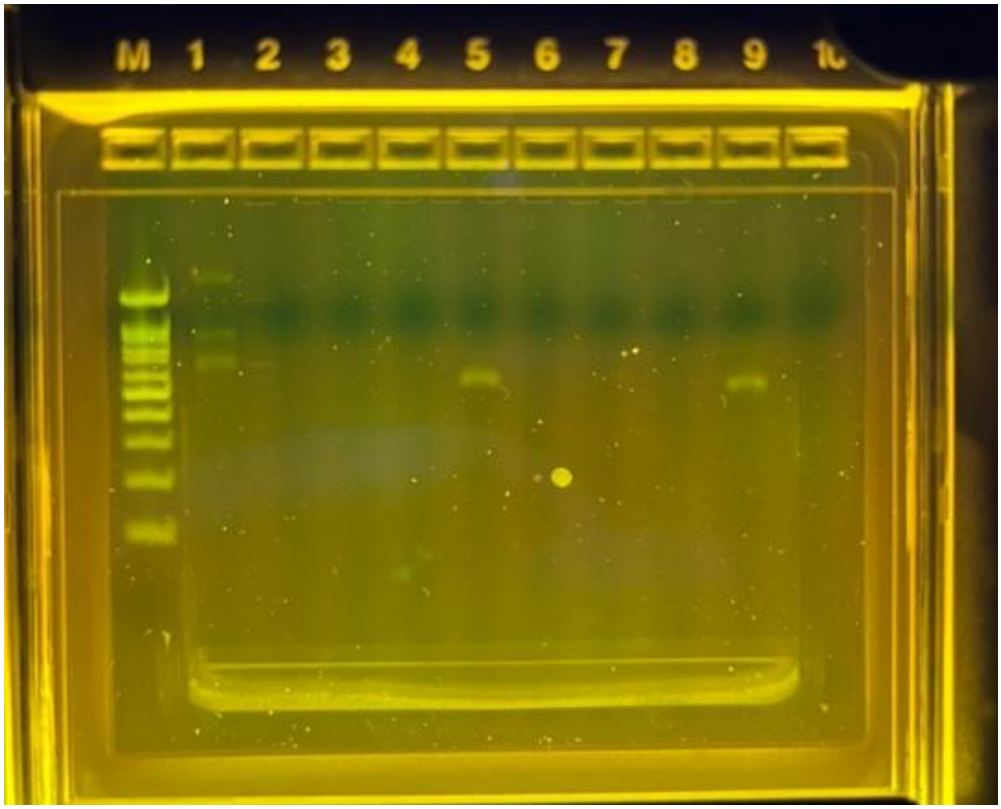

Figure 1. Agarose gel of juvenile extractions at BMSC. From left to right, ladder, positive control, negative control, sterile seawater (2nd control), Seawater, wash water, outer of juvenile (2 reps), entire homogenized juveniles (2 reps).

mucopolysaccharides that can inhibit DNA polymerase. Additional trials in which the positive control (which is known to work) was added into the juvenile samples also produced no visible DNA bands (Figure 2), confirming that inhibition was the issue

rather than an absence of bacterial DNA. The analyses of the 5 different species of marine invertebrate helped to determine if the lack of DNA from *N. ostrina* samples was specific to this species or was indicative of a broader problem with the extraction methodology. The results showed no band for echinoderms and ascidians (the species with increased mucus production), two bands in cnidarians, and a band for both crustaceans and mollusc (Figure 2).

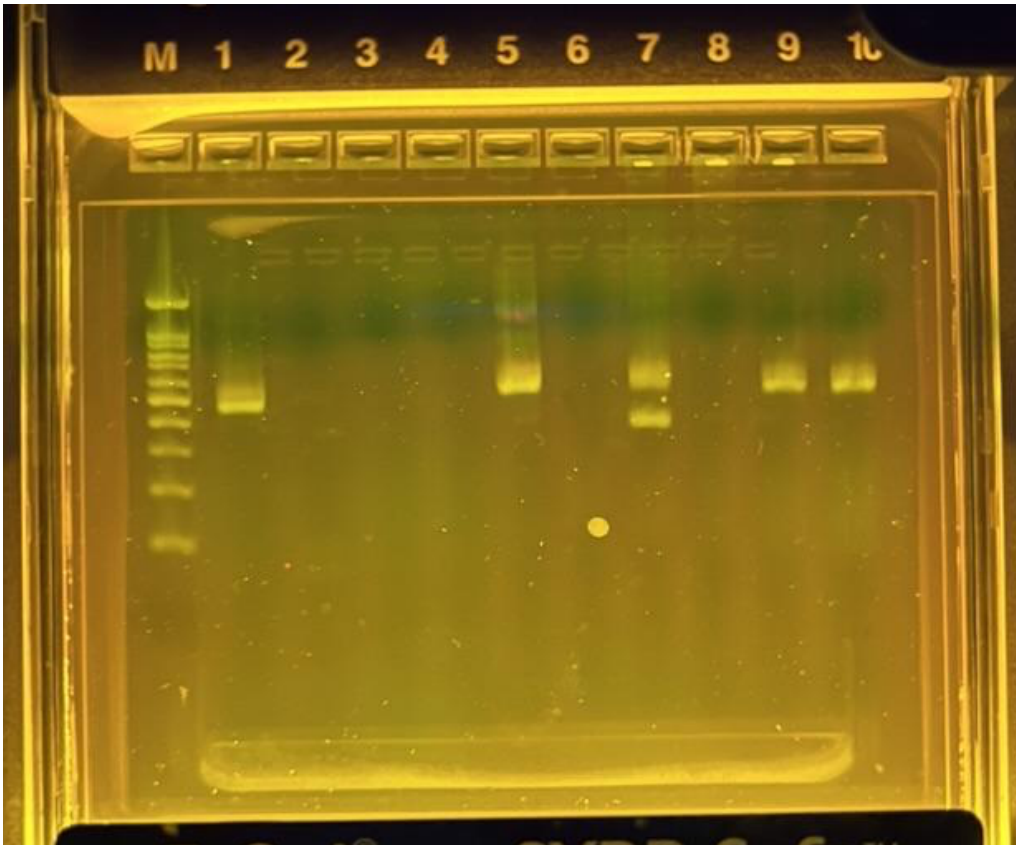

Figure 2. Agarose gel of juvenile extractions at BMSC. From left to right, ladder, positive control, negative control, homogenized juvenile spiked with positive control, juvenile unspiked, adult (N.ostrina), Echinoderm, Cnidarian, Ascidian, Crustacean, Mollusc (mussel).

The next DNA extraction trials, using the E.Z.N.A Mollusc & Insect DNA Kit, resulted in visible DNA bands for all samples of juvenile and adult *N. ostrina* (Figures 2,

3, 4). Bands of ~450 base pairs were visible, as expected for the amplification of the 16S rRNA gene sequence. The 16S rRNA gene has a known sequence length and, based on the primers we selected (341F and 806R), it should result in a band at ~450 base pairs long if bacterial DNA is present and has been amplified.

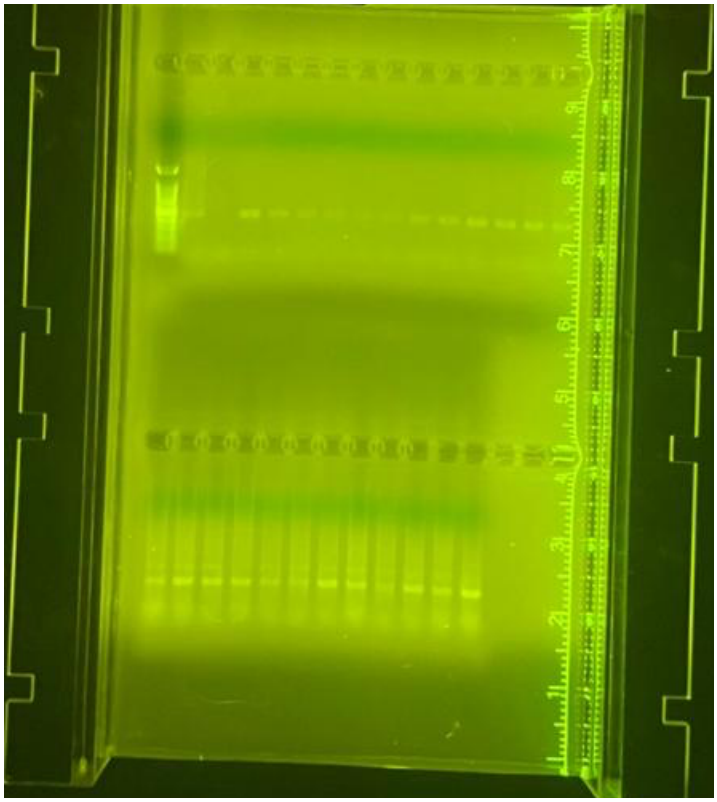

Figure 3. Agarose gel of diluted extractions from adult snails. The top row of the gel left to right, ladder, positive control, negative control and 12 replications of the outer microbiome. The lower row shows 12 replicates of the entire homogenized adults both outer and inner.

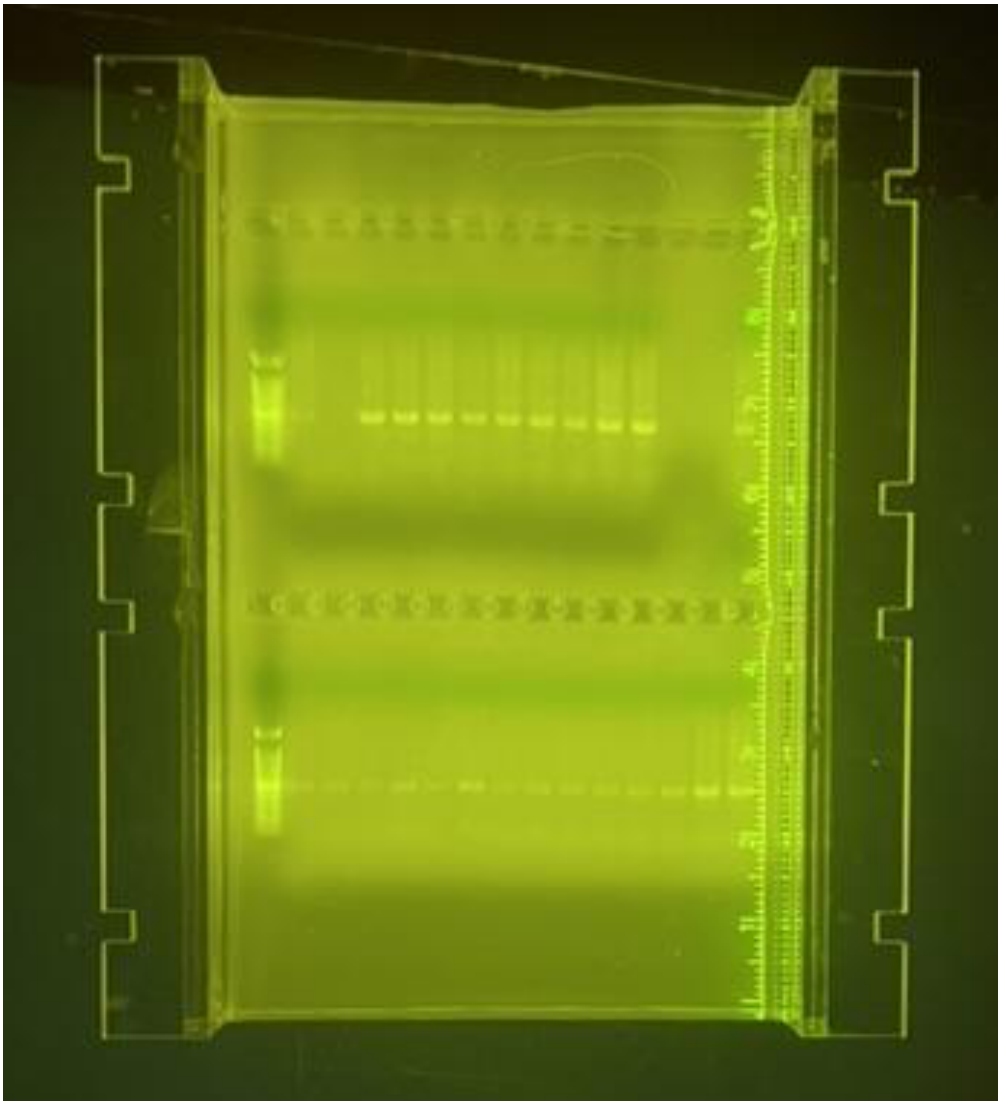

Figure 4. Agarose gel of extraction from juvenile snails. the top row in the gel left to right, ladder, positive control, negative control and 12 replications of the outer microbiome. The lower row shows the replications of the entire homogenized juveniles

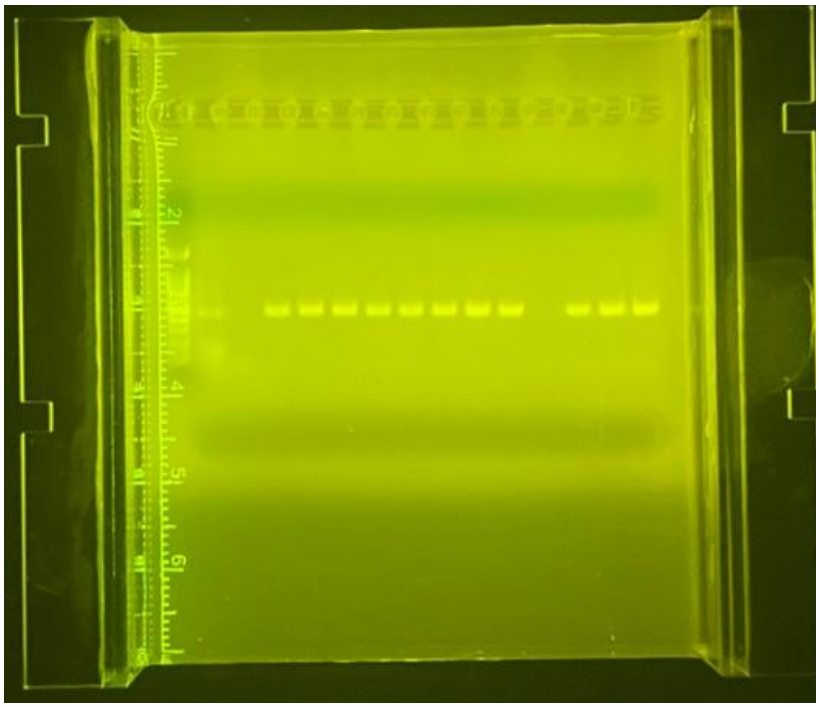

Figure 5. Agarose gel of extraction on seawater. the gel left to right, ladder, positive control, negative control and 12 replications of the outer microbiome. Well 9, is blank and did not get a good extraction possibly due to errors in the extraction process or insufficient DNA in that sample.

DISCUSSION

This study revealed that the E.Z.N.A Mollusc & Insect DNA Kit is more effective for extracting and amplifying microbial DNA from the outer surface of *Nucella ostrina* than the DNeasy PowerBiofilm Kit. Initial trials using the DNeasy PowerBiofilm Kit failed to produce consistently visible DNA bands on electrophoresis gels following PCR, suggesting that this kit is not able to remove inhibitors such as mucopolysaccharides during DNA extraction, adding challenges for the transcription of DNA during PCR amplification. As seen with other mucus-rich marine invertebrates such as echinoderms, DNA amplification was not successful even when spiked with positive controls, revealing the presence of inhibitors in the mucus of these organisms. The E.Z.N.A Mollusc & Insect DNA Kit includes

chloroform:isoamyl alcohol (24:1) step, which separates DNA and RNA from the other proteins, polysaccharides and organic compounds, allowing for cleaner isolation of DNA and RNA. This was essential in obtaining clear bands of~450 bp during gel electrophoresis that would confirm the successful amplification of the 16S rRNA gene sequence. The inclusion of the chloroform:isoamyl alcohol (24:1) step was responsible for the difference between these two kits. This suggests that the removal of mucopolysaccharides is critical for successfully extracting and amplifying microbial DNA from mucus-rich invertebrates.

Following the extraction and amplification of microbial DNA, the next step in this study would be to sequence the 16S rRNA gene in order to identify the bacterial phyla found on the outside of snails and compare the microbiome associated with the two life stages (juvenile & adult). In addition, by comparing the outer microbiome of the snails, the inner microbiome of the snails, the wash water microbes from the snails and the seawater microbes in their environment, we could determine if the outer microbiome is significantly different from the surrounding water and the inner microbiome. A comparative analysis here would also confirm whether the method of extracting the outer microbiome is working and what communities of microbes it contains.

This study demonstrates the importance of selecting appropriate DNA extraction methods when working to extract DNA from different conditions, such as the mucus-rich surface of *N. ostrina*. The E.Z.N.A Mollusc & Insect DNA Kit was shown to be the most effective in extracting amplifiable bacterial DNA from both juveniles and adult snails, largely due to its ability to remove mucopolysaccharides and other

possible inhibitors of PCR. The presence of distinct DNA bands at the expected size range confirms the successful isolation and amplification of the 16S rRNA gene, leading the way for future DNA sequencing of the microbial communities.

These findings contribute to the understanding of the microbial ecology associated with *N. ostrina,* but also provide a foundation for future research, including phyla characterization through sequencing to better understand interactions between marine invertebrates and their associated microbiomes. Understanding these relationships is crucial for assessing the potential impacts of environmental changes on the health and survival of marine invertebrates such as *N. ostrina*.

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