Spring 2020

Quantifying the Emanation and Decay of Environmental DNA from Three Marine Molluscs

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QUANTIFYING THE EMANATION AND DECAY OF ENVIRONMENTAL DNA FROM THREE MARINE MOLLUSCS

A Thesis

Presented to the

Faculty of

Moss Landing Marine Laboratories

California State University Monterey Bay

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

in

Marine Science

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by

Emily Rose Pierce

Term completed: Spring, 2020

CALIFORNIA STATE UNIVERSITY MONTEREY BAY

The Undersigned Faculty Committee Approves the

Thesis of Emily Rose Pierce:

QUANTIFYING THE EMANATION AND DECAY OF ENVIRONMENTAL

DNA FROM THREE MARINE MOLLUSCS

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DEDICATION

My thesis work is dedicated to my family for always believing in me and cheering me on. Also, to my friends for always being voices of reason, shoulders to cry on, and sources of a great laugh. Lastly, to all little girls (and boys) who get bullied for being science oriented, keep being the snail lady. One day, you could change the world (or, at least get a master's degree studying your childhood passion).

It is advisable to look from the tide pool to the stars and then back to the tide pool again.

John Steinbeck, *The Log from the Sea of Cortez*

ABSTRACT

Quantifying the Emanation and Decay of Environmental DNA
from Three Marine Molluscs
by
Emily Rose Pierce
Master of Science in Marine Science

California State University Monterey Bay, 2020

Environmental DNA (eDNA) is nucleic acids outside of living organisms found in air, soil, water, and ice. It is shed by organisms through waste and other bodily fluids, as well as cells sloughed off the outside of an organism. eDNA breaks down over time, especially when exposed to UV, heat, and bacteria. Scientists can analyze eDNA to identify organisms in an area, though the rates at which it is emanated and decayed seem to vary from organism to organism, complicating interpretation of results. The present study sought to quantify the rates of emanation and decay through a series of *in vitro* experiments for three species, *Mytilus californianus* (the California blue mussel), *Haliotis rufescens* (the red abalone), and *Lottia scabra* (the rough limpet). Using quantitative PCR (qPCR) to measure eDNA, I found that eDNA emanation rates varied based on species, size, and activity level, and that rates of decay can be influenced by bacterial activity and time under treatment. Small abalone released less eDNA than medium and large abalone over 24 hours, but limpets and mussels released the same amount of eDNA per species despite different wet weights. Similarly, inverted abalone with soft tissue exposed released more eDNA than in their normal posture, but this was not true for limpets or gaping *vs* non-gaping mussels. Lastly, eDNA degraded over time for all species, mostly in the first 24 hours, and bacteria affected abalone eDNA degradation. In eDNA degradation experiments, nonspecific PCR products decreased reliability of measurements over longer time periods. These data can be used by scientists and managers to interpret eDNA signals of these commercially or ecologically important molluscs to help protect these species and the communities in which they belong.

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ACKNOWLEDGEMENTS

First, thank you so much to Dr. Jonathan Geller for accepting me into his lab and for work as one of his student assistants throughout my time here. I could not have made it this far without Melinda Wheelock and Martin Guo who taught me so many laboratory techniques and greeted me daily with so much kindness and support. Melinda has been a source of advice for every struggle I had, and Martin constantly fixed my mistakes (i.e. rushing in to clean up glass when I knocked something over) and is one of the hardest working people I've ever met. In that same vein, thank you so much to Amanda Heidt and Emmet Haggard for being excellent role models, partygoers, slug lovers, and sea cucumber rescuers. I'll miss seeing both of you in lab at super weird times.

I could not have made it through my time at MLML without all of the friends I've made here. Felicia for allowing me to cry when I needed and for always making interesting looking art and models to brighten up every single day. Miya and Cindy for the numerous sleepovers, cuddle sessions, brunches, and for getting through physical oceanography with me. To all of the student body officers who served with me, thank you so much for always being patient and meeting with me early in the morning to try to make a difference on our community here at MLML. I've never had a lot of friends, being the weird snail girl, but I feel like every student in this community is someone I can call a friend, so thank you.

Of course, thank you so much to everyone who made my time at MLML what it was. Terra, Jane, Michelle, Jocelyn, Kathleen, Kris, Billy, James, Gary, Billy, Nilo, Sean, JD, and Brian, thanks for always being rays of sunshine and answering random questions I had. Similarly, thank you so much to the members of my thesis committee for supporting my research and training me in new skills. Specifically, Amanda Kahn, who always reminded me how exciting any result was and for getting mussels drunk with me, and Tom Connolly for being an amazingly kind and helpful human.

Last, but certainly not least, I could not have made it through this degree without my support system outside of MLML. All of my volunteers at the Exploration Center and the staff friends I've made… you all reminded me why I love science when everything was going wrong. You also made me laugh harder than I ever have and made a work environment that is so fun and safe I enjoy coming every single day. Also, to my community at New Life Nazarene Church who came into my life at a time I desperately needed. And of course, my family. Mom, dad, sister, boyfriend, cat… thank you so much for celebrating with me during the good times and allowing me to call you crying during the bad times. I love you all, thanks for sticking it out with me and always encouraging me.

Introduction

Species distribution is critical information for managers and scientists seeking to protect habitats and populations. For near-shore marine species, typical intertidal or subtidal survey methods can be costly and take a lot of time. Recently, conservation biologists have found environmental DNA (eDNA) useful to track the presence or absence of animals in an ecosystem. eDNA is DNA shed by organisms through waste, body fluids, or cells shed by the organism and can be found in water, air, ice, and soil (Haile et al., 2009; Lydolph et al., 2005; Thomsen and Willerslev, 2015). In brackish waters, scientists have used eDNA to track rare amphibians (Ficetola, et al., 2008; Thomsen et al., 2012). Scientists have also detected blue whales eDNA seawater up to two weeks after they've left an area (Foote et al., 2012). eDNA has been detected from molluscs in the Great Lakes region in a study looking specifically at invasive molluscs (Klymus et al., 2017). These studies highlight the versatility of using eDNA to detect the presence of organisms in a variety of bodies of water.

Molluscan species such as mussels, limpets, and abalone are critical to food webs in California nearshore ecosystems and as seafood dishes. Mussels, such as *Mytilus californianus*, are thriving intertidal fixtures, but some species of limpets (especially *Lottia gigantea*) and all species of abalone (*Haliotis* spp.) are threatened or endangered in California. This study will focus on *Mytilus californianus* (the California blue mussel), *Haliotis rufescens* (the red abalone), and *Lottia scabra* (the rough limpet). Limpets and abalone are important grazers in these nearshore ecosystems, keeping rocky areas clear of algae for recruitment of new organisms, and mussels filter water and serve as food sources for sea stars, including a keystone predator. Populations of these organisms

benefit not only the health of our oceans but also growth for California's economy; fisheries of the west coast of the United States alone produce almost $1 billion annually (NOAA West Coast Fisheries, n.d.). While mussels remain abundant, abalone and some limpet species have been threatened by overfishing and disease since the late 19th century (Roy et al., 2003; Taniguchi et al., 2000). Methods to track their populations through eDNA would be a useful tool for resource managers. However, the meaning of a positive signal of a species through eDNA, or the absence of such a signal, remains difficult to interpret.

DNA is quite stable, but certain physical factors can cause degradation (Allentoft et al., 2012; Dejean et al., 2011; Strickler et al., 2015; Willerslev et al., 2004). UV-B causes degradation of eDNA in water but UV attenuates with depth in water (Strickler et al., 2015). Bacteria and heat likely work together to degrade eDNA, as heat increases bacterial metabolic rate which may influence the rate the bacteria consume the DNA. Under experimental treatments, eDNA from aquatic vertebrates was detectable in water 14 to more than 58 days after removal of animals, though most degradation occurred within the first few days (Dejean et al., 2011; Strickler et al., 2015). The variation in accumulation and degradation rates means that study into these rates in more species and systems is vital to understanding the behavior of eDNA before it can be used for species monitoring.

The present study aims to fill this gap by characterizing behaviors that lead to ambient levels of eDNA, including long term and short term eDNA accumulation, and rate of degradation under normal environmental conditions. I used animals in natural and restrained postures (to maximize exposure of soft tissue) to assess accumulation rates in

the short term (less than 24 hours) and long term (greater than 24 hours). Using purified DNA, I looked at long term rates of DNA degradation in seawater under two different temperature treatments, 3 UV treatments, and in the presence or absence of bacteria. In the end, these data showed that all active animals release eDNA, but rates of eDNA shedding by abalone are substantially higher than by mussels or limpets. Furthermore, eDNA shedding happened at similar rates for each species but was greater at higher temperature and bacteria loads, and at higher UV intensity.

Research questions and hypotheses:

Question 1: How does eDNA production vary in three molluscan species based on body size?

Hypothesis 1.1: *Higher wet weight will lead to greater eDNA release within a species.*

Null hypothesis: Wet weight will not influence eDNA release within a species.

Hypothesis 1.2: The relationship between body size and amount of eDNA released will differ for each species.

Null hypothesis: The relationship between body size and the amount of eDNA released will not differ for each species.

Hypothesis 1.3: *Activity or posture will increase the amount of eDNA each individual releases.*

Null hypothesis: Activity level will not change the amount of eDNA each individual releases.

Question 2: How does eDNA degradation vary based on UV, heat, and bacterial activity?

Hypothesis 2.1: eDNA accumulated in the water from live organisms will decrease over time with greater UV, heat, and bacterial level.

Null hypothesis: eDNA accumulated in the water from live organisms will not change over time under different conditions of UV, heat, and bacteria.

Methodology:

Animal care and selection

All of the abalone used for this project were generously donated or purchased from Monterey Abalone Company. Mussels and limpets were collected on the Moss Landing Jetty (36.8078°, -121.7897°), Pigeon Point Lighthouse (37.1829°, -122.3944°), and Pleasure Point (36.9557°, -121.9717°). Using swabs of foot or body wall, I confirmed performance of molecular methods for each individual in my experiments by using the polymerase chain reaction (PCR) to amplify DNA using custom species-specific primers designed to avoid co-amplification of non-target DNA (Table 1). Each species amplified well only with the correct primer set through PCR, thus all animals in the study were able to be detected by these molecular techniques and non-specific amplification was avoided. Length, width, wet weight, and pedal area or gape angle were measured for each individual at the beginning of the experiment (Appendix A Tables 1a-1c) to sort the animals into different size classes. Pedal area was measured by allowing the abalone and limpets to relax on a piece of glass with a clear centimeter squared grid attached (Appendix A Figure 1). Pictures were taken from below and analyzed in ImageJ by comparing the amount of pixels within a square centimeter to a freehand drawn outline of the molluscan foot (Appendix A Figure 1). Similarly, to measure aperture, mussels were left in an aquarium tank until they opened; photographs of the openings were measured in ImageJ (Appendix A Figure 2).

Abalone, limpets, and mussels were kept alive in 56 L tanks with aerated, non-recirculating seawater at the Moss Landing Marine Laboratories. The tanks were cleaned of algal growth and waste weekly. Rocks with microalgae were included to feed the limpets, while abalone were fed thawed frozen *Macrocystis pyrifera*. Mussels were

sustained by natural particulates in the seawater system. A seawater system failure prior to experiments led to the death of some individuals, thus the study organisms consisted of survivors, potentially a nonrandom draw from the natural population but not biased across treatments.

During experiments, animals were placed in 473 mL (pint) sized jars with unfiltered seawater covered with Parafilm or metal lids. No water flowed through but a bubbler was kept in each jar. Animals were only isolated for a maximum of six days, at which point some animals became moribund and the experiments were ended. During these experiments animals were not fed. Jars were kept in a seawater table to regulate temperature between 11-13 ^{o}C.

Primer design and testing

Using the IDT PrimerQuest tool (https://www.idtdna.com/Primerquest/Home/Index) and twenty reference sequences per species acquired from GenBank (Appendix A Table 2), I designed three sets of forward and reverse primers for the polymerase chain reaction (PCR) for each study species. These primers were tested on DNA extracted from each species using the Qiagen DNeasy Blood and Tissue Kit (© Qiagen 2013-2018). I used a gradient PCR with annealing temperatures ranging from 45-65 ^{o}C, melting and extension temperatures of 94 and 72 respectively, and 27 cycles. Products were checked on a 3% MetaPhor agarose gel (Lonza) with a Fisher Ultra Low Range DNA Ladder to check for the predicted amplicon sizes. The optimal temperature was 56° C for each of the primer sets. Each primer set was designed to be species-specific and did not amplify other organisms

in this study. One set of primers with the clearest, brightest band on the MetaPhor gel was chosen for the rest of the experiments (Table 1).

Table 1: Primers used for this study.

PRIMER NAME	PRIMER SEQUENCE	ANNEALING TEMPERATURE
ABQPCRF1	CATCCTTAACCCTGCTCCTAAC	56 °C
ABQPCRR1	GCTAAGTCTACTGATGCTCCTG	56 °C
MUSQPCRF1	GGATGGACTATTTATCCACCTCTATC	56 °C
MUSQPCRR1	GAGAGCTAAGTCCTGCTAAGTG	56 °C
LIMQPCRF3	CCCTTCTTGTTGCCGCTATT	56 °C
LIMQPCRR3	GGTCGAAGAAGGCTGTGTTAAT	56 °C

DNA extraction

Following homogenization of the water sample by swirling or inverting a closed container three times, a fixed volume of water was filtered through a GF/F 0.7 μm, 25 mm diameter glass fiber filter (Deiner et al., 2015). Filters were then added to a 2 mL centrifuge tube submerged in the first two reagents from the Qiagen DNeasy blood and tissue kit (© Qiagen 2013-2020) and placed in a 56 ºC shaking incubator for 24 hours. I followed the manufacturer's recommendations during the following steps for DNA extraction except I decreased elution volume from their recommendation of 200 μL down to 80 μL unless otherwise stated. For every set of extractions I also did a blank extraction control to test for contamination of the extraction kit.

qPCR

qPCR was done using the StepOnePlus ™ Real-Time PCR System (ThermoFisher) and KAPA SYBR® FAST Universal 2X qPCR Master Mix (™ Life Technologies). Each reaction contained 7.8 μL of water, 10 μL of master mix, 1 μL of

template DNA, and 0.4 μL (10 μM) each of forward and reverse primers and High ROX. After heating to 95 °C for twenty seconds, each well was subjected to 40 cycles of 95 °C for three seconds to melt templates, 56 °C for twenty seconds to anneal primers, and 72 °C for thirty seconds to extend primers, with fluorescence read during the extension phase. After the 40 cycles completed, a melting curve was produced, heating in increments of 0.3 °C from 60 °C to 95 °C with fluorescence read at each increment.

Between fifteen and eighteen wells in the qPCR reaction plate were DNA standards used to make a standard curve relating fluorescence and starting template DNA concentration. DNA standards were made from genomic DNA that was quantified using QuantIT PicoGreen and diluted ten-fold 5-6 times in nuclease free water. To ensure precision, each of these 5 or 6 dilutions were repeated in the plate three times. One no-template control was also included in each plate. The rest of the wells contained eDNA extraction product with unknown DNA concentrations.

Analysis of the data generated from the qPCR run included the following quality controls: ensuring that each of the unknown samples conformed well to the standard curves, that negative controls from extractions and PCR showed no sign of DNA, and that the melting curve for each individual of the same species were similar. DNA concentration data was imported into R for statistical analysis.

24 hour eDNA accumulation assay

Individuals of each species were divided up into three size classes: small, medium, and large (Table 2) based on length and were separated into bleach-cleaned mason jars. Each jar contained one individual and was filled to the brim with water from the Moss Landing Marine Laboratories seawater pump, then covered in Parafilm. Four

organisms of each size class were used, for a total of 12 samples. A bleached air bubbler was connected to an air line and placed into each mason jar to ensure that the environment did not become hypoxic. The jars were kept in the aquarium room in a bath of non-recirculating seawater, keeping them near ambient ocean temperatures throughout the experiment. The time that the animal was placed into a jar filled with water was recorded. A 15 mL sample was taken after the 24 h time period by filtering the water through a GF/F filter.

To test if activity or posture affected eDNA release, medium sized organisms were placed into a glass beaker with 60 mL of water. A 15 mL sample of seawater was taken after 2 h of unconstrained behavior (apart from being in a jar) during which their behavior was videotaped continuously and used to track animal behavior. For the experimental portion of the experiment, fourteen mussels were slightly propped open with a 32 mm x 1.5 mm diameter stainless steel rod between their valves to prevent closure and placed in separate 200 mL glass beakers. Fourteen limpets were placed upside down to encourage foot and head motion, and they were unable to right themselves. Fourteen abalone were also placed upside down, using 1.5 cm diameter Velcro circles that were glued near the apex of their shell, on the bottom of a 200 mL glass beaker containing 60 mL of seawater. The same animals were left for 2 h while being videotaped. To analyze the differences between individuals acting normally and individuals in a different behavioral state, I used a paired t-test on the DNA concentrations generated from qPCR.

Table 2: Size classifications for each species.

	SMALL	MEDIUM	LARGE
LIMPET	0.9-1.2 cm	1.3-1.5 cm	1.7-2.1 cm
MUSSEL	1.7-2.2 cm	2.6-3.2 cm	4.4-5.1 cm
ABALONE	1.3-1.7 cm	2.2-2.5 cm	4-4.4 cm

Degradation assay

DNA was extracted as described above from foot tissue of four limpets, abalone, and mussels and pooled to create a stock solution of DNA that could be equally aliquoted into the treatment plates. The extraction method was the same as others in this study, but I added 200 uL of elution buffer at the final step to increase the volume and yield of DNA extracted. I used qPCR, using methods and standards described above, to confirm that DNA concentrations were detectable in these stocks. qPCR was chosen over other methods to quantify DNA because I had extra wells in qPCR plates during quantification of activity study eDNA. To each of the wells on a 6-cell well plate I added 10 mL of seawater and sufficient DNA to achieve a starting concentration of about 6 ng/uL. I then took an initial DNA concentration reading by immediately processing one replicate.

For this experiment, change over time in DNA concentration was measured in seawater from the MLML seawater system that passes through a sand filter, and from seawater that was passed through a 0.4 μm filter to remove particles and then autoclaved to kill any bacteria. I added 10,000 units of penicillin (ThermoFisher #15140122) to 500 mL for a concentration of 20 units/mL to the filtered and autoclaved seawater to keep bacterial growth at bay. Both of these water treatments were aliquoted into 17 mL culture wells before the stock DNA was added.

Three levels of UV light and two temperatures were used to expose the plates to conditions mimicking a natural environment. Maximum tide pool temperatures in Santa Cruz, CA, near to where limpets and mussels were collected can exceed 20 °C (Leong et al., 2018). The culture well plates were placed in cold (10 °C) and room temperature (~20 °C) rooms. UV-B in the Northern Hemisphere ranges from 0-250 mW/m^2 and reaches its peak near middle of summer (Kerr et al., 1994). Three UV levels were achieved by placing a UV lamp (REPTI ZOO Reptile Full Spectrum UVA + UVB Sun Lamp, 100 W) above the cell wells, which were arranged on vertically spaced shelves to experience 60 mW/m^2 and 24 mW/m^2 UV-B. A zero detectable UV treatment was achieved by placing the plates in a double lined cardboard box . After 0.25, 0.5, 0.75, 1, 2, 3, 4, 7, 14 and 21 days, 10 mL of water was removed and immediately filtered as described above. The filter was then extracted following the extraction method above.

To see what effect heat, UV, bacterial level, and elapsed time have on eDNA degradation, I used a Randomized Block ANOVA after collecting the data and comparing the AIC values for multiple models. The data were blocked by temperature, UV, and bacterial treatment in the analysis. The randomized block ANOVA is ideal for data where each block undergoes the same multiple treatments. This design decreases the amount of experimental error by looking at the differences between the blocks and is ideal for data that are linearly related in some way.

Flow cytometry

To measure the filter feeding clearance rate of the mussels for these experiments, I used a culture of *Porphyridium* spp., a single-celled red algae, along with flow cytometry to measure the change in cell concentration over 24 hr. I measured cell

concentration at the beginning of the study and put 1 mL of the culture into 12 jars containing the same mussels from the 24 hour accumulation study and one jar with no animals present to measure the change in cell concentration 24 hours in the absence of grazing. Jars were kept in the aquarium room at the ambient temperature of the seawater system (11-13^{o} C), each with a bubbler placed into it to keep cells suspended and oxygen levels up. Jars experienced some natural light due to the small windows to this room and some brief moments of artificial light, but this was consistent across treatments. 8 mL samples of water were taken from each of the jars after 24 hours and analyzed again using flow cytometry. Any cell growth in the control jar was subtracted from the final clearance rate.

I also used flow cytometry to analyze the amount of bacterial growth, if any, during the degradation study. One mL of abalone DNA- treated water from each degradation plate was preserved in 0.5% formaldehyde, then stored them in the refrigerator until the samples could be analyzed at the same time. For the analysis, I used an Attune acoustic focusing cytometer from Applied Biosystems. The entire milliliter sample was used and placed into an individual cuvette along with 2 µL of 100X SYBR Green. Samples were left to incubate on the counter for 10 minutes before 100 µL of each sample was run through the flow cytometer at a rate of 25 µL per minute. We also ran a negative control of MilliQ water. The flow cytometer was first calibrated with 1 µm beads, which helped set the x axis gate for particle size. The y axis gate selects for nucleic acid containing particles. The gates were consistent across samples.

Modeling

To assess how eDNA might spread in the ocean, I created a model in R using the package Plotly (Sievert, 2018). The overall goal of the model is to see how far eDNA might be able to travel over a 24-hour period. In the model, an animal is placed on a pier piling and sheds eDNA at a rate taken from experimental values in this study. The model represents ocean movement in the winter, when waves are larger, and in the summer, when waves are much calmer. The wave data came from USGS wave databases and represent maximum wavelength and height. These data were used to calculate the Stokes drift, or average shoreward wave particle velocity from Denny (1988)

$$u = \left\{\frac{\pi H^2}{4L}\right\}\left\{\frac{\cosh\,(2ks)}{sinh^2(kd)}\right\}\omega$$

where H is maximum wave height, L is maximum wavelength, k is the wavenumber (related to the wavelength), s is the position in the water column, d is the bottom depth, and ω is the angular frequency. Stokes drift has been shown to be a major contributor to onshore movement of particles in the nearshore (Monismith and Fong, 2004). Tides are modeled as a sine wave with the same period as semidiurnal tides. The model includes a factor for simple diffusion by creating a random number, representing the "random walk" idea of diffusion (Figure 1). The random number was a combination of two randomly generated numbers multiplied by each other; the first was a random number between 0 and 1, the second was a random integer between -1 and 1. Under these conditions, the particle could move up to one meter up or down in an hour. Lastly, eDNA is shed at a constant rate, but disappears over time due to degradation by heat, bacteria, and UV.

The data, code, and implications of this model can be found in various locations throughout this document. The Stokes drift calculation, a major driver in the movement

of particles towards shore, is presented in the results. Additional factors that may move particles toward or away from the shore but are not included in the model, such as upwelling and tidal excursion, can be found in the discussion. Lastly, the code to run the model with stokes drift and diffusivity can be found in the appendix (Appendix B).

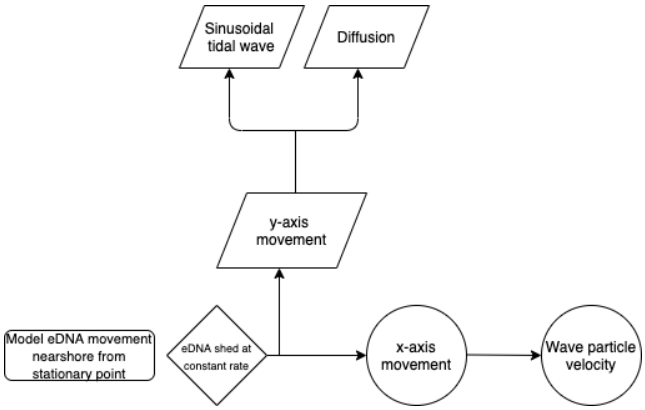

Figure 1: a simplified diagram of how different oceanic forces affect eDNA movement in the water column for use in this code.

Results

1.1 and 1.2 Animal size or weight and eDNA released (accumulation study)

Larger animals generated more eDNA than small animals when normalized to wet weight of each animal. I found significant differences between small and large individuals in the amount of eDNA released (Two-Way ANOVA F=7.414, Tukey HSD, p=0.00274) and medium and large individuals (Two-Way ANOVA, Tukey HSD, p=0.0179) over a 24 hour period. In general, abalone generated more eDNA than limpets or mussels (Figure 2). There was a significant difference of eDNA exuded between *Haliotis sp.* and *Lottia sp.* when normalized by wet weight (Two-Way ANOVA F=9.675, Tukey HSD, p=0.000396) and *Mytilus* sp. and *Haliotis* sp. (Two-Way ANOVA, Tukey HSD, p=0.0255) but not between *Mytilus* and *Lottia* (Figure 3). Values for measured eDNA concentrations can be found in Table 3.

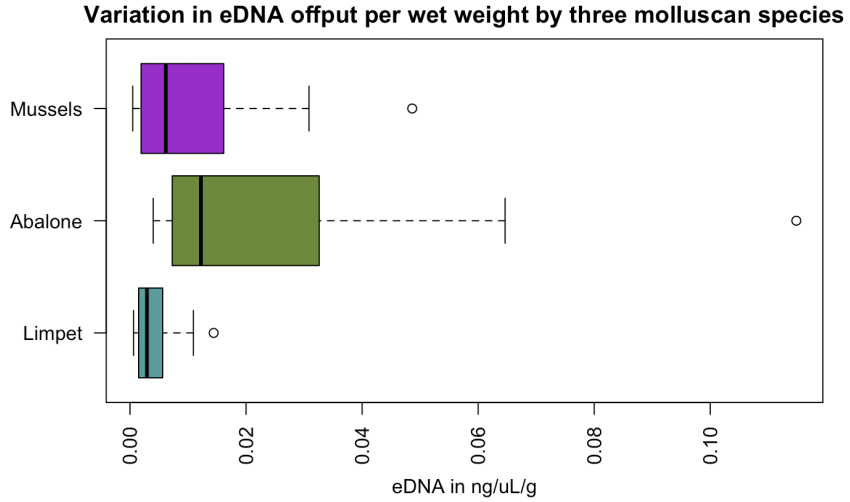

Figure 2: eDNA shed by mussels (*Mytilus californianus*), abalone (*Haliotis rufescens*), and limpets (*Lottia scabra*) normalized by wet weight. The bar is the median and the whiskers represent the lower and upper quartiles. Open circles represent outliers.

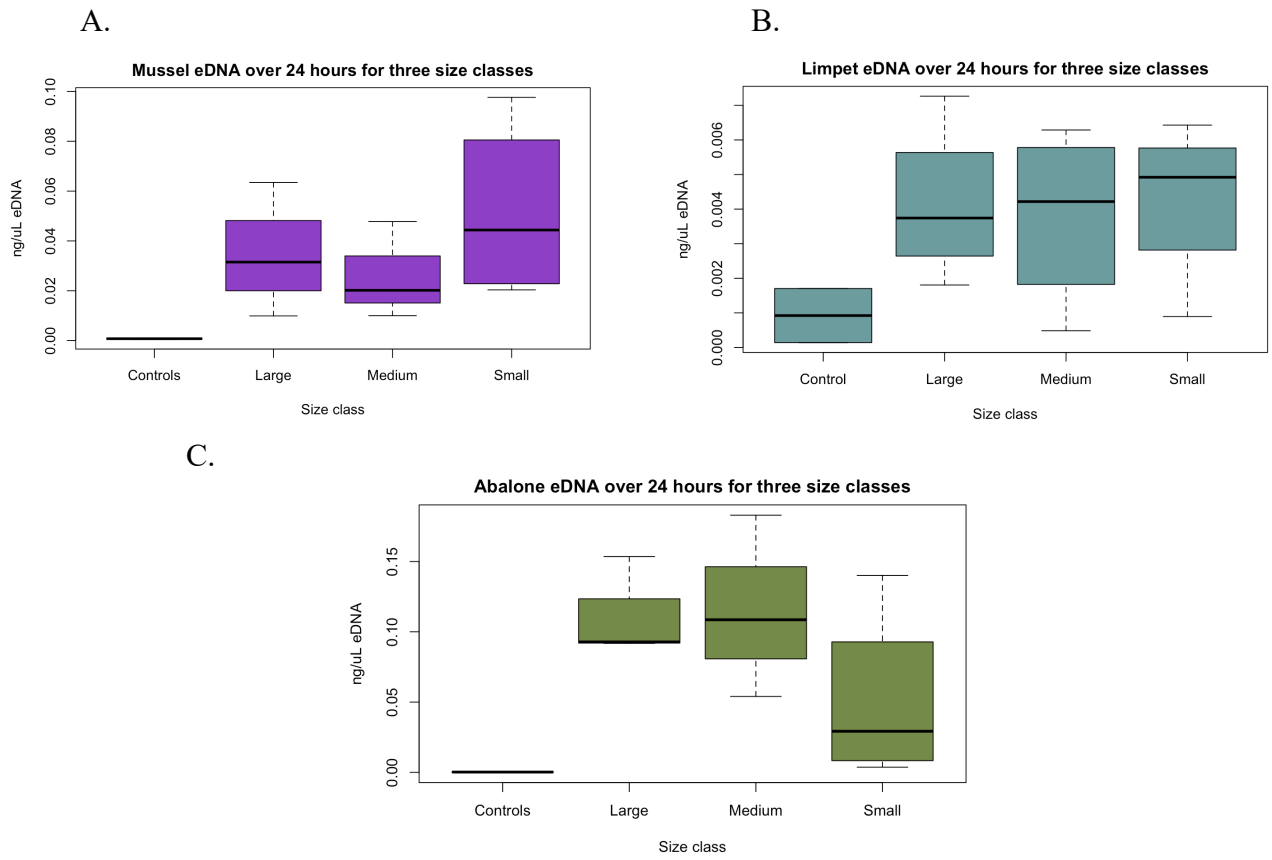

Figure 3: Boxplots showing eDNA released by each organism over 24 hours grouped by their size classes. Mussels (*Mytilus californianus*), (a) do not release eDNA based on size, nor do limpets (*Lottia scabra*) (b). Small abalone, (*Haliotis rufescens*), put off less eDNA than medium and large abalone (c)

Table 3. Average eDNA emanated in 24 hours with standard deviation in ng/μL

	Abalone	***Limpet***	***Mussel***
Small	*0.0855*±0.05	*0.0043*±0.004	*0.0517*±0.04
Medium	*0.1208*±0.05	*0.0038*±0.003	*0.0259*±0.02
Large	*0.1666*±0.2	*0.0053*±0.003	*0.0341*±0.02

1.3 Activity level and eDNA release

Some animals escaped treatment by removing aperture wedges (mussels) or breaking free and righting themselves (abalone). Specifically, four mussels escaped

treatment but three of them lasted at least half the time, four abalone escaped and two of them lasted at least half the time, and no limpets escaped treatment. Abalone with exposed tissue excreted more eDNA than normally situated abalone (Paired T-Test, t=-1.8137, p=0.04643) (Figure 4). This pattern did not hold for *L. scabra* or *M. californianus*; both species released very little eDNA during this period (Figure 5). Despite size differences, all mussels cleared the same amount of water over the 24-hour time period (Appendix A Figure 3)

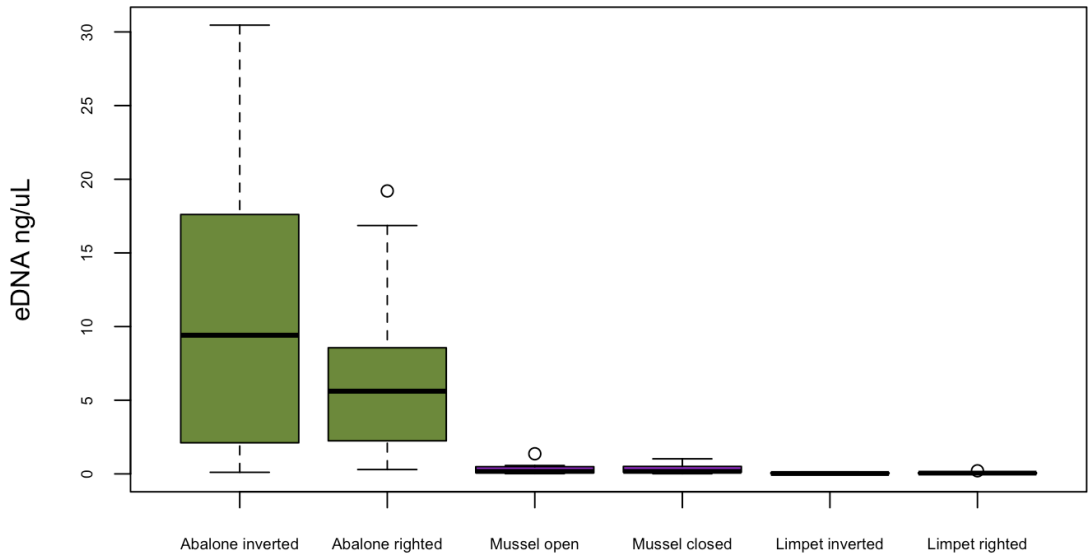

Figure 4: Boxplot showing the amount of eDNA shed from inverted and righted individuals of each species. The y-axis shows eDNA shed in ng/µL. As these were paired data and the statistics were based on the comparison between one individual in different treatments, I did not normalize these data to wet weight. The bar is the median and the whiskers represent the lower and upper quartiles. Open circles represent outliers. Bracketed bars with asterisk indicates a significant difference.

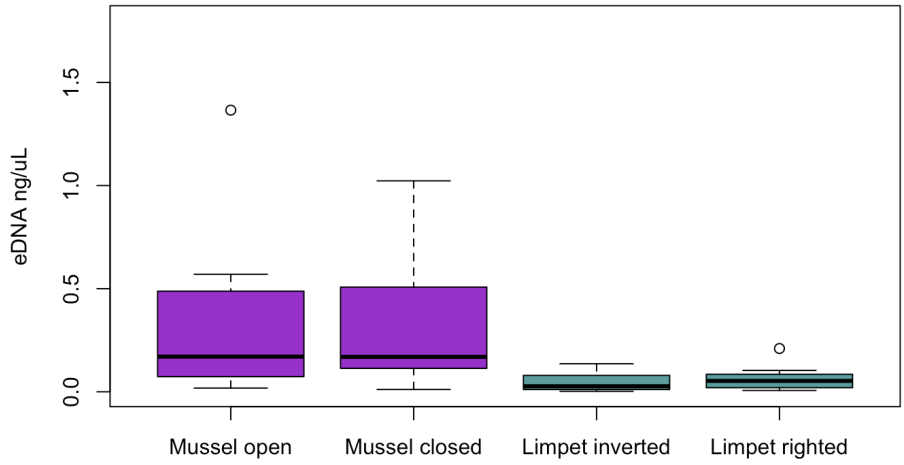

Figure 5: The same graph as above, zoomed in on the mussel and limpet data to show the differences on a smaller scale.

2.1 eDNA degradation rates

This study quantified the degradation of spiked eDNA over time in sea water under various environmental treatments. eDNA concentrations decreased over time, especially in the first 24 hours in each of the treatments, though the factors that influenced this degradation varied from species to species (Figure 6). For the first 48 hours when the most degradation occurred, elapsed time affected mussel eDNA (Randomized Block ANOVA $F_{1, 66}$=9.209, p=0.00344) (Appendix A Table 6) and limpet eDNA (Randomized Block ANOVA $F_{1, 81}$=12.468, p=0.000686) (Appendix A Table 7). For abalone eDNA, bacterial activity (Randomized Block ANOVA $F_{1, 81}$=5.111, p=0.02645) (Appendix A table 5) and elapsed time (Randomized Block ANOVA $F_{1, 81}$=7.343, p=0.00821) affected the rate of degradation. According to the flow cytometric measurements, the antibiotic, autoclaved, filtered seawater started at bacterial concentrations around 12,000 ppm. Aquarium room seawater had concentrations of

about 50,000 ppm. By the end of the experiments, treated and ambient seawater had risen to about 20,000 and about 70,000 respectively.

Figure 6a Abalone

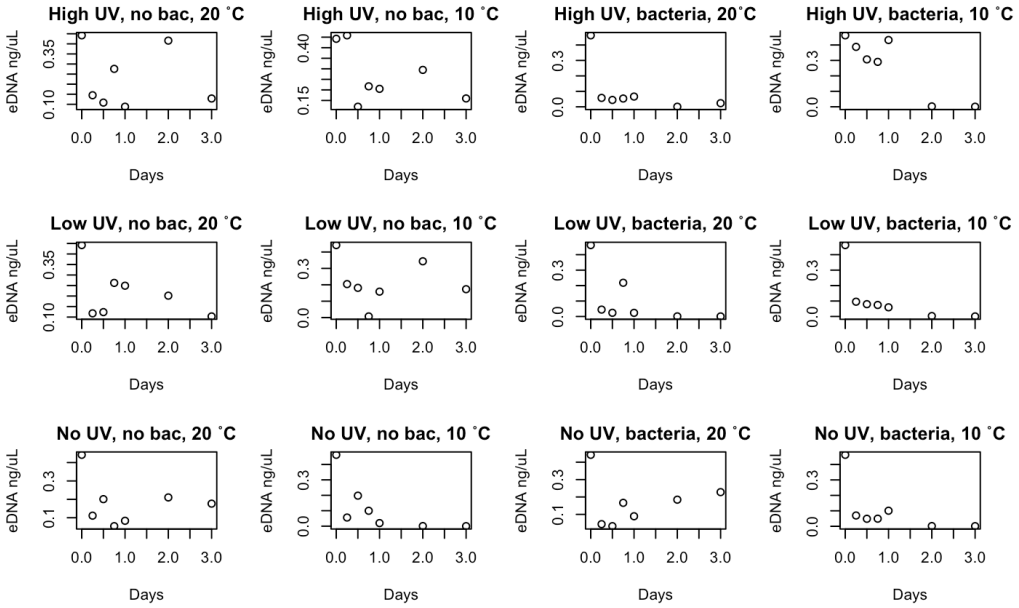

Figure 6b Mussel

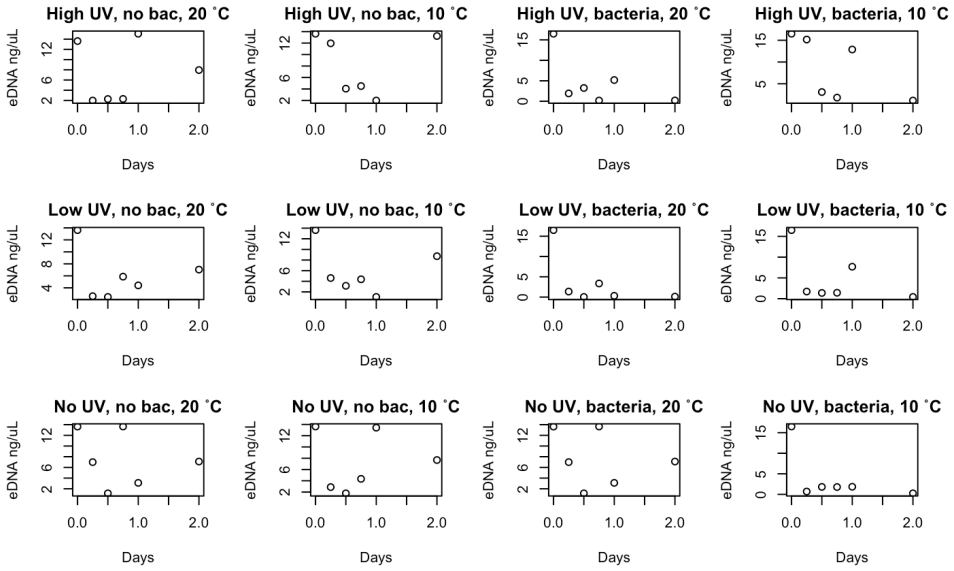

Figure 6c Limpet

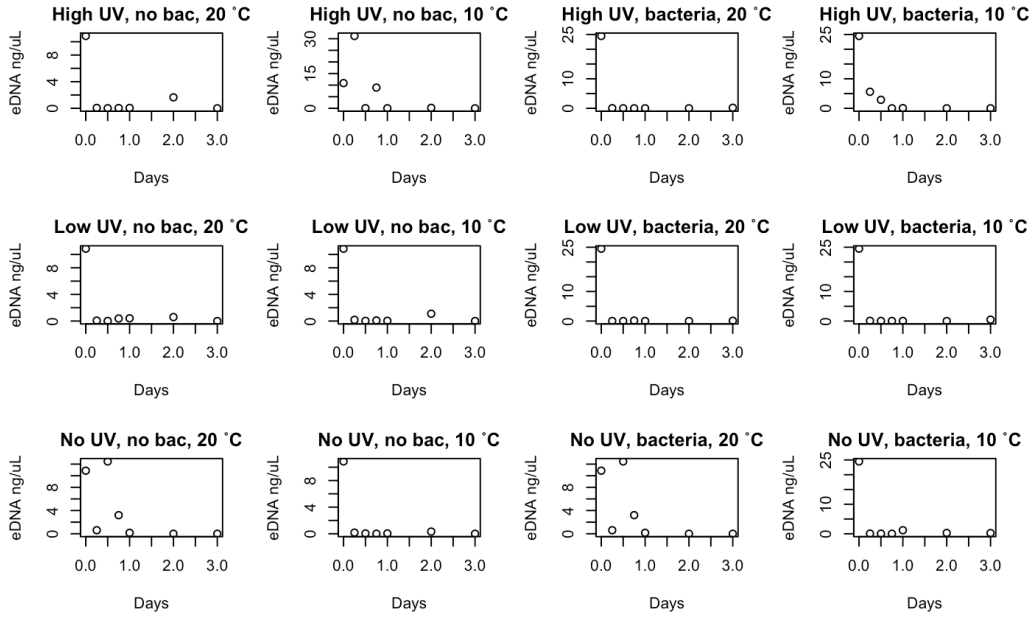

Figure 6 a, b, c: Degradation plots for each of the study species. Each graph represents the remaining DNA since the initial addition at day 0. In the title of each graph, you can see the UV treatment, bacterial conditions, and temperature. In general, the top row graphs are all high UV, middle is low UV, bottom is no UV. The right two columns are samples with reduced bacteria, the left two are samples with ambient bacteria. The first and third column are samples stored at a warmer temperature, second and fourth were stored at the cooler temperature.

To put these values into context, I calculated the half-life of the eDNA based on the concentrations at time zero and day 1 across all treatments (Table 4). Half of the abalone and mussel eDNA degraded twelve hours after addition, whereas limpet DNA degraded more rapidly.

Table 4. Average eDNA half-life for each species

	Abalone	**Limpet**	**Mussel**
Half-life (hours)	**12.77**	**3.577**	**16.91**

Following each run of the qPCR machine, a melting curve is generated by measuring the temperature at which the PCR product melts on a range from 65 °C to 95 °C (Figure 7). The melt curve can be used to compare length and sequence of PCR product without sequencing or running a gel. Melt curves from qPCR showed an

increase in variation of melting temperatures in more degraded samples, suggesting amplification of non-target fragments (Figure 8).

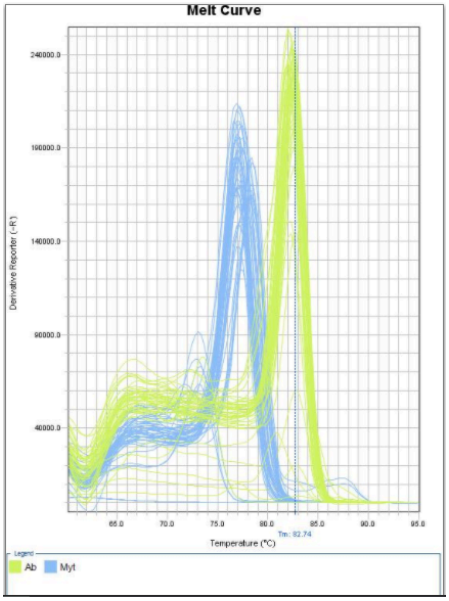

Figure 7: A typical melt curve for two targets, green is PCR product from the abalone *Haliotis rufescens*, blue is PCR product from the mussel *Mytilus californianus*. Single major peaks for each species indicate that the majority of PCR product is a single target (COI).

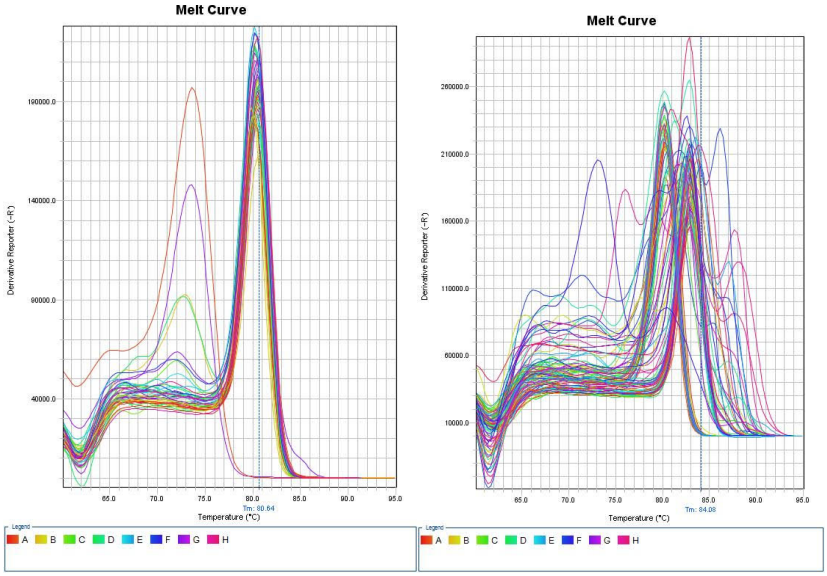

Figure 8: Two melt curves from the degradation experiments of abalone (*Haliotis rufescens*) eDNA. Left are qPCR melt curve results from day zero through day three, right are melt curve results from day 4 through 21. Different colors represent the row that each sample was measured in on the 96 well qPCR plate (A-H). Peaks at melt temperatures higher or lower than the expected temperature (~80 °C) indicate accumulation of non-specific products.

Modeling:

The basic model generated here suggests that eDNA can travel away from the point of origin quickly, even in the summertime when waves are smaller. Velocities were calculated based on USGS significant wave heights (Table 3) at a region just south of Moss Landing (36.7636 º, -121.8173 º), where some of the largest waves are observed within Monterey Bay (Erikson et al., 2014). in the winter and summer, eDNA could move up to 0.377 m/s towards shore in the winter and up to 0.065 m/s in the summer. This five-fold difference in speed can be attributed to the larger waves and longer wavelength in the winter. This means that eDNA particle transport in the ocean could be up to 234 m/h in the summer and up to 1357.2 m/h in the winter towards shore. Thus, an animal's eDNA could be detected hundreds if not thousands of meters away from where the animal actually is, even if the eDNA degrades quickly. Diffusion and tides also affected particle movement, though based on this model, Stokes drift is the main contributor to shoreward movement.

Table 5: Variables to calculate towards shore wave velocity

	Maximum Wave Height (H)	**Maximum Wavelength (L)**	**Period (T)**
Summer	1.92 m	99.801 m	10.7 s
Winter	5.05 m	150.32 m	15.6 s

Discussion

eDNA analyzed with molecular techniques is becoming an increasingly popular way to detect species in the environment. However, detection of eDNA may say little about the abundance or proximity of individuals of a species, depending on its rate of production, persistence, and dispersion. This study was intended to investigate these issues for three molluscan species. Abalone were found to release eDNA at a rate consistent with body size and posture. Smaller abalone exuded less eDNA than medium and large sized abalone. Further, abalone forced to expose their soft tissue released more eDNA than abalone in a relaxed, normal posture. These results were not consistent for limpets and mussels; size and posture had no effect on eDNA release. eDNA rates of production vary between species and these rates should be assessed for other species before attempts to quantify abundance or proximity in the field.

eDNA persistence was measured under treatments of three UV levels, two temperatures, and two bacterial treatments. Elapsed time affected eDNA breakdown for nucleic acids from all species and most of that degradation occurred in the first 24-hours. Bacterial activity affected degradation of abalone eDNA, but none of the other treatments had a statistical influence on eDNA persistence for abalone, limpets, or mussels. Variation in measured eDNA concentrations from degraded eDNA and experimental error added noise to the analysis of these data so refined methods and more replicates in future studies may find more of an effect from UV and heat on eDNA degradation.

eDNA accumulation over 24 hours

The purpose of this experiment was to address whether wet weight of an animal affects the amount of eDNA they release. I found that size class affected how much eDNA each species emanated with large animals exuding the most eDNA. Between species, I found that abalone exuded more eDNA than limpets and mussels, but mussels and limpets exuded a similar amount of eDNA. Few other studies have looked into specific eDNA accumulation rates, but one study found that over a 1-2 day period, 3 *Cyprinus carpio* (carp) put off more eDNA than one fish (Takahara et al., 2012). This suggests that for some species, biomass is correlated to the amount of eDNA released, even amongst higher organisms such as vertebrates, though it seems to vary between species and should be measured for each target.

Movement of water for ventilation or filter feeding may play roles in some of these differences, as well as physical differences between species. Physical differences, such as associated microbes, activity level, body geometry, amount of exposed tissue or another inter-species mechanism likely drive this pattern and were investigated in this study. Careful measurements of mussel aperture and abalone and limpet pedal area showed that although mussels have a lot of surface area internally, they do not open very wide and are not exposed all the time (Appendix A Tables 1a-1c). Limpets and abalone had comparable pedal area to weight ratios but in most cases I noticed the abalone traveled further during the 24-hour period than the limpet, though these observations are anecdotal as no cameras were set up to measure distance moved. Metabolic rate between species could affect the amount of eDNA released and further studies should quantify the metabolic rates of study organisms (Takahara et al., 2012). Finally, DNA in feces might

be affected by the length of alimentary tract, rate of cells sloughing into the tract, and rate of fecal production (Klymus et al., 2015; Turner et al., 2015).

eDNA accumulation with activity

To test how activity levels might affect eDNA emanation rates, I subjected experimental animals to conditions that forced their soft tissue to be exposed such as keeping them inverted (abalone and limpets) or keeping their valves open (mussels). I found that abalone forced to expose their soft tissue released more eDNA than righted abalone, but this pattern was not true for limpets and mussels. Although this experimental design forced activity through the exposure of soft tissue, animals did move a lot during the tests while inverted or propped open. I attempted other treatments to cause animals to be hyperactive such as addition of a predator or food, but small predators were ineffective, and algae inhibited DNA extractions.

Using a paired t-test, I found that abalone exude more eDNA when "active," but mussels and limpets exuded the same amount of eDNA whether active or passive. The intention was for the wedge to increase time that mussels spent ventilating, but instead may have created an unnatural stress that actually reduced respiration and feeding rates, therefore leading to no difference in eDNA exudation between the treatments. For example, mussels have been shown to change growth rates and reproduction rates quickly during the stress of field transplantation, meaning they have a quick response to abnormal situations and may have changed metabolic processes during this stressful experiment (Petes et al., 2007).

Limpets appeared passive and unable to ever right themselves and would die within a few days if left prone on their shells in the tanks during the experimental

period. Abalone readily righted themselves if unrestrained, and during this experiment made attempts to flip over despite being attached with Velcro. This difference in activity level between limpets and abalone may have led to the difference in amount of eDNA shed seen in this experiment. Tests of metabolic rates for each species may inform future eDNA studies of these species.

eDNA degradation

I treated 10 mL water samples containing DNA from the three species to three levels of UV, two concentrations of bacteria, and two temperatures for a maximum of 21 days to see how quickly the eDNA would degrade. Most of the degradation occurred in the first 24 hr. DNA was detectable by PCR up to 21 days after the addition of DNA, but those full data are not presented here because unexplained variation between qPCR trials and samples complicated analysis of results. Variability in measured eDNA concentrations increased with more degraded samples (Appendix A Figure 10). 77 samples and 18 standards were arrayed on a single 96-well qPCR plate; therefore, two runs were necessary for the entire experiment. In the second qPCR run, the DNA concentrations jumped to near starting concentrations. Either randomizing time points across different runs of the qPCR, or utilizing a higher-capacity instrument, would help manage such experimental error.

Assumptions in these experiments were that SYBR green qPCR reactions were specific to the COI gene. Validation experiments showed that PCR primers were specific to the intended target, and that PCR reactions were relatively free of artifacts such as primer-dimer that can contribute to the total SYBR green-stained double stranded DNA. However, the melt curves suggest that as time goes on in the degradation study, non-

target double stranded DNA is produced. Unexpected spikes in measured DNA concentrations may be due to primer dimerization or a concatenation of partial PCR products. Use of SYBR green qPCR may yield inaccurate estimates of eDNA concentrations unless extraneous fluorescence can be subtracted. Probe-based qPCR (e.,g., Taqman) might be a better tool to measure amplifiable eDNA in the presence of degradation products.

Elapsed time since DNA addition affected limpet and mussel eDNA degradation, while both elapsed time and bacterial count affected abalone DNA degradation rate. UV did not seem to have an impact on eDNA degradation over the 21-day period. This may be because the range of UV in this experiment ranged from 60 mW/m^2 to 0 mW/m^2 but natural average summer Northern hemisphere maximum UV reaches as high as 250 mW/m^2 (Kerr et al., 1994). Future study should use a larger range of UV treatments during degradation experiments.

Heat alone at the levels in this study would not be enough to completely degrade DNA, because DNA is stable at temperatures far above and below that used in this experiment. The literature suggests that heat acts to degrade eDNA by increasing the metabolic rate of bacteria and enzyme activity that would break the DNA down (Dejean, et al., 2011; Strickler, et al., 2015). Bluegill sunfish and Idaho giant salamander exhibited similar degradation rates to these molluscs, where 90% of eDNA was degraded in about 24 hours with no stressors or natural light levels, respectively (Maruyama et al., 2014; Pilliod et al., 2014). American bullfrogs and European flounder eDNA reached 90% degradation after about seven days under light and temperature treatments, suggesting great variation in eDNA degradation rates for chordates (Strickler et al., 2015;

Thomsen et al., 2012). More species-specific studies of eDNA degradation are imperative to understanding variation and causation of differences in eDNA breakdown rates. Composition of eDNA may also affect degradation, free DNA or eDNA in feces may degrade faster due to lack of protection and/or bacterial level, though further study is needed to confirm or deny this.

Modeling

In order to show how eDNA might spread in a nearshore ecosystem, I designed a movement model in R where eDNA concentrations and metadata can be added. With my calculations of diffusivity, Stokes drift, and other factors, I was able to show that eDNA can travel far from its point of origin and still be detectable. The model showed that eDNA emanated from an animal moves quickly towards shore and may accumulate in very shallow areas. Depending on how close the animal in question is to the shore, the swash zone or beach area may be an excellent place to sample for eDNA as it likely accumulates there. Furthermore, it must be noted that because eDNA can travel over 1000 m in an hour, it may be difficult to pinpoint the exact location of an animal based on eDNA, especially on days with larger waves. However, these values represent maximum wave heights during summer and winter, there may be days in the summer with small waves, where other forces control the velocity. For example, upwelling winds generated in the spring and summer lead to surface water being pulled offshore, which may lead to eDNA traveling away from the shore. Data from Monterey Bay suggest that upwelling may move water up to 2 cm/s or up to 72 m in one hour (Drake et al., 2005). Though offshore transport may be of smaller magnitude than potential onshore transport, wind direction should be considered when predicting eDNA position. Tidal excursion may

also impact the toward shore movement of particles, moving a maximum of 1.7 km in a tidal period, though tidal currents move in an ellipse, so much of this distance is alongshore movement (Petruncio, 1993). This value was calculated with a tidal amplitude of 12 cm/s, an expected value for tidal amplitude from the area I calculated Stokes drift for (Peteruncio, 1993).

The half-life of eDNA will also influence how far away from the organism the eDNA will be detectable. Calculated half-life values are comparable to similar studies with carp, where eDNA half-life was calculated to be 6.31 h (Maruyama et al., 2014). As the particles move, they also become more dispersed in the water column. Half-life may represent a feasible radius of detection of an organism due to how the particles would dilute and degrade, though lower limits to detection using qPCR should be considered. For limpets and assuming no losses during extraction, the low half-life calculated in this study would lead to a potential range of detection up to 835 m in the summer and 4845 m in the winter, calculated based on modeled toward shore velocities. Mussels and abalone eDNA have a longer half-life, leading to a potential toward shore detection range of 2960 m in the summer and 22.96 km in the winter for mussels, and 2988 m in the summer and 17.33 km in the winter. Other eDNA studies have suggested that dilution can decrease detectability of eDNA even 25 m away from the target in stream ecosystems, where between 56% and 83% of PCR samples couldn't detect freshwater pearl mussels (Stoeckle et al., 2015). These limits to detection should be studied in the natural environment due to the complexity of fluid dynamics and variability of eDNA concentration measurements.

Diffusivity or the diffusion coefficient was an important calculation in my model to help particles move up and down in the water column. During the half-life of the eDNA, particles may have moved an average of 3.03 m for abalone, 3.49 m for mussels, and 1.61 m for limpet up or down in the water column. Calculated diffusivities in the ocean range from about $3x10^{-4}$ m^2/s near to surface down to about $1x10^{-4}$ m^2/s in the deep ocean (Arzel and de Vediere, 2016, Cronin et al., 2015). The average calculated diffusivity for my model was near $2x10^{-4}$ m^2/s, putting it at a similar magnitude to calculated values in the ocean.

Had my results suggested that UV or heat affect eDNA degradation, these factors could have been added to the model. Future study should include these factors as the swash zone can be a high UV, high heat, and even high shear force area of the ocean. As a particle moves up and down in the water column, the temperature and UV it is exposed to may slow degradation rates. Freshwater studies have shown that eDNA persists longer in more turbid lakes, where UV can't penetrate as deep (Eichmiller et al., 2016).

Applicability and future study

Overall, this study showed the viability of detecting marine molluscan species using eDNA with molecular tools in a laboratory setting. Other studies have shown that eDNA can be detected in the field, which should be the next step for this research. For molluscs, scientists have looked for eDNA from invasive species such as quagga and zebra mussels, *Dreissena spp.*, the New Zealand mudsnail *Potamopyrgus antipodarum*, and laver spiral shell *Peringia ulvae* (Ardura et al., 2015; Clusa et al., 2017; Goldberg et al., 2013; Peñarrubia et al., 2016; Williams et al., 2017). These bivalves and gastropod are commonly spread to different locations on vessels, either on the hull or in ballast

(Ardura et al., 2015; Bij de Vaate, 2010; Johnson and Carlton, 1996). Once a population has been established, they spread quickly, clogging municipal water transportation pipes and pumps, affect phytoplankton and therefore the productivity of lakes, and also may outcompete indigenous invertebrates (Cohen and Weinstein, 1998). For this reason, the detection of these molluscs using eDNA must work when the molluscs are at low density in the system and they are more easily eradicated.

For *Haliotis* spp., all seven species in California (*H. rufescens, H. corrugata, H. cracherodii, H. folgens, H. sorenseni, H. kamtschatkana, H. walallensis*) are now found at low densities. Following the rise of the abalone fishery in the late 1940's, *H. rufescens* and *H. corrugata* were caught commercially and later *H. cracherodii*, *H. folgens*, and *H. sorenseni* were added to the mix and subsequently overharvested (Taniguchi et al., 2000). In the late 1980's several species of abalone were also affected by Withering Foot Syndrome, an infectious disease which began affecting abalone around the Channel Islands due to increasing water temperatures (Lafferty & Kuris, 1993). These stressors together have kept California abalone populations at low levels. Due to other studies that suggest molluscan eDNA can be detected at low levels, the detection of abalone eDNA in coastal waters may help scientists and managers find and protect endemic abalone populations (Hawk and Geller, 2018).

Lottia spp. are also threatened by human harvest for consumption. Preferential harvest of larger individuals in a population can lead to overall shrinking size among remaining individuals (Castilla & Duran 1985; Branch & Moreno 1994; Jackson & Sala 2001). Even species that are not harvested tend to be smaller in environments where other species are harvested (Jackson & Sala 2001; Keough et al. 1993). One study

compared *L. gigantea* size measurements from museum data and data from Cabrillo National Marine Reserve (CNMR) (an area where species collection is prohibited) to data collected in the field at beaches in Los Angeles, Orange, and San Diego counties and found that *L. gigantea* at the human-impacted sites were significantly smaller than previous *L. gigantea* collected in the field and those at CNMR (Roy, et al., 2003). Again, human harvest is having an impact on these gastropods and eDNA may provide insights into species range, which may benefit from further protection to retain larger individuals within the populations.

Mytilus spp. are found worldwide and while they are not threatened, they can be invasive due to their ability to adapt to different conditions and prevalence in nearshore ecosystems. Due to the complexity of the *Mytilus* genome, mussels can also hybridize with each other and spread out, further complicating the picture (Braby & Somero, 2005). *M. trossulus*, a native species, can hybridize with *M. galloprovincialis*, sometimes hybridizing along a gradient of salinity and temperature. eDNA may be useful to assess population gradients and to detect new invasive species of mussels with the proper primers.

There are many applications and further study to be done with these data, but all require careful technique. Following in the recommendation of Dejean et al. (2011), the most important aspect of any eDNA study is to ensure that one has the most specific primers for the study species (Dejean et al., 2011). This includes testing the primers on multiple targets and optimizing PCR conditions. Furthermore, because eDNA may be in very low concentrations, care must be taken to extract the samples without contamination. Dejean et al. (2011) recommends having a dedicated room to extracting

and working with rare DNA, similar to what one might have for ancient DNA. Lastly, processing the maximum number of samples possible in each batch is crucial to maintaining consistency of these results. This was an issue I ran into in my thesis, as, due to time and budget constraints I was only able to take one sample per treatment for the degradation study. This may have contributed to high variability in estimated eDNA concentrations, compounded by qPCR mismeasurement of amplification artifacts in degradation experiments. If budget is not a consideration, replicates of treatments should be assessed.

Though processing eDNA samples in the laboratory requires a lot of care, collecting samples to test for presence or absence using eDNA can be fairly simple. In the UK, researchers are putting citizen scientists to work, collecting water samples from 35 ponds to sample for a threatened newt, *Triturus cristatus* (Biggs et al., 2015). The newt lives in turbid waters, making it difficult to find using normal survey methods. Eighty-six volunteers were given basic instructions for how to collect water samples and collected 30 mL samples from ponds. Over 90% of samples analyzed resulted in accurate detection of the great crested newt. Citizen science projects like this could prove useful in the future of this project to map the regions where molluscs live on the California coast.

Conclusions

This study is the first to look at the emanation and decay rates of eDNA from *H. rufescens*, *L. scabra*, and *M. californianus*. It showed promising evidence that eDNA from these molluscan species can be detected at low levels with the correct primers and PCR conditions, making this research plausible for field studies. Furthermore, it was found that eDNA from these species can be detected at least three weeks following the removal of the organism from the system. Generally, eDNA was at its highest concentration in the first 24 h, while older eDNA falls to a very low level even though it was detectable for many days. This behavior can provide an opportunity to distinguish the recent presence of an animal versus a lingering older signal. Detected eDNA should be used to detect the presence of species of interest in the marine environment, though more research is needed to assess the temporal and spatial scope of the method for more species due to variation in the rates of emanation and degradation

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APPENDIX A

SUPPLEMENTAL FIGURES AND TABLES

Supplemental figures and tables:

Table 1. Study animal morphometrics (In ID, L=large, M=medium, S=small)

Tables 1a, b, c: These tables contain metadata for experimental animals. The animal name represents the species, size class, and individual number within a size class. Measurements of length were measured in centimeters and wet weight was measured in grams.

Table 1A: *Haliotis rufescens* morphometrics

Individual ID	Wet weight (g)	Width (cm)	Length (cm)	Pedal area (cm^2)	Pedal area/wet weight
1AbL1	14.82	2.8	4.3	6.402698864	0.4320309625
1AbL2	13.506	2.4	4	7.361430396	0.5450488965
1AbL3	17.524	3	4.4	6.79702381	0.3878694253
1AbL4	12.06	2.4	4.1	9.42821558	0.7817757529
1AbM1	3.382	1.4	2.4	2.350584795	0.6950280294
1AbM2	2.834	1.2	2.2	1.617024448	0.570580257
1AbM3	4.041	1.5	2.5	3.417360285	0.845671934
1AbM4	3.275	1.3	2.3	3.038877551	0.9279015423
1AbS1	1.22	0.8	1.5	1.461080586	1.197607038
1AbS2	1.172	0.8	1.5	1.201358093	1.025049568
1AbS3	0.927	0.6	1.3	1.457407407	1.572176276
1AbS4	1.465	0.9	1.7	1.443885449	0.9855873371

Table 1B: *Lottia scabra* morphometrics

Individual ID	Wet weight (g)	Width (cm)	Length (cm)	Height (cm)	Pedal area (cm^2)	Pedal area/wet weight
SL1	0.644	0.9	1.1	0.4	0.1792850519	0.2783929377
SL2	0.589	0.6	0.9	0.4	0.4539241623	0.7706692059
SL3	0.612	0.9	1.2	0.4	0.480142213	0.7845460996
SL4	0.349	0.4	0.9	0.3	0.4118209118	1.180002613

ML1	0.889	0.9	1.3	0.7	0.6082405935	0.6841851446
ML2	1.179	0.9	1.4	0.4	0.9686256504	0.8215654372
ML3	1.019	1	1.5	0.4	1.275415445	1.251634391
ML4	0.794	0.7	1.3	0.5	0.5250365497	0.6612551004
LL1	2.831	1.7	2.1	1.7	0.9778669043	0.3454139542
LL2	2.501	1.2	1.8	0.9	1.186177249	0.474281187
LL3	2.127	1.4	1.9	0.7	1.903422619	0.8948860456
LL4	2.653	1.2	1.7	0.7	1.086655405	0.4095949512

Table 1c: *Mytilus californianus* morphometrics

Individual ID	Length (cm)	Width (cm)	Depth (cm)	Wet weight (g)	Aperture (degrees)
MS1	2	1	0.5	2.683	5.072
MS2	1.7	0.6	0.5	2.35	4.348
MS3	2	1	0.6	3.024	4.925
MS4	2.2	1	0.6	3.165	3.987
MM1	2.6	1.2	0.9	5.493	4.158
MM2	3.2	1.5	1	7.05	4.229
MM3	3	1.3	0.9	5.749	4.321
MM4	3	1.4	1	5.176	4.328
ML1	4.4	1.8	1.5	15.829	3.351
ML2	5.1	1.7	1.7	20.904	4.592
ML3	4.7	2	1.7	21.399	4.712
ML4	4.8	1.8	1.6	17.335	4.089

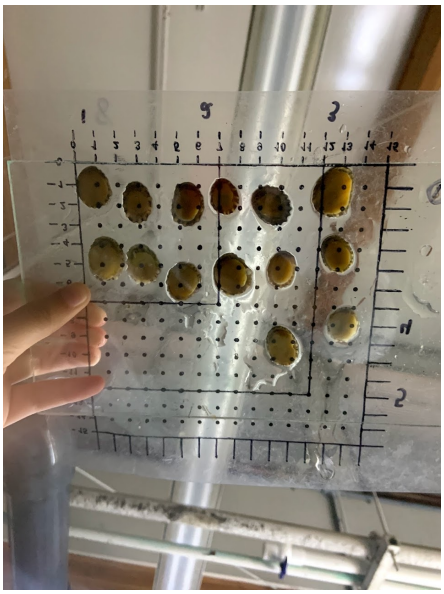

Figure 1: A photograph showing how pedal area was measured for abalone and limpets. Each dot square is a square centimeter so using ImageJ I was able to compare the amount of pixels within the square centimeter to a freehand drawn outline of the molluscan foot.

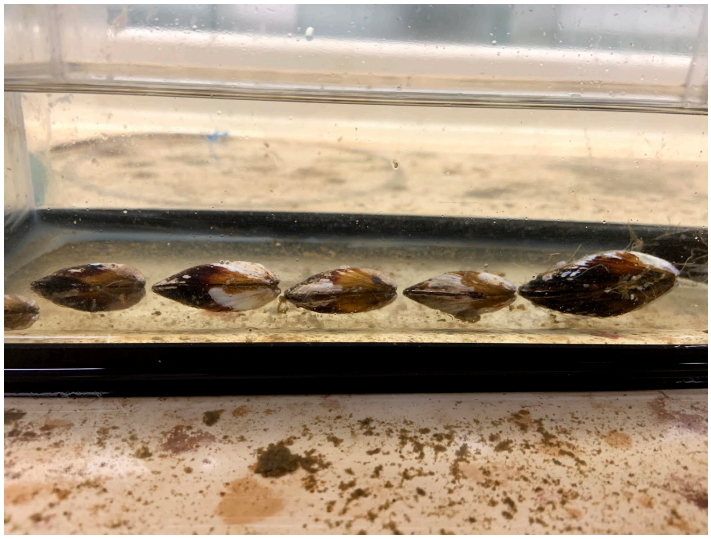

Figure 2: A photograph showing how shell aperture was measured for each experimental mussel. Animals were placed in a line into an aquarium tank with running water and a bubbler oriented and watched for 2 hours. As each mussel opened, images were taken to be analyzed with the "angle" tool in ImageJ.

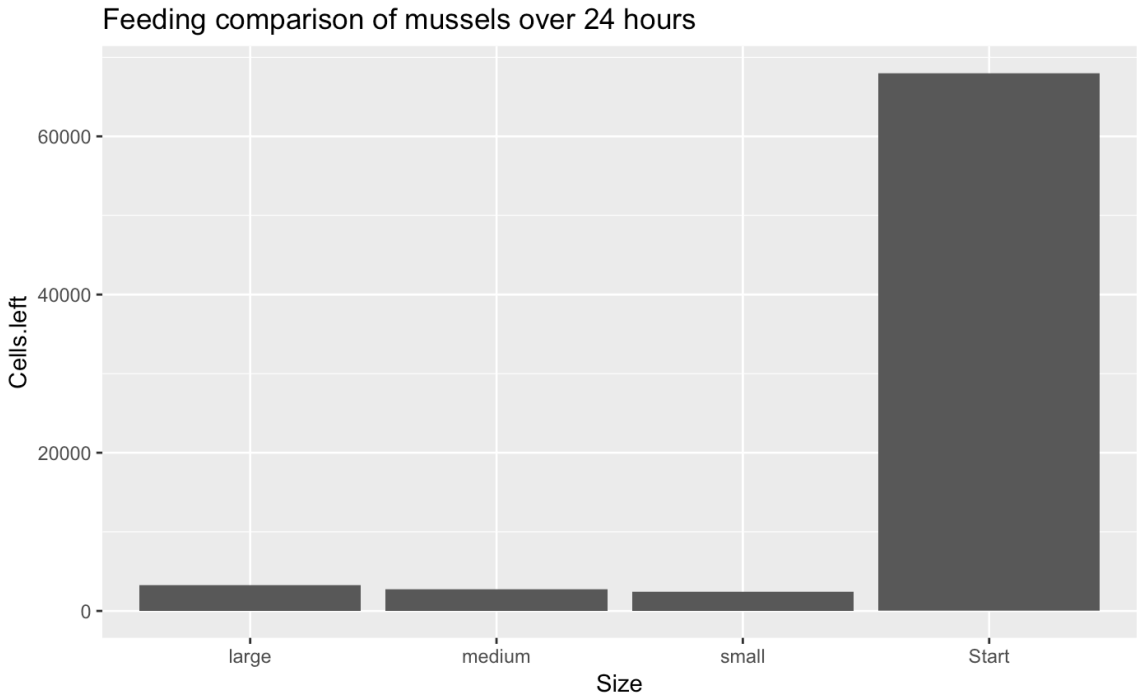

Figure 3: A barplot showing the amount of cells cleared by large, medium, and small mussels over 24-hours compared to the starting concentrations.

Table 2. Genbank accession numbers for COI sequences used in primer design.

Haliotis rufescens	EU636201.1, DQ297549.1, DQ297547.1, DQ297545.1, DQ297543.1, DQ297541.1, DQ297540.1, DQ297535.1, DQ297533.1, DQ297531.1, DQ297526.1, DQ297525.1, DQ297524.1, DQ297523.1, DQ297522.1, DQ297521.1, DQ297509.1, DQ297508.1, DQ297510.1, DQ297507.1
Mytilus californianus	MK091880.1, MK037177.1, MF544563.1, KF643561.1, KF643953.1, KF643867.1 KY454037.1, KY454036.1, MG431277.1, MG431276.1, MG431275.1, MG431274.1, MG431273.1, MG431268.1, MG431266.1, MG431264.1, MG431262.1, MG431260.1, MG431258.1, MG431257.1, GQ902240.1
Lottia scabra	KJ006004.1,KJ006003.1,KJ006002.1,KJ006001.1,KJ006000.1,KJ005999.1,KJ005998.1,KJ005997.1,KJ005996.1, KJ005995.1, KJ005994.1, KJ005993.1, KJ005992.1, KJ005991.1, KJ005990.1, KJ005989.1, KJ005988.1, KJ005987.1, KJ005986.1, KJ005985.1

Table 3. eDNA per weight vs size class and species (normalized for weight)

	Degrees of Freedom	**Sum of Squares**	**Mean Square**	**F value**	**P value (p<0.05)**
Size Class	2	14.17	7.084	7.414	0.002337 *
Species	2	18.49	9.245	9.675	0.000544 *
Residuals	31	29.62	0.956		

Shapiro-Wilk normality test	
w=0.98382	p=0.865

Tukey HSD post hoc test	**Comparison**	**p-value**
Size class	Medium-Large	0.0179464 *
	Small-Large	0.0027368 *
	Small-Medium	0.7436693
Species	Lottia-Haliotis	0.0003960 *
	Mytilus-Haliotis	0.0255458 *
	Mytilus-Lottia	0.2647470

Table 4. Activity and eDNA off put results

One tailed Paired t-test	**t-value**	**degrees of freedom**	**p value (p<0.05)**
Haliotis rufescens	-1.8137	13	0.04643 *
Mytilus californianus	0.16845	13	0.5656
Lottia scabra	0.86752	13	0.7993

Table 5. Degradation – abalone

Randomized Block ANOVA					
	Degrees of freedom	Sum of squares	Mean square	F value	p value ($p<0.05$)
Bacterial treatment	1	0.1685	0.16851	5.111	0.02645 *
UV treatment	2	0.1744	0.08721	2.645	0.07712
Temperature	1	0.0362	0.03625	1.099	0.29751
Day since addition	1	0.2421	0.24209	7.343	0.00821 *
Residuals	81	2.6704	0.03297		

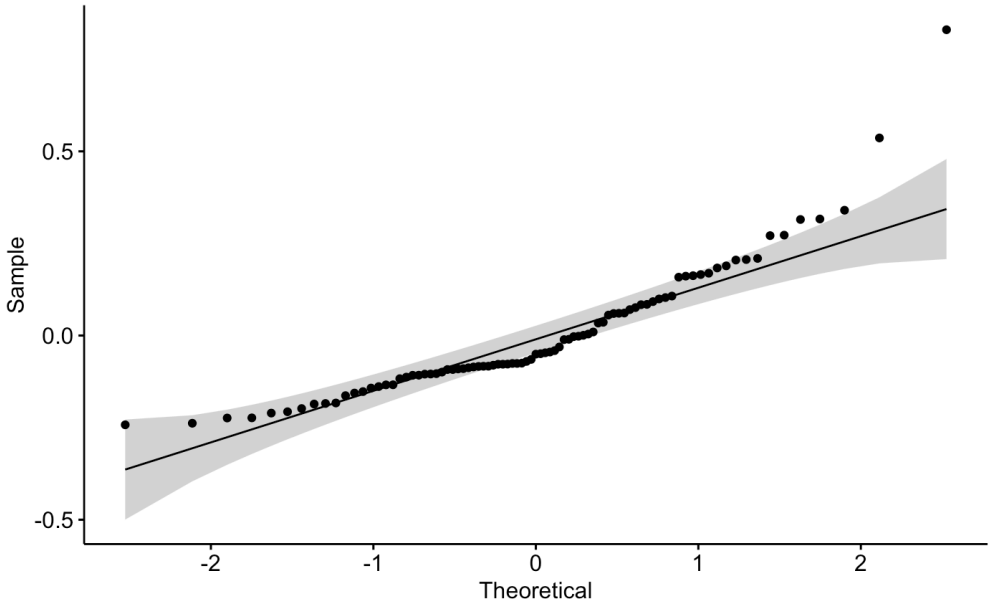

Figure 4. A plot showing the normality of the residuals from the randomized block ANOVA on *M. californianus*. As long as the majority of the black dots are within the gray region, the residuals are normal enough for the assumptions of the statistical test to be met.

Table 6. Degradation – mussel

Randomized Block ANOVA					
	Degrees of freedom	Sum of squares	Mean square	F value	p value ($p<0.05$)
Bacterial treatment	1	60.2	60.75	2.189	0.14379
UV treatment	2	48.1	24.05	0.874	0.42220
Temperature	1	6.4	6.37	0.231	0.63220
Day since addition	1	253.5	253.48	9.209	0.00344 *
Residuals	66	1816.8	27.53		

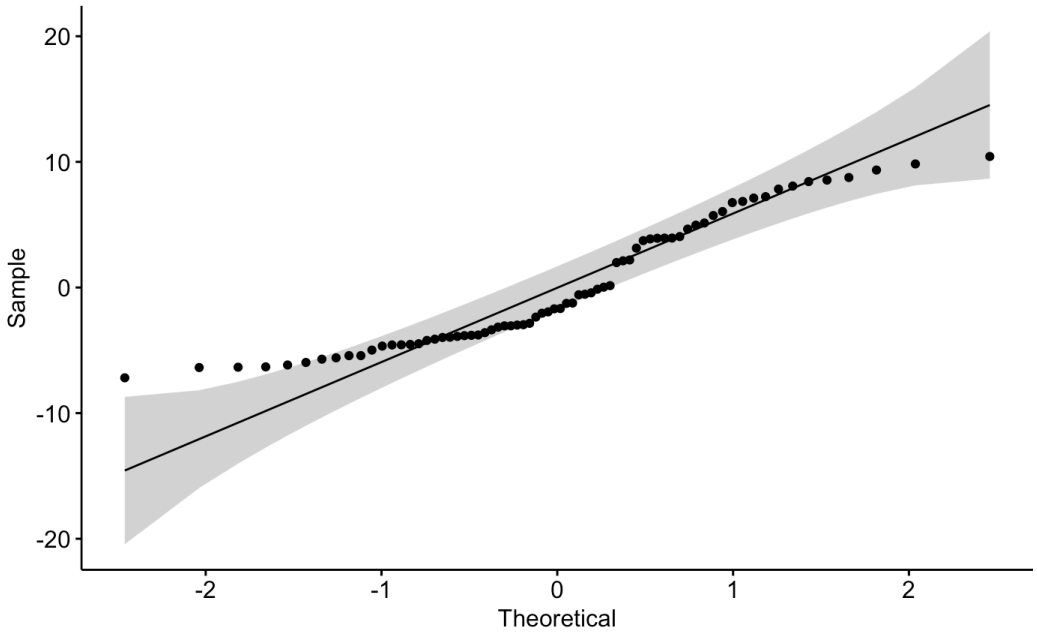

Figure 5. A plot showing the normality of the residuals from the randomized block ANOVA on *H. rufescens*. As long as the majority of the black dots are within the gray region, the residuals are normal enough for the assumptions of the statistical test to be met.

Table 7. Degradation – limpet

Randomized Block ANOVA					
	Degrees of freedom	Sum of squares	Mean square	F value	p value ($p<0.05$)
Bacterial treatment	1	49	48.6	0.986	0.323568
UV treatment	2	45	22.7	0.461	0.632343
Temperature	1	8	7.8	0.158	0.692321
Day since addition	1	614	614.0	12.468	0.000686 *
Residuals	81	3989	49.2		

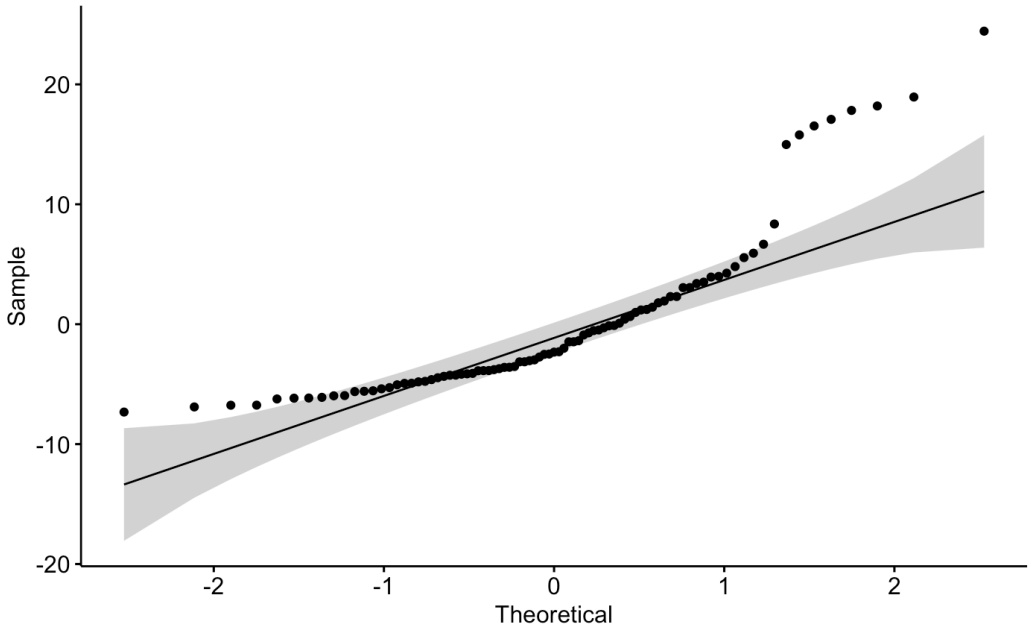

Figure 8. A plot showing the normality of the residuals from the randomized block ANOVA on *L. scabra*. As long as the majority of the black dots are within the gray region, the residuals are normal enough for the assumptions of the statistical test to be met.

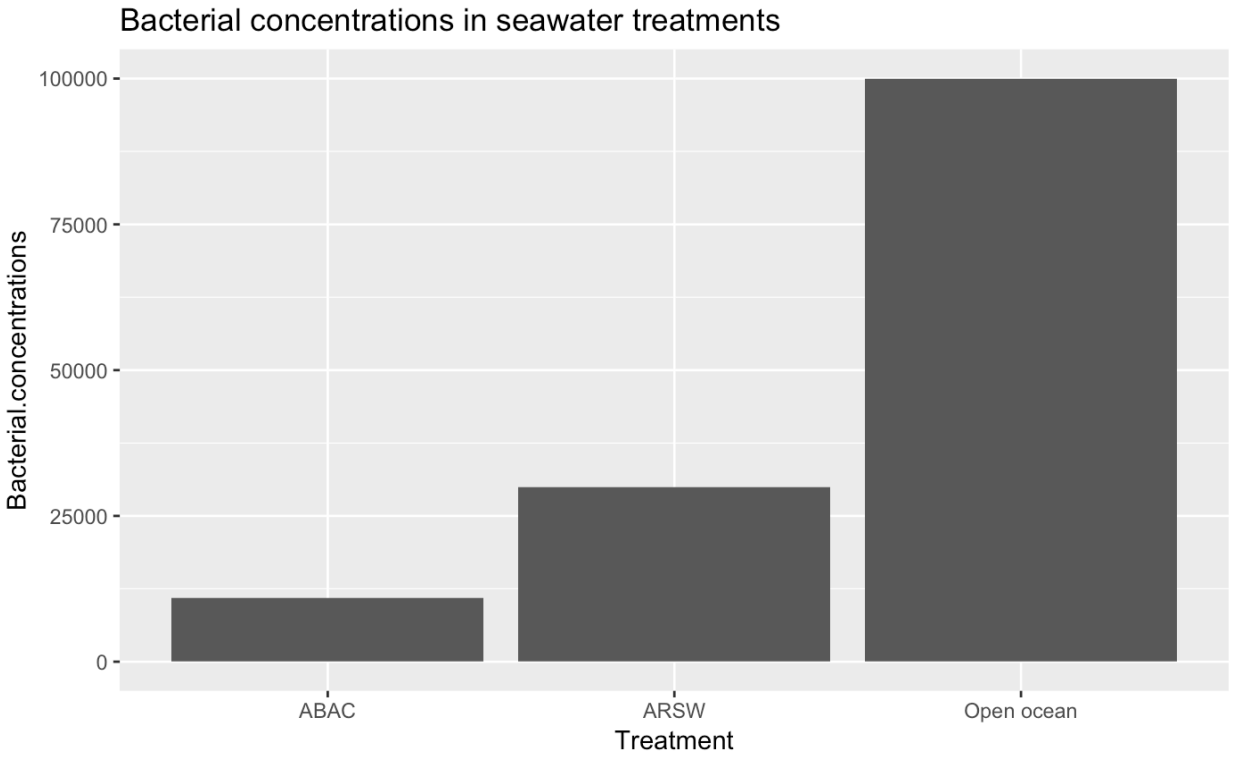

Figure 9. Bacterial concentrations of antibiotic autoclaved seawater, aquarium room sea water, and open ocean seawater measured by the flow cytometer at the beginning of the experiment. Concentrations were measured as ppm.

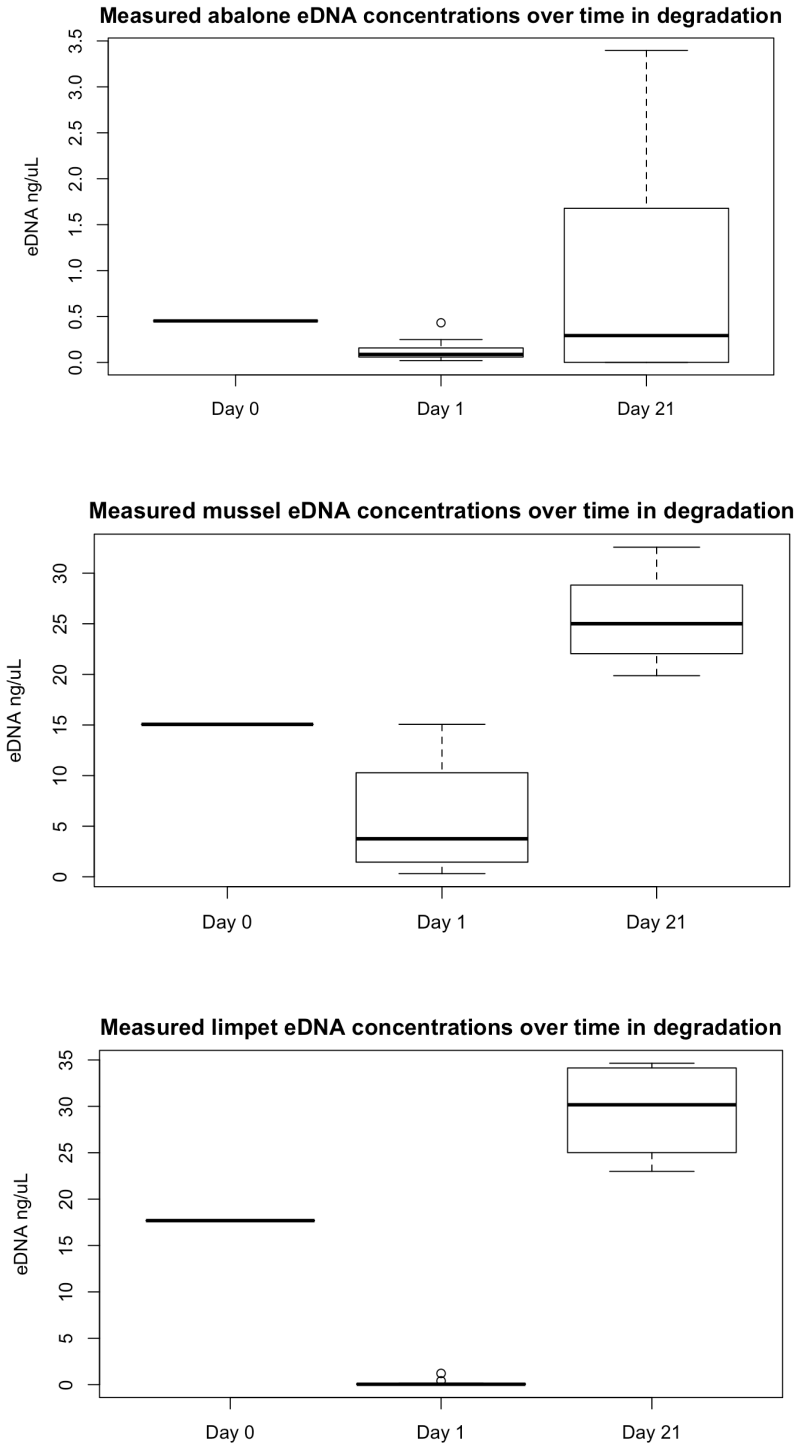

Figure 10. Measured eDNA concentrations during the start and end of the degradation study. Concentrations after day 2 or 3 were measured on a separate qPCR plate, potentially leading to error in measurements. Furthermore, creation of double stranded DNA from degraded nucleic acids or primers could have led to spikes in measured DNA concentrations.

APPENDIX B

ENVIRONMENTAL DNA MOVEMENT CODE

```
#You can read in a file containing the data, generated in a loop or by hand.  Make sure this csv is in the same directory you use R in.
modeldata=read.csv("ThesismodeldataS.csv", header=TRUE)
library(plotly)
df <- modeldata
#Many of the following can be edited once your data is loaded in.  I loaded in data containing X (towards shore velocity), Diffusiony (tides and diffusion), age (age of the particle since creation), and DNA particle (a number representing each individual particle)
base <- modeldata %>%
  plot_ly(x = ~X, y = ~Diffusiony, size = ~age, color = ~age,
          text = ~DNA.particle, hoverinfo = "text") %>%
  #setting the range and axes labels
  layout(xaxis = list(type = "linear", range = c(-3,7000), title="Distance traveled towards shore"), yaxis = list(range = c(0, 10), title="Height from bottom (Depth)"), title="eDNA movement in summer")
base %>%
  #This section creates the slider on the bottom of the graph so you can see the model at different times
  add_markers(data = modeldata, frame = ~timenumeric, title="time") %>%
  hide_legend() %>%
  animation_opts(frame = 1000, transition = 0, redraw = FALSE)
```