Spatial Dispersion of Red Abalone (*Haliotis rufescens*) Environmental DNA (eDNA) in a Controlled Marine Environment and Applications of eDNA to Monitor Critically Endangered Abalone (*Haliotis* spp.) Populations in the Wild

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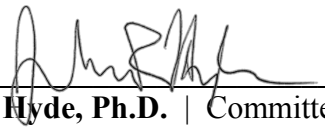

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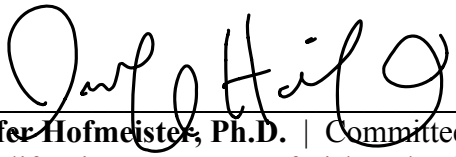

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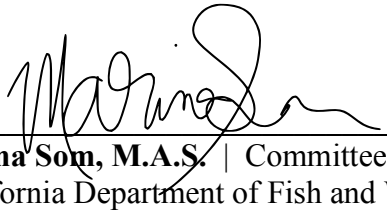

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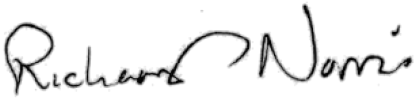

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Abstract

The analysis of environmental DNA (eDNA) from water samples is improving species monitoring by offering enhanced detection of rare, cryptic, and endangered taxa over traditional survey methods. This study aimed to investigate the dispersion of red abalone (*Haliotis rufescens*) eDNA in a controlled marine environment and assess the feasibility of detecting presence of abalone (*Haliotis* spp.) eDNA in the ocean. Genus-specific primers were used to amplify red abalone eDNA, and multiple experiments revealed the eDNA permeated a two million liter volume of seawater within 18 hours of introduction. Field validation was conducted with seawater samples from two locations where abalone are known to occur along the California coast, and both samples amplified presumed abalone eDNA using the same genus-specific primers. Environmental DNA is a promising tool to detect the presence of cryptic and endangered abalone species in the ocean, with the potential to complement and strengthen current visual survey methods.

Introduction

Southern California is a hotspot for abalone (*Haliotis* spp.) diversity with all seven northeast Pacific species occurring there. Abalone in this region have been fished for thousands of years and their populations supported high-value fisheries until the latter half of the twentieth century (CDFG, 2005; Cox, 1962; Tegner, 1989). Starting in the 1970's, populations declined drastically from disease, predation, and overexploitation by commercial and recreational fishing, which prompted a moratorium on all California abalone fishing south of San Francisco by 1997 (CDFG, 2005). The white abalone (*H. sorenseni*) experienced a particularly dramatic population decline and received Federal Endangered Species Status in 2001.

Commercial harvesting of white abalone began to increase in 1967 and supported a short, intense fishery where landings peaked in 1972. By 1978, the catch declined so severely that reporting the species in landings data was no longer required (Hobday & Tegner, 2000). Despite this extreme reduction in white abalone catch, the fishery wasn't closed until decades later in 1996 (Hobday & Tegner, 2000). White abalone populations continued to decline in California even after the closure, and became the first marine invertebrate listed as endangered under the Endangered Species Act (ESA; 66 FR 29046, 29 May 2001). Closing this fishery led to a loss of fishery-dependent data and provided a need to monitor white abalone populations through increased fishery-independent survey methods. Data on white abalone abundance is essential not only to identify areas suitable for additional surveys, but to also support conservation management plans in locating appropriate sites to outplant captive-bred juvenile white abalone in order to increase wild population numbers (DiNardo, 2018).

Since the early 2000s, remotely operated vehicle (ROV) and SCUBA survey methods have been consistently used to monitor white abalone populations. While ROV surveys have shown the continued decline of white abalone at deeper locations around California's offshore islands and outer banks (Butler et al., 2006; Stierhoff et al., 2012; Stierhoff et al., 2014), both ROV and SCUBA survey methods have their limitations. These methods require a large investment of effort while often finding few or no white abalone (Neuman et al., 2018 & 2019; Obaza et al., 2018). A more cost effective, less time consuming, and more sensitive method can help to better

evaluate the status of white abalone in the wild, especially as populations dwindle and resources to conserve species are reduced.

Over the past two decades, filtering DNA that is shed or excreted from an organism into its surrounding environment, known as environmental DNA (eDNA), has proven successful in detecting species without direct observation (Ficetola et al., 2008; Foote et al., 2012; Jerde et al., 2011; Schmelzle & Kinziger, 2016). The applications of eDNA vary widely from monitoring entire ecosystems and species biodiversity (Kelly et al., 2014a; Port et al., 2016; Thomsen et al., 2012a & 2012b), while being sensitive enough to detect the presence of invasive or cryptic taxa that are difficult to sample and at low densities (Jerde et al., 2011; Niemiller et al., 2018; Pilliod et al., 2013; Schill & Galbraith, 2019). Studies show the analysis of eDNA from water samples is more effective, efficient, and of lower cost than other survey methods used (Lugg et al., 2017; Schmelzle & Kinziger, 2016; Thomsen et al., 2012a & 2012b). In freshwater ecosystems, detectable eDNA can persist over days or weeks (Dejean et al., 2011; Thomsen et al., 2012b), whereas eDNA degrades faster in seawater with turnover occurring in mere hours to days (Dell'Anno & Corinaldesi, 2004; Sassoubre et al., 2016; Thomsen et al., 2012a). These quick degradation rates in seawater allow for eDNA surveys to reflect contemporaneous data of species present in the marine environment, providing crucial information for applications in monitoring.

The spatial dispersion of eDNA released from an organism is vital for estimating the location of an individual. In river systems, studies found that eDNA from caged brook trout (*Salvelinus fontinalis*; Jane et al., 2015) and planktonic crustaceans (*Daphnia longispina*; Deiner & Altermatt, 2014) can be detected from hundreds of meters downstream to as far as 12 km from the animal respectively, whereas eDNA of caged striped jack (*Pseudocaranx dentex*) was detectable mostly within 30 m of the source (Murakami et al., 2019). The smaller the distribution of eDNA, the more useful it is in estimating an animal's location. For a rare and cryptic taxon like white abalone, utilizing eDNA in this context provides a promising method to quickly and accurately identify a rare animal's general location and guide focused ROV and SCUBA survey efforts.

We examined the spatial distribution of red abalone (*H. rufescens*) eDNA in a controlled laboratory setting. Red abalone was used as a proxy species in place of white abalone, in order to avoid placing unnecessary stress on an endangered species. The goal was to evaluate how far eDNA was horizontally and vertically dispersed from an abalone in a two million liter experimental tank. Field validation was conducted on the ability to detect abalone eDNA at locations along the California coast where they are known to occur. Genus-specific primers were used to test for abalone eDNA presence in all genetic sampling, and we did not try to detect specific species at this time.

Methods

Tank Experiment

Experiments assessing red abalone eDNA distribution were conducted in La Jolla, CA, at NOAA's Southwest Fisheries Science Center (SWFSC) in the facility's Ocean Technology Development Tank. This tank holds two million liters of seawater and measures 10 m wide by 20

m long by 10 m deep. The seawater in this tank is on a 12-hour re-circulation cycle and passes through a combination of sand filters, ultraviolet radiation, ozone, and degassing units. The temperature of the tank was 19°C for the duration of all experiments. A Van Dorn style horizontal water sampler was used to collect 2L water samples analyzed in these experiments. Prior to placing a single red abalone in the tank, a 2L water sample was collected from the bottom center of the tank, serving as a control to ensure there was no residual abalone eDNA present. Individual red abalone were randomly selected from in-house populations and we used a different red abalone for each experiment. We aimed to use abalone of similar size for the experiments to avoid confounding variables. Those selected had an average shell length of 94.0 ± 11.2 mm and a weight of 134 ± 45 g. For each replication, an individual animal was placed in a PVC pipe framed cage, measuring 33 cm x 27 cm x 13 cm, with plastic mesh sides and an acrylic bottom. The caged abalone was placed on the center of the tank floor at 10 m depth and starved for the duration of the experiment. Water samples were collected 18 hours after initial placement of the caged abalone for each experiment.

For the first experiment, water samples were collected from the tank bottom at distances of 0.5 m, 1 m, and 2 m from the center of the caged abalone (Figure 1A). Additional water samples were collected on the top of the cage (0 m) and 1 m above the cage. The second, third, and fourth tank experiments utilized an expanded sampling range: water samples were collected along the tank bottom at 1 m, 2 m, 5 m, and 10 m distances from the center of the caged abalone; directly on top of (0 m) and 1 m above the cage; and surface water samples were taken directly above the animal and at each center edge of the tank (Figure 1B). Samples were first collected at distances furthest from the abalone and continued inward (i.e., 10 m, 5 m, 2 m, 1 m, 0 m). Four tank experiments were performed in total, each separated by at least one week, and a total of 57 water samples including four controls were collected for eDNA analysis. After a water sample was collected from the tank, it was poured into a 2L capacity Nalgene™ amber HDPE bottle for short term storage. The bottles were kept on ice in a cooler and filtered within six hours of collection. Bottles were decontaminated with a 10% bleach solution and rinsed four times with tap water before each use.

Field Sampling

Seawater samples were collected on March 31, 2020 at approximately 11:00am PST at two locations off the coast of Point Loma in San Diego, CA. Both seawater samples were collected from the seafloor one hour after a low tide cycle, using a 2L Van Dorn style horizontal water sampler. A strong surface current and red tide were observed at both locations on that day. The first site chosen, referred to as White Abalone Outplant site, was the location of the first juvenile white abalone outplant attempt, where approximately 1,600 juvenile white abalone were released in November 2019. This site also contains adult white abalone, one pink abalone (*H. corrugata*), and several adult and juvenile red abalone based on diver surveys in February 2020. A 2L seawater sample was collected at this site from the seafloor at a depth of ~20 meters. The second location, New Hope Rock, has been known to have a presence of red, pink, and possibly green abalone (*H. fulgens*), but has not been recently surveyed. A 2L seawater sample was collected from the seafloor at a depth of ~12 m. Both water samples were stored in a cooler with ice packs and filtered within six hours of collection.

Water Filtration

Prior to filtration, all surfaces, filtering equipment, and the outside of the water bottles were decontaminated with DNA Away™ (Molecular Bioproducts). Water samples collected from the tank experiments and field sites were vacuum filtered through single-use 47mm diameter cellulose nitrate membrane filters (nominal pore size 0.45 µm, Sterlitech). Each filter was folded inward, placed in a 1.5 ml conical microcentrifuge tube, and stored at -20ºC until DNA extraction. A clean filter membrane was removed from a packaged filter column, placed in a 1.5 ml conical microcentrifuge tube, and treated identically to the other samples as an extraction control for each experiment. Instruments were decontaminated with DNA Away™ between each sample to prevent cross-contamination.

Primer Design

Mitogenomes for the seven abalone species were downloaded from GenBank (accession numbers NC_036928 and KX260956) or mined from genome sequences (Masonbrink et al., 2019) and aligned using Sequencher version 5.2. The genome was surveyed by eye for short regions of 150-300 base pairs (bp) that contained sufficient variability to differentiate all species, and universal primers were designed to amplify these regions. The choice of small fragments was done both to increase the ability to detect degraded DNA and to be amenable for metabarcoding approaches using next generation sequencing methods (e.g. Miya et al., 2015). After initially screening several primer pairs for abalone (Table 1), forward primer Abalone-eDNA_F2 and reverse primer Abalone-eDNA_R were chosen for downstream analyses; together they amplified a 341bp size fragment of the 5' end of the mitochondrial cytochrome c oxidase subunit I gene. These primers were designed to work across all local species of abalone and were tested and shown to amplify DNA from white, red, pink, and black abalone (*H. cracherodii*) housed at NOAA SWFSC. The specificity of these primers for non-abalone taxa is currently untested, though no exact match was found for both primers using the NCBI BLASTn algorithm. Species-specific primers were also designed for white abalone to be utilized in detecting presence of this species from eDNA in the ocean, however, these primers have yet to be tested for specificity. Due to these limitations, the genus-specific primers were also used to amplify abalone eDNA from the water samples collected at the field sites.

DNA Extraction

DNA was extracted from the filters using a modified protocol from the Qiagen DNeasy Blood and Tissue Kit. For DNA extraction, 400 µl Milli-Q water, 180 µl Buffer ATL, and 20 µl Proteinase K were added to the 1.5 ml tube containing the filter, briefly vortexed, and incubated at 56°C on a heat block for 30 minutes. After incubation, the filter and solution were transferred to a 5 ml centrifuge tube, and 600 µl of both Buffer AL and 100% ethanol were added and vortex mixed. Due to the larger volume needed to submerge the filters in extraction buffer, it was necessary to run sequential 600 µl volumes of the solution through the spin column for each sample. Capture tubes were centrifuged, flowthru was disposed, and the process repeated until there was no liquid remaining. Further downstream processing of DNA followed the manufacturer's protocol. DNA was eluted in a final volume of 100 µl Buffer AE.

PCR Amplification

DNA extracted from tank experiment water samples were amplified according to PCR Protocol 1: each 10 µl PCR reaction contained 5 µl HotStarTaq Plus Master Mix (Qiagen), 0.25 µl of each primer (10 µM), 0.25 µl BSA (20 mg/ml), 1.0 µl CoralLoad Concentrate (Qiagen), 1 µl of DNA template, and 2.25 µl RNase-free water (Qiagen). PCR conditions were as follows: initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 60 seconds, 72°C for 60 seconds, and a final extension step of 72°C for 3 minutes. No template controls were used in each PCR amplification set to test for contamination. PCR products were verified after electrophoresis on a 2% agarose gel stained with ethidium bromide.

Earlier work (L. Martin, unpublished data) had shown that giant kelp (*Macrocystis pyrifera*) present in tanks led to co-extraction of PCR inhibitors in water samples, most likely polysaccharides and polyphenols. Though this was not an issue in these tank experiments, samples collected from within the Point Loma kelp forest exhibited significant PCR inhibition. To overcome sources of inhibition, 80 µl of the eluted DNA extracted from the field samples were cleaned using a OneStep™ PCR Inhibitor Removal Kit (Zymo Research). In addition to PCR Protocol 1, a second method was used on the field sample DNA to compare the effectiveness of different PCR protocols when inhibitory compounds are present. PCR Protocol 2 was performed in a 10 µl reaction volume: 1 µl of 2mM of each dNTP, 1 µl 10X PCR buffer (670 mM Tris-HCl pH 8.8, 166 mM $(NH_4)_2SO_4$, 100 mM β-mercaptoethanol, 20 mM $MgCl_2$), 0.1 µl *Taq* DNA polymerase (NEB, 5 units/µl), 0.25 µl of each primer (10 µM), 0.25 µl BSA (20 mg/ml), 1.0 µl CoralLoad Concentrate (Qiagen), 1 µl of DNA template, and 5.15 µl RNase-free water (Qiagen). Thermal cycling conditions consisted of an initial denaturation step at 94°C for 2 min 30 sec, followed by 35 cycles of 94°C for 30 sec, 60°C for 60 sec, 72°C for 60 sec, and a final extension step of 72°C for 3 min. Both cleaned and uncleaned field sample DNA were amplified according to the two PCR protocols in reactions using 1 µl of 1:10 template DNA dilutions, 1 µl and 2 µl template DNA, and run with both 35 and 45 thermal cycles. Furthermore, eight individual PCR reactions of the field site water samples were spiked with 1 µl of clean red abalone DNA into the reaction volume, serving as an internal positive control to test for inhibition (Table 3). No template controls were used in each PCR amplification set to test for contamination. PCR products and fragment size were verified on a 2% agarose gel stained with ethidium bromide.

Results

Tank Experiments

For the first tank experiment, abalone eDNA amplified with PCR Protocol 1 in all eight water samples that were collected 18 hours after the cage was set at the bottom of the tank (Table 2; Figure 2A; Figure 3). Abalone eDNA was not detected in the control water sample taken prior to the addition of the animal. The extraction control and no template PCR control did not amplify the target DNA, indicating there was no contamination during lab work. Due to all samples showing presence of abalone eDNA, sampling distances were expanded for the second, third, and fourth experiments.

PCR results from the second experiment showed only a single, very faint band from the water sample collected directly over the caged abalone (Table 2; Figure 4A). These samples were re-run according to both PCR protocols using 45 cycles to increase sensitivity. PCR Protocol 1 showed contamination in the tank control and these results were not used (Table 2; Figure 4B). PCR Protocol 2 successfully amplified target DNA in 10 of the 15 experimental water samples collected, at the following horizontal distances from the caged abalone: twice at 1 m, 2 m, 5 m, twice at 10 m; 1 m above the cage; and at surface samples collected at both 10 m distance, and directly above the cage (Table 2; Figure 4C). PCR Protocol 2 did not amplify target DNA in the initial amplification that showed abalone eDNA presence at 0 m.

The third tank experiment successfully amplified abalone eDNA with PCR Protocol 1 in all fifteen water samples collected (Table 2; Figure 2B; Figure 5). All controls did not amplify target DNA, showing no evidence of contamination.

The fourth tank experiment amplified abalone eDNA with PCR Protocol 1 in fourteen out of fifteen water samples collected (Table 2; Figure 2C; Figure 6). The sample that did not amplify target DNA was collected at the surface, 10 m from the center of the tank. The tank control showed a very weak band on the gel, however, the rest of the samples that amplified eDNA displayed stronger bands (Figure 6). The no template control did not amplify target DNA.

Field Sampling

From multiple combinations of PCR protocols with differing amounts of template DNA, water samples collected from both field sites showed abalone eDNA amplification using 2 µl template DNA and 45 cycles using PCR Protocols 1 and 2 separately (Table 3; Figure 7). The White Abalone Outplant site sample amplified eDNA using PCR Protocol 1, whereas the New Hope Rock sample amplified eDNA using PCR Protocol 2. Target DNA was not amplified in the no template controls in the PCR reactions.

Discussion

While we initially thought abalone may have a limited and confined eDNA detection range due to their sedentary lifestyle, our experiments suggest otherwise. The results of the tank experiments indicate that eDNA from a single, relatively small abalone, can distribute detectable eDNA throughout a volume of two million liters over a fairly short time period and can be detected from $1/10^6$ of this volume. While our tank experiments allowed for eDNA detection up to 10 m from the abalone, ocean currents and tides have the potential of carrying abalone eDNA farther from the source, which may increase its detectable range outside of 10 m. Field validation was also possible, despite inhibition from marine algae, indicating a potential for utilizing eDNA survey methods to assess abalone presence in the wild.

The first tank experiment incorporated a smaller sampling range than the third and fourth experiment, and all three of these experiments produced PCR bands of similar strength from abalone of similar size. Had the first experiment utilized the expanded sampling range, it would most likely have indicated abalone eDNA presence throughout the entire tank as it did for the third and fourth experiments.

The second experiment initially resulted in one sample indicating presence with a weak signal from the water sampled directly above the cage (Figure 4A, lane 6). Due to the contrasting results from the first experiment, the DNA samples were re-run with two different PCR methods, along with an increase in the number of cycles and various amounts of template DNA to boost sensitivity. PCR Protocol 1 showed contamination in the tank control sample, so these results were not used (Figure 4B, lane 1). The contamination was most likely a result from lab work and not from the DNA sample since it did not amplify with any other set. PCR Protocol 2 amplified different water samples, but only two bands on the gel were strong and the rest were fairly weak (Figure 4C). The second experiment's results were removed from analysis due to inconsistent and conflicting PCR outcomes, and to avoid spurious conclusions.

Previous studies suggest that eDNA is more likely to be found within the water depth stratum in which the organism occurs (Minamoto et al., 2017; Murakami et al., 2019). Based on the spatial distribution hypothesis in these studies, the water sampling range was designed to detect eDNA presence distributed horizontally from the abalone, and surface water samples were not initially included. However, due to the 12-hour cycle of mixing and turnover of the tank water, there was no delineation between horizontal or vertical eDNA dispersion. Though flow is low, we cannot rule out that the tank flow helped to distribute eDNA more uniformly throughout the tank than in a more static system. While we did not quantify eDNA from the water samples in these experiments, the 18-hour residence time of the abalone in the test tank was sufficient to distribute a strong genetic signal throughout two million liters of seawater. Additional field studies should be conducted that test the spatial distribution hypothesis by incorporating a regular vertical sampling from a known source in addition to horizontal samples. Though these tank experiments show potential for significant dispersion of eDNA, further work should also evaluate decay rates of abalone DNA in seawater, as this is likely a large factor in defining the ultimate dispersal kernel.

Our small sample size of field results showed potential for eDNA to be used to detect presence of abalone in the ocean without direct observation. For this pilot survey of field sampling sites, areas were chosen based upon *a priori* knowledge that abalone inhabit those locations. White Abalone Outplant site was recently stocked with juvenile white abalone, and also contains adult white abalone, juvenile and adult red abalone, and pink abalone. New Hope Rock has been known to have a presence of red, pink, and possibly green abalone, but has not been recently surveyed. Future next generation metabarcoding sequencing of the water samples collected from the field sites will allow us to evaluate not only the abalone species detected and the number of sequence reads per species, but also determine if there was any interference by non-abalone taxa. As the White Abalone Outplant site has been most recently surveyed, we can then compare the metabarcoding data from the water samples to the field survey data and evaluate the utility of eDNA from a metabarcoding approach.

Environmental DNA surveys have proven valuable in detecting rare and cryptic species in freshwater systems (Jerde et al., 2011; Pilliod et al., 2013; Thomsen et al., 2012b), however, few studies have been conducted in a marine environment, especially on invertebrates. In the ocean, eDNA is affected by multiple factors including distribution by currents and tides, dilution, mixing, sources of inhibition, and faster degradation rates relative to freshwater systems. It's

important to note that the amplified abalone eDNA from the field did not produce PCR bands as strong as the tank experiments under the same PCR conditions. This indicates that there may be very low template DNA or inhibitory compounds due to the aforementioned factors present in marine environments. Prior experiments (L. Martin, unpublished data) have shown that *Macrocystis pyrifera*, a primary food source for abalone, contributes to significant inhibition when trying to amplify abalone eDNA from aquaria. When *Macrocystis* was removed from these tank experiments, abalone eDNA produced very strong signals. Therefore, we know that when collecting water samples from a location with giant kelp, genetic techniques should be optimized to minimize inhibition. For the water samples collected from the field sites, inhibition was minimized by cleaning the DNA samples with the Zymo OneStep™ PCR Inhibitor Removal Kit, and by adding BSA to the PCR reactions. We did not default treat water samples from the tank experiments with the inhibitor removal kit since a macroalgae food source was not included with the abalone in the cage. Furthermore, three out of four of the reactions spiked with red abalone DNA using PCR Protocol 2 did not amplify any DNA with the genus-specific primers, whereas the spiked reactions did amplify abalone DNA when using PCR Protocol 1. This indicates not only that inhibition was truly a limiting factor in those reactions, but that PCR Protocol 1 utilizing Qiagen's HotStarTaq Plus Master Mix may be a more sensitive reagent capable of overcoming the adverse effects of PCR inhibitors. Successful amplification of abalone eDNA from both field sites did occur, showing that we were able to reduce inhibitors from the ocean using multiple techniques. Due to the likelihood of both low concentration of abalone eDNA and strong presence of inhibitory compounds in kelp forests, further work should be done to identify optimal DNA extraction methods and protocols to maximize assay sensitivity.

Utility of eDNA to improve abalone surveys

Current survey methods to detect abalone in the wild utilizing ROVs and SCUBA divers are both limited by visual detection of the cryptic animal in addition to taxonomic expertise. While divers have the advantage of being more mobile on the seafloor and have better visual inspection capabilities, they are restricted by air time and depth. ROVs are able to overcome those constraints but are sometimes unable to access suitable habitats (i.e., dense kelp forests) because of the risk of entanglement, and video footage may fail to detect species on account of the ROV's inability to move objects and perform close, detailed observations. Juvenile abalone may also go undetected by the human eye due to their small size and typical behavior of inhabiting small, protected crevices (Carson et al., 2019). Together, these factors contribute to imperfect detection, hindering the performance of species distribution models, especially when detectability is already low (Lahoz-Monfort et al., 2014). Combining the high sensitivity and specificity provided by eDNA survey methods with current methods would help increase detection of abalone.

Despite its potential benefits as a monitoring tool, eDNA, like other survey methods, has its caveats. Environmental DNA detected from water samples fails to identify its source (e.g. feces, mucus, epithelial cells, etc.), the life stage or size of the animal, or even its status of being alive or dead. Environmental DNA is also susceptible to false detection. False positive eDNA detection could result from amplification of ancient DNA attached to sediment (Turner et al., 2015), detection of the organism when the target DNA is carried far from the source such as in predator excrement, or contamination of samples in the field or laboratory (Darling & Mahon,

2011). False negative detection can result from inhibition, increased dilution, or high current or direction of flow dispersing eDNA from the source (Darling & Mahon, 2011; Jane et al., 2015). False positives/negatives can lead to an over/under estimation of species presence; therefore, adequately accounting for sources of error and an accurate interpretation of detection rates would increase the reliability of eDNA for monitoring and management.

In order to overcome aspects of imperfect detection from eDNA, visual surveys can be paired with eDNA detection surveys to estimate the probability of acquiring false detection (Guillera-Arroita et al., 2017; Schmidt et al., 2013; Smith & Goldberg, 2020). Such calibration studies are common in ecological surveys when new sampling methods are employed (Lacoursière-Roussel et al., 2016; Pont et al., 2018). Additionally, site occupancy models derived from presence/absence data already account for imperfect detection, and they can also calculate the number of eDNA samples necessary to obtain reliable cumulative detection probabilities (Schmidt et al., 2013). Coupling abalone ROV and SCUBA surveys with eDNA detection surveys would provide a great opportunity to utilize statistical methods to effectively address detection rates of abalone, in addition to calculating the number of eDNA samples required for a high detection probability. This would improve confidence in detecting abalone through eDNA and increase trust in the method with conservation managers.

As technology rapidly advances, laboratory constraints with this survey method are decreasing. The recent introduction of a backpack water filtration system and a handheld quantitative PCR (qPCR) unit provides a portable method for the eDNA detection of species. The Biomeme portable handheld qPCR system delivers rapid on-site detection of species within 60 minutes and has shown results comparable to those obtained in the lab (Nguyen et al., 2018; Thomas et al., 2019). This portable system has been used to rapidly detect invasive species on-site (Thomas et al., 2019), and as the system is improved to increase sensitivity and decrease inhibition (Sepulveda et al., 2018), a portable eDNA detection unit in conjunction with species-specific primers could provide near instantaneous results to guide diver and ROV abalone surveys while out in the field.

In the United States, the U.S. Fish and Wildlife Service has been using eDNA as a complementary survey method since 2013 as an early detection tool for several species of invasive Asian carp in the Great Lakes. Any positive detection of Asian carp from eDNA in the Great Lakes or nearby river basins warrants an immediate response from the monitoring program to help inform where traditional methods should be used for further assessment. Likewise, eDNA surveillance could be used to complement and guide visual survey methods to locate white abalone in the wild for population assessments in accordance with the White Abalone Recovery Plan (NMFS 2008). Furthermore, pursuant to section 7 of the ESA, any federal agency activity, such as coastal development or construction and wastewater discharge, that may affect an endangered white or black abalone is required to consult with the National Marine Fisheries Service to ensure their action will not jeopardize the existence of a listed species. Environmental DNA surveillance could be a quick way to initially assess the presence/absence of cryptic endangered white and black abalone in the area of concern to help streamline efforts. All seven abalone species in southern California and the recovery of their depleted populations is managed by the Abalone Recovery and Management Plan. Similarly, eDNA surveys could be a relatively quick and cost-effective tool providing abalone species information from a single water sample

to assess presence along the California coast and the offshore Channel Islands. While not meant to replace traditional abalone survey methods, using eDNA to assess an area could be a valuable complementary tool to prioritize resource allocation.

Future efforts

Future research in abalone eDNA shedding and decay rates in the lab will provide insight as to how much eDNA is being released and how long it persists in the environment, allowing for a more accurate assessment of how far abalone eDNA can travel from the source. Further field experiments can be conducted on SCUBA to evaluate the horizontal and vertical spatial distribution of eDNA from an abalone to understand how their eDNA interacts with the environment. Additionally, the samples collected from this study's tank experiments can also be evaluated with qPCR to identify how much eDNA was collected at each sample in every replicate, and if the distribution of eDNA was uniform throughout the tank. We utilized genus-specific primers in our experiments, which is useful in assessing all abalone species present in water samples from field sites. The development and testing of species-specific primers will aid in the direct detection of individual species, which will be most useful to quickly survey for endangered white and black abalone species to inform conservation managers of their presence. Furthermore, metabarcoding techniques in aquarium experiments will allow us to analyze eDNA from different ratios of multiple abalone species and evaluate if rare species can be detected among common abalone species.

Conclusion

This project served as an initial pilot study investigating the potential of using abalone eDNA as a survey method. The tank experiments demonstrate that abalone are capable of dispersing eDNA throughout a two million liter volume, and can be detected from $1/10^6$ of this volume. Field validation was possible at two locations where abalone are found and demonstrated the need to optimize laboratory protocols in order to minimize inhibition and maximize detection probability. To use eDNA as an effective and accurate monitoring tool to complement current abalone survey methods, further research is needed to understand the relationship between the organism and its eDNA, the relationship between eDNA and the environment, and to improve confidence in eDNA species detection. Accurate interpretation of abalone eDNA could ultimately facilitate in locating abalone for visual surveys and provide presence-absence data for more accurate species distribution models. As research advances, utilizing eDNA will enhance efforts to monitor and protect endangered and recovering abalone species.

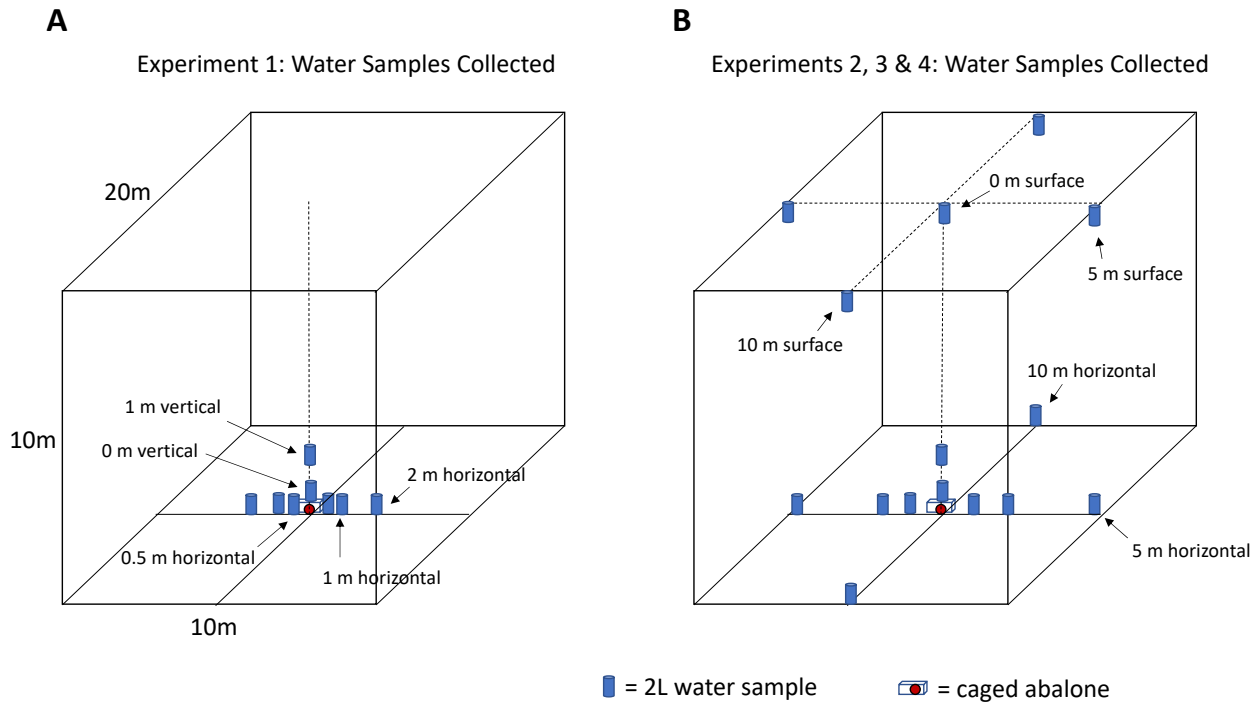


Figure 1. Experimental sampling design. Depicted are the water samples collected at measured distances from the caged red abalone (*Haliotis rufescens*) for each experiment conducted in the technology tank. Water samples, represented by the blue cylinder, were collected 18 hours after initial placement of the caged animal. **A)** 2L water samples collected in Experiment 1: at distances 0.5 m, 1 m, and 2 m horizontally from the caged abalone in opposite directions; and 0 m and 1 m above the cage. **B)** Experiments 2, 3, and 4 collected 2L water samples from an expanded range: at distances 1 m, 2 m, 5 m, and 10 m horizontally from the caged abalone in opposite directions; 0 m and 1 m above the cage; and surface samples collected directly above the animal, and at each center edge of the tank.

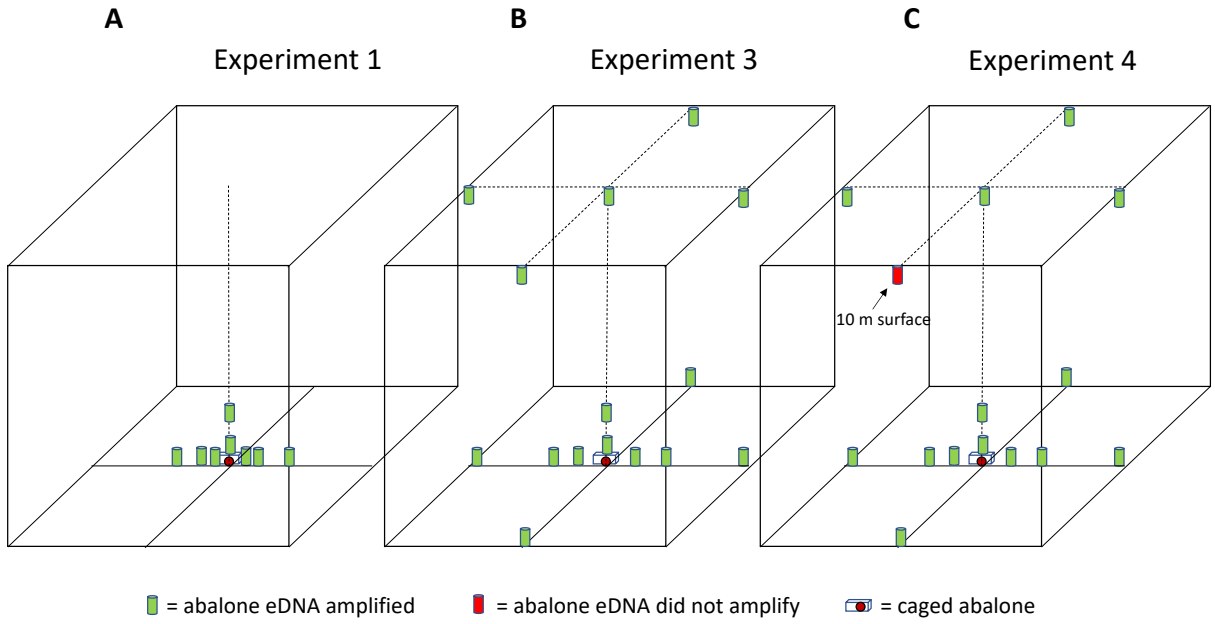


Figure 2. Results of abalone eDNA amplified in tank experiments. PCR results for the amplification of abalone (*Haliotis* spp.) eDNA from water samples collected for tank Experiments 1, 3, and 4. Results from Experiment 2 were omitted due to contamination and inconsistent outcomes. **A)** Experiment 1 showed positive amplification of abalone eDNA in all water samples collected, as indicated by the green cylinders. **B)** Experiment 3 showed positive amplification of abalone eDNA in all water samples collected using the expanded sampling range, as indicated by the green cylinders. **C)** Experiment 4 showed positive amplification of abalone eDNA in fourteen out of fifteen water samples collected for the expanded sampling range. The only sample that did not amplify abalone eDNA, as indicated by the red cylinder, was from a sample collected on the surface, 10 m from the center of the tank.

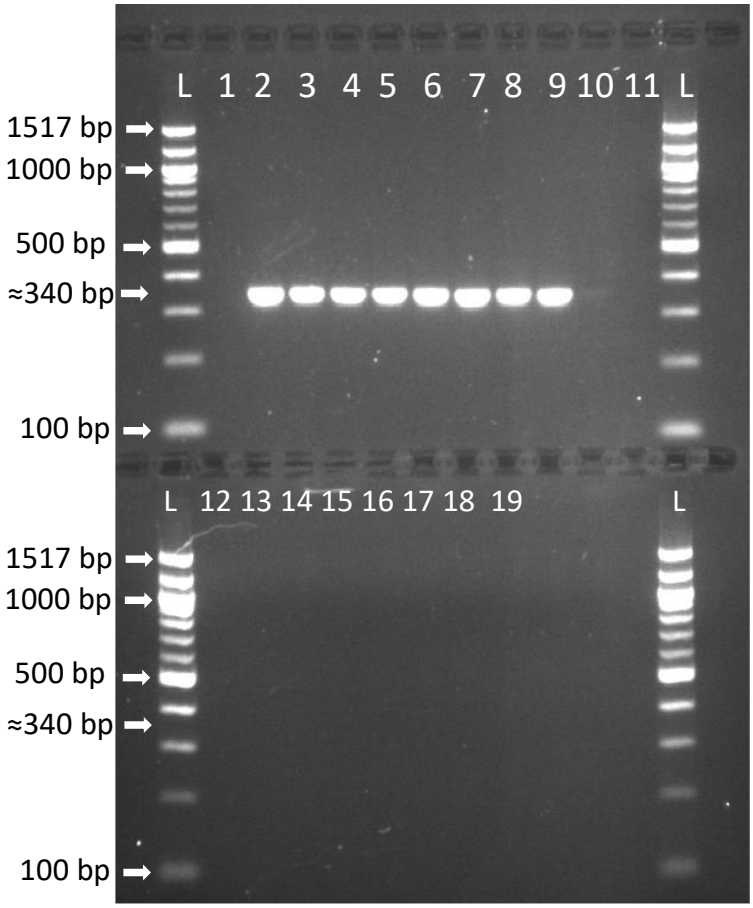
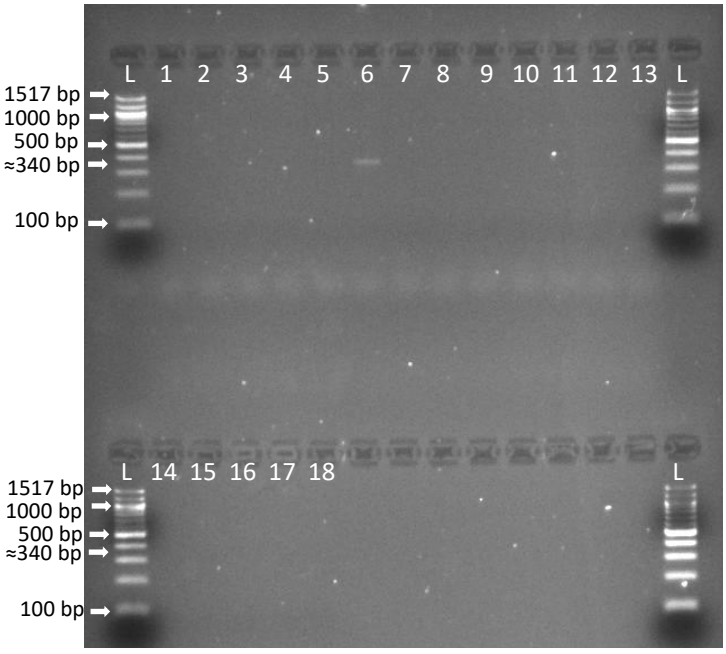


Figure 3. Agarose gel electrophoresis of amplified abalone eDNA from Experiment 1 and field site water samples. PCR products from tank Experiment 1 and initial trial of field site water samples using PCR Protocol 1 were visualized on a 2% agarose gel after a 30 minute migration at 120 volts. 100 base pair (bp) DNA ladders were run in the left and right outside lanes. Amplified products using abalone (*Haliotis* spp.) genus-specific primers were ≈340bp. Lane identification key: 1 = tank control, 2 = 0 m, 3 = 1 m vertical, 4 = 0.5 m horizontal, 5 = 1 m horizontal, 6 = 2 m horizontal, 7 = 0.5 m horizontal, 8 = 1 m horizontal, 9 = 2 m horizontal, 10 = New Hope Rock (NHR) field site (very faint band), 11 = NHR 1:10 dilution, 12 = NHR with Zymo Inhibitor Removal Kit (IRK), 13 = NHR 1:10 dilution with IRK, 14 = White Abalone Outplant (WAO) field site, 15 = WAO 1:10 dilution, 16 = WAO with IRK, 17 = WAO 1:10 dilution with IRK, 18 = extraction control, 19 = no template control.

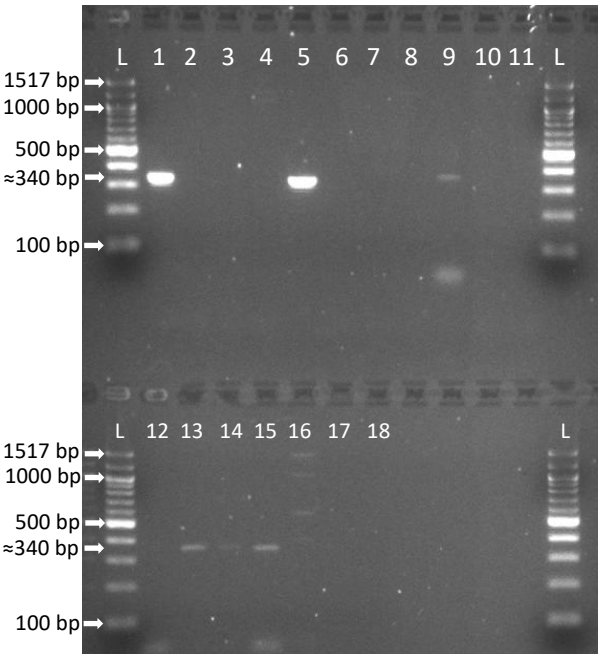
A

Experiment 2



B

Experiment 2 re-run



C

Experiment 2 re-run

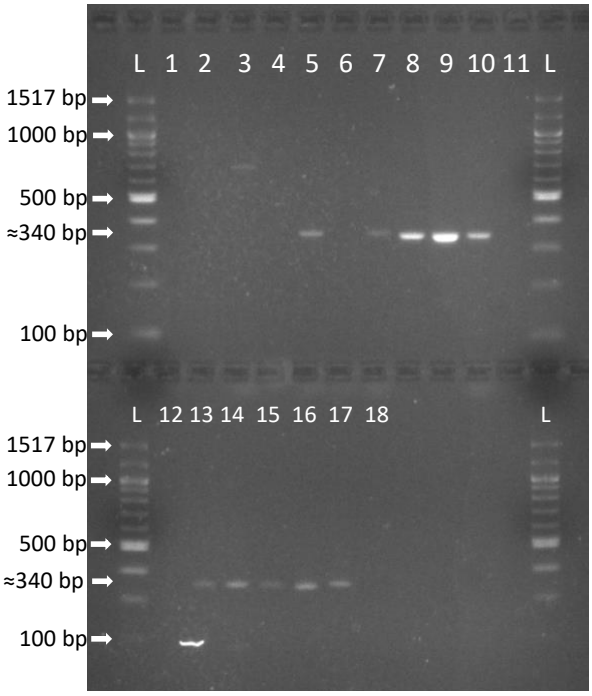


Figure 4. Agarose gel electrophoresis of amplified abalone eDNA from Experiment 2. PCR products from tank Experiment 2 were visualized on a 2% agarose gel after a 30 minute migration at 120 volts. 100 base pair (bp) DNA ladders were run in the left and right outside lanes. Amplified products using abalone (*Haliotis* spp.) genus-specific primers were ≈340bp. **A)** PCR products from tank Experiment 2 water samples using PCR Protocol 1. Lane identification key: 1 = tank control, 2 = extraction control, 3 = 5 m horizontal, 4 = 2 m horizontal, 5 = 1 m horizontal, 6 = 0 m, 7 = 1 m vertical, 8 = 1 m horizontal, 9 = 2 m horizontal, 10 = 5 m horizontal, 11 = 5 m from surface center, 12 = 5 m from surface center, 13 = 10 m from surface center, 14 = 10 m horizontal, 15 = 10 m from surface center, 16 = 10 m horizontal, 17 = surface above cage, 18 = no template control. **B)** PCR products from tank Experiment 2 water samples re-run using PCR Protocol 1. Lane identification key is the same as in A. **C)** PCR products from tank Experiment 2 water samples re-run using PCR Protocol 2. Lane identification key is the same as in A.

Experiment 3

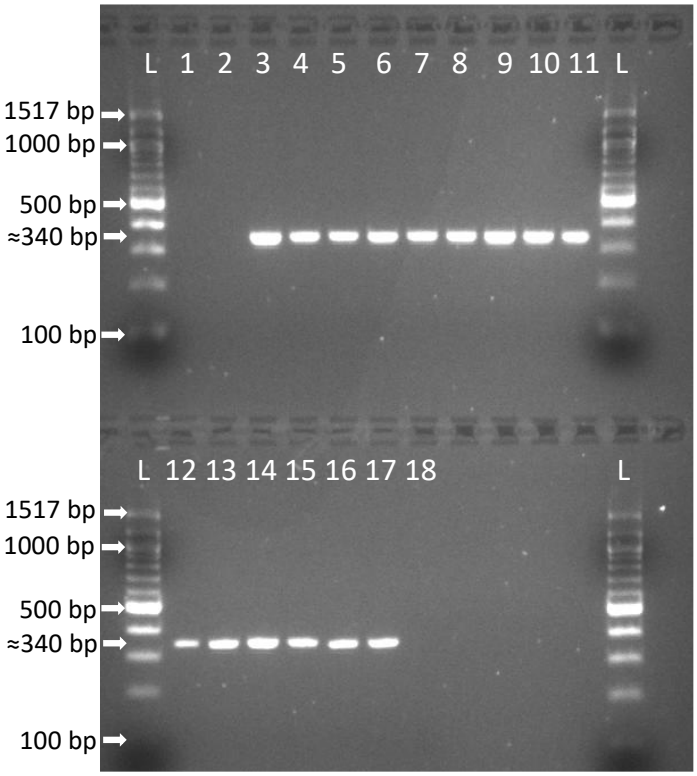


Figure 5. Agarose gel electrophoresis of amplified abalone eDNA from Experiment 3. PCR products from tank Experiment 3 water samples using PCR Protocol 1 were visualized on a 2% agarose gel after a 30 minute migration at 120 volts. 100 base pair (bp) DNA ladders were run in the left and right outside lanes. Amplified products using abalone (*Haliotis* spp.) genus-specific primers were ≈340bp. Lane identification key: 1 = tank control, 2 = extraction control, 3 = 0 m, 4 = 1 m vertical, 5 = surface above cage, 6 = 10 m from surface center, 7 = 10 m horizontal, 8 = 5 m from surface center, 9 = 5 m horizontal, 10 = 2 m horizontal, 11 = 1 m horizontal, 12 = 1 m horizontal, 13 = 2 m horizontal, 14 = 5 m horizontal, 15 = 5 m from surface center, 16 = 10 m horizontal, 17 = 10 m from surface center, 18 = no template control.

Experiment 4

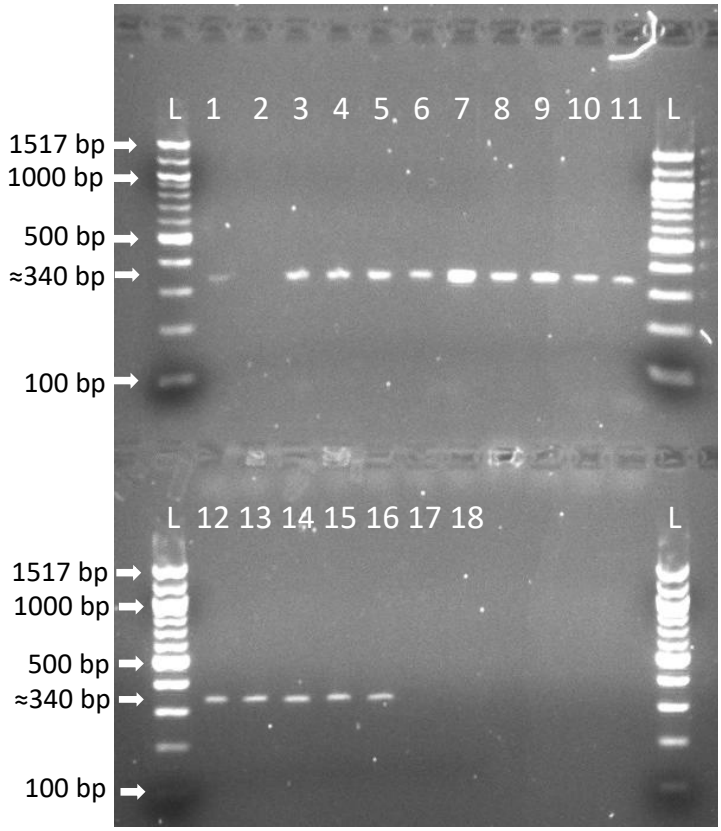


Figure 6. Agarose gel electrophoresis of amplified abalone eDNA from Experiment 4. PCR products from tank Experiment 4 water samples using PCR Protocol 1 were visualized on a 2% agarose gel after a 30 minute migration at 120 volts. 100 base pair (bp) DNA ladders were run in the left and right outside lanes. Amplified products using abalone (*Haliotis* spp.) genus-specific primers were ≈340bp. Lane identification key: 1 = tank control, 2 = extraction control, 3 = 0 m, 4 = 1 m vertical, 5 = surface above cage, 6 = 10 m from surface center, 7 = 10 m horizontal, 8 = 5 m from surface center, 9 = 5 m horizontal, 10 = 2 m horizontal, 11 = 1 m horizontal, 12 = 1 m horizontal, 13 = 2 m horizontal, 14 = 5 m horizontal, 15 = 5 m from surface center, 16 = 10 m horizontal, 17 = 10 m from surface center, 18 = no template control.

Field Sites

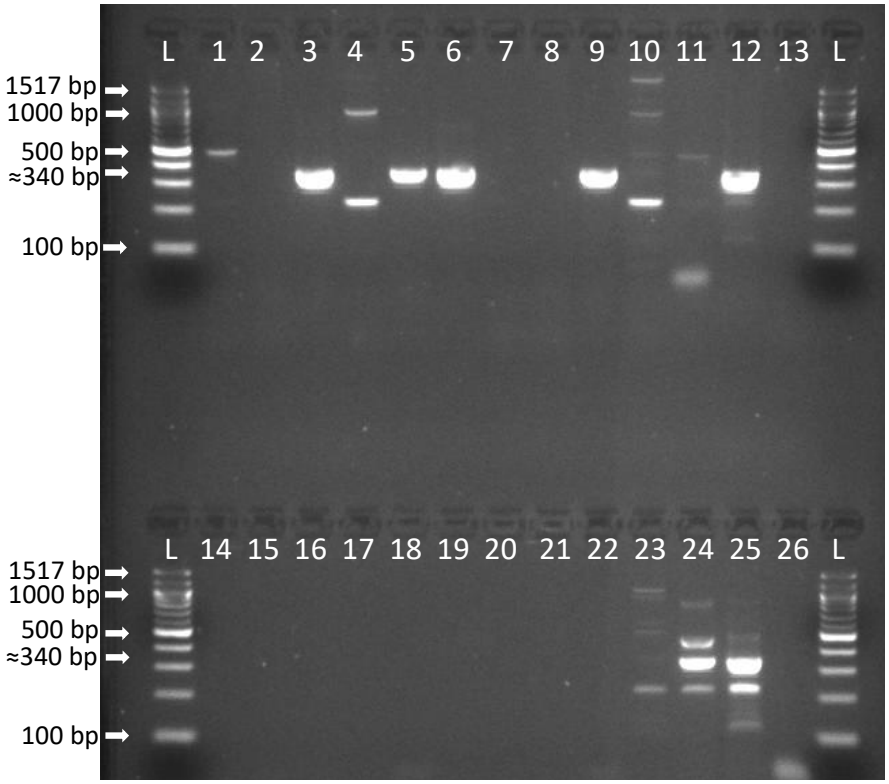


Figure 7. Agarose gel electrophoresis of amplified abalone eDNA from field sites. PCR products from field site water samples were visualized on a 2% agarose gel after a 30 minute migration at 120 volts. 100 base pair (bp) DNA ladders were run in the left and right outside lanes. Amplified products using abalone (*Haliotis* spp.) genus-specific primers were ≈340bp. PCR products from field site water samples using PCR Protocol 1 with 45 cycles and PCR Protocol 2 with 45 cycles are in lanes 1-13 and 14-26 respectively. Lane identification key: 1 = 1 µl New Hope Rock (NHR), 2 = 2 µl NHR, 3 = 1 µl NHR spiked with 1 µl *H. rufescens* DNA, 4 = 1 µl NHR with Inhibition Removal Kit (IRK), 5 = 2 µl NHR with IRK, 6 = 1 µl NHR with IRK spiked with 1 µl *H. rufescens* DNA, 7 = 1 µl White Abalone Outplant (WAO), 8 = 2 µl WAO, 9 = 1 µl WAO spiked with 1 µl *H. rufescens* DNA, 10 = 1 µl WAO with IRK, 11 = 2 µl WAO with IRK, 12 = 1 µl WAO with IRK spiked with 1 µl *H. rufescens* DNA, 13 = no template control, 14 = 1 µl NHR, 15 = 2 µl NHR, 16 = 1 µl NHR spiked with 1 µl *H. rufescens* DNA, 17 = 1 µl NHR with IRK, 18 = 2 µl NHR with IRK, 19 = 1 µl NHR with IRK spiked with 1 µl *H. rufescens* DNA, 20 = 1 µl WAO, 21 = 2 µl WAO, 22 = 1 µl WAO spiked with 1 µl *H. rufescens* DNA, 23 = 1 µl WAO with IRK, 24 = 2 µl WAO with IRK, 25 = 1 µl WAO with IRK spiked with 1 µl *H. rufescens* DNA, 26 = no template control.

Table 1

Primer sequences designed to amplify abalone DNA.

Primer name	Sequence
Abalone-eDNA_F	5'- TTG CCA CAA TCC ACG GTG -3’
Abalone-eDNA_R	5'- GCT TTY GTT CAT CGG GCG T -3’
Abalone-eDNA_F2	5'- GTA GAC ACH CGT GCC TAC TTC -3’
White/Pinto_R	5'- GTC TTA TCT TGG TGG ATT TCT TTA C -3’
White-ab_F1	5'- TTG AAG TCG CTC GGG CTG ATA T -3’
White-ab_F2	5'- AGT CGC TCG GGC TGA TAT -3'

Table 2

PCR Protocols used and results for each tank experiment

Experiment	PCR Protocol	# samples detecting presence	Sample distances (m) with presence
Tank Experiment 1	PCR Protocol 1	8/8; strong bands	horizontal: 2 x 0.5, 2 x 1, 2 x 2 vertical: 0, 1
Tank Experiment 2	PCR Protocol 1	1/15; faint band	vertical: 0
Tank Experiment 2	Re-run Protocol 1: 45 cycles 2 µl DNA	Contamination in control	
Tank Experiment 2	PCR Protocol 2: 45 cycles 2 µl DNA	10/15; 2 strong bands, 8 weak bands	horizontal: 2 x 1, 2, 5, 2 x 10 vertical: 1 surface: 0, 2 x 10
Tank Experiment 3	PCR Protocol 1	15/15; strong bands	horizontal: 2 x 1, 2 x 2, 2 x 5, 2 x 10 vertical: 0, 1 surface: 0, 2 x 5, 2 x 10
Tank Experiment 4	PCR Protocol 1	14/15; strong bands	horizontal: 2 x 1, 2 x 2, 2 x 5, 2 x 10 vertical: 0, 1 surface: 0, 2 x 5, 10

Table 3

PCR protocols used and results for each field site

Field Site	PCR Protocol, cycles, and DNA template	# samples detecting presence
White Abalone Outplant	PCR Protocol 1: 35 cycles; uncleaned DNA: 1 µl of 1:10 dilution, 1 µl; DNA cleaned with inhibition removal kit (IRK): 1 µl 1:10 dilution, 1 µl	0/4; Inhibition
White Abalone Outplant	PCR Protocol 2: 35 cycles; uncleaned DNA: 1 µl of 1:10 dilution, 1 µl; DNA cleaned with IRK: 1 µl 1:10 dilution, 1 µl	1/4; very faint band for 1:10 diluted DNA cleaned with IRK
New Hope Rock	PCR Protocol 1: 35 cycles; uncleaned DNA: 1 µl of 1:10 dilution, 1 µl; DNA cleaned with IRK : 1 µl 1:10 dilution, 1 µl	1/4; very faint band for uncleaned 1 µl DNA
New Hope Rock	PCR Protocol 2: 35 cycles; uncleaned DNA: 1 µl of 1:10 dilution, 1 µl; DNA cleaned with IRK : 1 µl 1:10 dilution, 1 µl	0/4; Inhibition
White Abalone Outplant	PCR Protocol 1: 45 cycles; uncleaned DNA: 1 µl, 2 µl, 1µl + 1 µl red abalone (*Haliotis rufescens*) DNA as internal positive control; DNA cleaned with IRK: 1 µl, 2 µl, 1µl + 1 µl red abalone DNA	0/4; Inhibition
White Abalone Outplant	PCR Protocol 2: 45 cycles; uncleaned DNA: 1 µl, 2 µl, 1µl + 1 µl red abalone DNA; DNA cleaned with IRK: 1 µl, 2 µl, 1µl + 1 µl red abalone DNA	1/4; strong band with 2 µl DNA cleaned with IRK
New Hope Rock	PCR Protocol 1: 45 cycles; uncleaned DNA: 1 µl, 2 µl, 1µl + 1 µl red abalone DNA; DNA cleaned with IRK: 1 µl, 2 µl, 1µl + 1 µl red abalone DNA	1/4; strong band with 2 µl DNA cleaned with IRK
New Hope Rock	PCR Protocol 2: 45 cycles; uncleaned DNA: 1 µl, 2 µl, 1µl + 1 µl red abalone DNA; DNA cleaned with IRK: 1 µl, 2 µl, 1µl + 1 µl red abalone DNA	0/4; Inhibition

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