UNIVERSITY OF CALIFORNIA

Los Angeles

The Use of Environmental DNA in Evaluating Conservation Efforts within Southern California's Marine Waters

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Environment and Sustainability

by

Tanner Waters

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ABSTRACT OF THE DISSERTATION

The Use of Environmental DNA in Evaluating Conservation Efforts within Southern California's Marine Waters

by

Tanner Waters

Doctor of Philosophy in Environment and Sustainability

University of California, Los Angeles, 2023

Professor Robert A. Eagle, Co-Chair

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Environmental DNA metabarcoding (eDNA) is an evolving methodology of biodiversity monitoring. By leveraging shed and excreted DNA from organisms in a marine environment, researchers can perform DNA amplification and sequencing to identify what species were present at a site without the need for visual identification. This thesis uses eDNA to evaluate conservation efforts in Southern California as well as further establish and refine eDNA as a survey tool. In Chapter 1, I evaluate seagrass beds in three distinct geographic settings in Southern California. We demonstrate that nearly half of community composition is associated with whether seagrass was found off the mainland, off an island, or in an embayment. In addition, when compared to traditional monitoring, eDNA was able to identify roughly ~50% to 400% greater number of fish species at each site. In Chapter 2, I create a novel eDNA droplet digital PCR assay for the detection of the invasive species *Caulerpa prolifera* that was found in

Newport Bay in 2021 and found it to have the lowest eDNA steady state of any previously tested species. We demonstrate the critical importance of benchmarking eDNA assays before their use by managers and highlight some limitations for using eDNA in species detection. In Chapter 3, we perform two years of monthly sampling of MPAs using community scientists and UCLA researchers. We find that MPAs are distinct from nonMPAs in multivariate space for both fish and metazoan communities and that metazoan communities in MPAs are more diverse and stable compared to nonMPAs. We also show the scalability that eDNA has in biodiversity monitoring through the incorporation of volunteers into field sampling. Together, these chapters better our understanding of marine conservation in Southern California and further demonstrate the utility and limitations of eDNA metabarcoding for monitoring marine biodiversity.

The dissertation of Tanner Waters is approved.

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2023

DEDICATION

To my mother, my father, and my twin brother.

Your love and inspiration makes everything possible.

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Vita

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Introduction

Threats to our global oceans

More than 80% of the ocean is unmapped and unobserved. This fact is often used to conjure up images of a dark and mysterious world deep in the ocean still left to be discovered. However, we don't need to continue exploring to know that humans are already having an outsized negative impact on our oceans and the things that live in it. Global sea surface temperatures have increased by an average of 0.11◦C per decade in the last 100 years (IPCC, 2013), the ocean's pH has acidified by 0.1 since the start of the industrial revolution (IPCC, 2013), hypoxia dead zones now cover more than 245,000 km^2 of ocean (Diaz and Rosenberg, 2008), and over 5 trillion plastic pieces pollute our waters (Eriksen et al., 2014). These singular and cumulative impacts have left no area of the ocean unaffected by humans (Halpern et al., 2008).

These changes are having a direct impact on marine ecosystems and the organisms that live in them. Fish are changing their body size (Audzijonyte et al., 2020) and shifting their reproduction and development (Pankhurst and Mudnay, 2010), marine calcifying organisms are experiencing reduced survival, growth and calcification (Kroeker et al., 2010), and phytoplankton are decreasing in size which is altering their contribution to nutrient cycling (Mousing et al., 2014; Winder and Sommer, 2012). Beyond the morphological and physiological effects on species, our impact on the global ocean has led to the extinction of over one third of marine mammals, sharks and corals (IPEBS, 2019), an average species range shift of 72 km/decade (Poloczanska et al., 2013) and a projected turnover over 60% of current biodiversity (Cheung et al., 2009). There is no doubt that species diversity is changing in our oceans and will continue to do so.

California's oceans

The study system for this thesis is the Southern California Bight (SCB). The SCB is a bend in the shoreline of Southern California from Point Conception to south of San Diego and includes the Channel Islands. The Bight is a uniquely diverse ecosystem due to upwelling that occurs in the system, the interaction of the California Current and Counter Current which creates the Southern Californian Eddy, and the wide range of habitats that are found here from kelp forests, rocky reefs, seagrass, and estuaries. These factors lead this to be a species rich area known as a biodiversity hotspot (McGinnis, 2006).

Despite this designation, the waters of Southern California are under threat of ocean acidification (Schiff et al., 2019), warming sea surface temperatures (Phillips and Sievanen, 2018), hypoxia (McClatchie et al., 2010), overfishing (Koslow et al., 2015), and pollution (Bay et al., 2003). It is known as one of the most threatened marine biodiversity hot spots globally (McGinnins, 2006). Alteration of the physical, chemical and biological parameters of the ocean put these species at risk. These issues directly threaten the health and likelihood of survival of the species that inhabit the SCB. Without intervention, we risk altering the community composition of our ecosystems and losing these unique species and habitats to ongoing anthropogenic impacts. In order to evaluate the ever-changing nature of our oceans, biodiversity monitoring needs to be implemented at wide scale and high frequency.

Biodiversity monitoring

Conventional biodiversity monitoring employs a number of different methodologies depending on the ecosystem and taxa of interest. Each methodology carries its own pros, cons, and biases that must be acknowledged when surveying species richness and diversity. Marine

researchers may use dredges, trawls, or baited traps; nets, plankton pumps or long-lines for pelagic communities; scuba surveys, mounted video, or ROV photographs for epifaunal community; and sediment cores for infaunal communities (Costello et al., 2016). These methods, although traditionally used, make monitoring biodiversity difficult. Many larval and juvenile stage organisms are too morphologically similar to differentiate and are unable to be resolved in surveys (Thomsen and Willerslev, 2015). Other organisms, known as 'cryptic' species, are able to evade detection through means of camouflage or hiding (Stoof-leichsenring et al., 2012). Even organisms that are easily observable require that researchers have an intimate taxonomic knowledge of the species they're studying in order to properly identify them. This makes biodiversity monitoring less accessible. Some conventional methods, such as scuba surveying or fishing, require specific environmental conditions to be able to complete the survey which can lead to a bias in temporal frequency (Thomsen and Willerslev, 2015). Other sampling techniques, such as bottom trawling, are destructive to the environment and cause harm to the environments and organisms that are being studied (Costello et al., 2016). These pitfalls have led researchers to try and find new ways of studying biodiversity.

Environmental DNA

One such technique described by Pace et al. (1987) aimed to analyze microbial populations using ribosomal RNA found in the environment. This idea evolved into the concept of environmental DNA metabarcoding (eDNA). Organisms produce DNA that can be later detected within the environment via a water, soil, or sediment sample. This can happen through the exfoliation of cells, excretion of feces and urine, release of gametes during reproduction or any other activity that releases cells and free-floating DNA into the environment (Barnes and Turner, 2016).

Monitoring with eDNA takes advantage of this free-floating environmental DNA by collecting it in water samples and trapping it in a filter. To identify the species represented in the water sample, taxa-specific genes are amplified using PCR. These genes, known as barcodes, can be used to identify taxa as specific as a single species to as broad as 'metazoans. Once the barcodes are amplified, the DNA is sequenced and compared against a reference database. Since sequences are species dependent, matching the sample DNA sequence with a list of known sequences allows researchers to identify the organisms that were present in the environment. Environmental DNA provides a non-invasive, cost effective and logistically simple way to sample species richness that has been shown to not only match but outperform conventional sampling methods in species identification when directly compared (Hänfling et al., 2016; Shaw et al., 2016; Yamamoto et al., 2017; Fediajevaite et al., 2021).

Environmental DNA shows exceptional promise as a biomonitoring tool (Deiner et al. 2017), but questions on its applications still remain. While there have been studies on the spatial extent of eDNA's signal and its persistence in the environment, many questions on the shedding, degradation, and fate and transport of eDNA remain unresolved (Thomsen and Willerslev, 2015). This is impacted by our limited understanding of how physical, chemical, and biological factors influence the degradation of environmental DNA and how eDNA's different physical states impact its spatial and temporal extent. Previous work has shown that DNA will remain in the environment and get transported until it degrades (Harrison et al. 2019). Degradation is dependent on DNA form and is influenced by temperature, pH, DO, light, enzymatic activity, and mechanical degradation (Barnes et al., 2014; Eichmiller et al., 2016; Lance et al., 2017; Strickler et al., 2015; Weltz et al., 2017). Depending on these factors, environmental DNA usually lasts between one day to one week (Minamoto et al., 2017; Sassoubre et al., 2016;

Thomsen et al., 2012) but can last up to three weeks or longer under conditions which slow decay (Eichmiller et al., 2016). In this time, marine eDNA can be transported generally between 30-150m but up to multiple kilometers depending on the dynamics of the environment (Murakami et al., 2018; O'Donnel et al., 2017; Yamamoto et al., 2016). Understanding the fate and transport of eDNA dynamics is critical for the accurate interpretation of detected eDNA signals. Thus improving our understanding of both the spatial and temporal resolution of eDNA approaches in the field as well as understanding the physiological mechanisms that control eDNA generation and degradation are critical for the application of eDNA as an effective monitoring tool.

This specifically impacts our ability to quantify abundances from eDNA. Deriving abundance information from eDNA signatures obtained in the field requires researchers to have a complete understanding of the impact that the processes from eDNA shed, to collection, to laboratory amplification and analysis has on the data. Numerous studies have shown environmental DNA to be positively correlated with biomass (Doi et al., 2016; Pilliod et al., 2013; Takahara et al., 2012), but with generally mixed results. These studies have mostly taken a correlative approach linking final sequence reads to species abundance in the field. This doesn't account for the underlying processes that influence the capture of eDNA or its characteristics in the environment. Modeling studies have begun to help us understand how our steps from extraction to sequencing in the laboratory influence observed results (Shelton et al. 2023, Silverman et al. 2021, Gold et al. 2023b). However, to extend such frameworks beyond controlled laboratory settings (Gold et al. 2023a), additional work in understanding both spatial and temporal signatures as well as species-specific DNA shedding and decay rates in the marine environment are needed.

In order for environmental DNA to become a mainstream biodiversity monitoring tool it needs to continue being refined and show that it's a viable methodology for effectively surveying different ecosystems. Chapter one studies five distinct seagrass beds in Southern California by taking seasonal eDNA samples at each site. We compare three geographical regions which seagrass is found in Southern California- in embayments, off the mainland and off islands- to understand how their fish communities differ from one another. We ground truth this work by comparing our eDNA surveys to conventional scuba surveys. This project will not only demonstrate eDNA's capabilities and resolution within coastal marine ecosystems but will allow us to understand the importance of both preserving different geographic seagrass meadows. The second chapter creates a novel eDNA assay for the detection of an invasive algae, *Caulerpa prolifera*, found in Newport Bay. Through both field and tank based experiments we test the limitations of eDNA's use in invasive species detection. This work will highlight the importance of benchmarking novel eDNA assays in the laboratory and field and improves our understanding of the role of species-specific physiology on eDNA detections. Furthermore, this work will be crucial in understanding the proper application of eDNA for time-sensitive invasion work. The third chapter is a two-year monthly study of two marine protected areas and two unprotected areas in Malibu, CA. This chapter uses the power of community scientists, in collaboration with Heal the Bay, to study how MPA protections are influencing the fish and broader metazoan communities. Together, these projects will help to better our understanding of the environmental DNA within our coast, its ability to describe community composition of various ecosystems and demonstrate its accessibility and scalability to the general public. By doing so, we help to squarely place environmental DNA as the next frontier for biodiversity monitoring.

CHAPTER 1

Environmental DNA metabarcoding reveals distinct fish assemblages supported by seagrass (Zostera marina and Zostera pacifica) beds in different geographic settings in Southern California

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Abstract

Seagrass beds are disappearing at a record pace despite their known value to our oceans and coastal communities. Simultaneously, our coastlines are under the constant pressure of climate change which is impacting their chemical, physical and biological characteristics. It is thus pertinent to evaluate and record habitat use so we can understand how these different environments contribute to local biodiversity. This study evaluates the assemblages of fish found

at five *Zostera* beds in Southern California using environmental DNA (eDNA) metabarcoding. eDNA is a powerful biodiversity monitoring tool that offers key advantages to conventional monitoring. Results from our eDNA study found 78 species of fish that inhabit these five beds around Southern California representing embayment, open coastal mainland and open coastal island settings. While each bed had the same average number of species found throughout the year, the composition of these fish assemblages was strongly site dependent. There were 35 fish that were found at both open coast and embayment seagrass beds, while embayment seagrass sites had 20 unique fish and open coast sites had 23 unique fish. These results demonstrate that seagrass fish assemblages are heterogenous based on their geographic positioning and that marine managers must take this into account for holistic conservation and restoration efforts.

Introduction

Seagrass ecosystems are ecologically, economically, and culturally significant in California. They provide dozens of ecosystem services including acting as juvenile fish nurseries [1], providing invertebrates habitat and food [2], protecting coastlines [3], stabilizing sediment [4], purifying water [5], sequestering carbon [6] and mitigating ocean acidification [7]. Seagrass beds are also known to be one of the most productive ecosystems on earth and hotspots for biodiversity in coastal systems [1,8]. They have been estimated to provide ecosystem services of over $19,000 per ha of meadow per year [9]. This places the worth of their ecosystem services higher than tropical forests, coral reefs, and mangroves per unit area [9]. However, over 29% of seagrass has disappeared globally since 1879 [10] and over 90% within certain parts of California [11]. The trend of seagrass loss has accelerated from 0.9%/year prior to 1940 to 7%/year since 1990 [10]. In order to fully evaluate the impact of this seagrass loss and provide

justification for restoring these environments, it is necessary to evaluate the extent of the services they provide.

Seagrass beds are able to support high levels of biodiversity because they provide three-dimensional structure to an otherwise bare soft bottom seafloor. This vegetation provides a foundation for algae and epibionts to grow, which creates the basis of the ecosystem's food web. Associated seagrass species feed on the seagrass blades and associated epiphytes live on the blades. These species also use the seagrass' physical feature as protection from predators [12]. Fish diversity in particular is high within these habitats due to their dependence on seagrass as a nursery habitat [1]. Seagrass provides structural complexity for fish to attach their eggs to and for juvenile fish to hide from predators. While seagrass is known to increase survival rates compared to bare sand, seagrass is also shown to increase juvenile growth more than bare habitats and other structured habitats [13].

These beds are under constant stress of changing ocean conditions such as increased temperature, eutrophication, physical damage/removal and fishing pressures, Surveys done just ten years ago in the area may already be outdated in providing us with an understanding of the currently supported marine biodiversity [14-18]. Thus, there is a growing need to routinely monitor seagrass beds not just within embayments, but also on the open coast and Channel Islands within Southern California.

In order to assess the community composition of seagrass beds, this study employs environmental DNA (eDNA) metabarcoding. eDNA is the methodology of collecting free-

floating DNA and cells that have been excreted or secreted from organisms [19]. This DNA is extracted and certain gene regions, known as barcodes, are amplified and sequenced to reveal species presence or absence for broad-scale biodiversity, predator diet analysis and trophic interactions [20]. eDNA retains some key advantages in biodiversity monitoring. Firstly, eDNA can differentiate between morphologically similar species [20]. This is especially important in seagrass beds that are used as nurseries where visual surveys may be unable to identify juveniles down to the species level [20]. Conventional surveys require taxonomic identification by an expert which could introduce errors from possible misidentification. Secondly, eDNA has been shown to better detect rare and cryptic species that are more easily overlooked in conventional methods including highly camouflaged and sediment inhabiting taxa that are difficult to detect using visual surveys [20]. Thirdly, eDNA sampling is logistically less complex in the field than visual surveys, which allows researchers to take a greater number of samples across broader spatial and temporal ranges [21]. Lastly, eDNA has often been demonstrated to be cheaper, more sensitive and able to detect more species when directly compared to traditional methods of biodiversity monitoring [22].

Environmental DNA has been shown to be a powerful tool when surveying seagrass habitats. Researchers have previously employed this method via water column collection [23-28] and sediment sampling [29-30]. A number of these seagrass studies have demonstrated that when directly compared to a conventional survey method, eDNA was able to detect a higher number of species [23-25]. Other studies emphasized the importance of using concurrent eDNA and conventional survey techniques in revealing the full scope of biodiversity [26, 27, 30]. Despite the literature support of eDNA's use in seagrass monitoring, there has been no eDNA surveys

done on fish communities of Southern California seagrass beds, which sit in a very specific biogeographic position of a productive upwelling region for both island and mainland populations. In Southern California, some of the seagrass population is within marine protected areas and others within heavily human impacted urban coastal environments.

Environmental DNA approaches do have known limitations that warrant consideration. First, it is important to note the influence of taxonomic assignment on data output and interpretation. The accuracy of taxonomic assignments is largely driven by two features: barcode choice and reference database completeness. For example, a commonly used barcode used for fish diversity globally, the MiFish Universal primer set, is unable to resolve the majority of fish in the *Sebastes* (Rockfish) genus [31], an environmentally and commercially important species in California. Thus, without the use of an additional barcode, the MiFish Universal primer set fails to resolve *Sebastes* species. While only a small number of rockfish are known to inhabit Southern California seagrass beds, it is still worth noting that their species level resolution is not possible utilizing this marker set alone. Likewise, accurate taxonomic assignment can only be achieved with comprehensive reference barcode databases that contain sequences for all monitored species [32]. Fortunately, extensive efforts have been made in the California Current Large Marine Ecosystem to sequence the vast majority of marine fishes [33].

In addition to taxonomic assignments, interpretation of eDNA metabarcoding data is influenced by detection probabilities. Detection probabilities are a function of both the total concentration of DNA in the environment and assay efficacy [34]. The total concentration of DNA in the environment is a function of shedding rates, degradation [35], and fate and transport in marine

systems [36, 37] while assay efficiency for a given taxa is a function of methodological choices including volume filtered, inhibition, and PCR driven amplification bias among many others [38, 39]. Despite these limitations and biases, here we use well established marine eDNA assays with demonstrated efficacy in Southern California coastal marine ecosystems [40-44].

This study tests the utility of eDNA methods to provide seasonally resolved fish survey information in five *Zostera sp*. beds around Southern California with diverse biogeographic contexts like heavily human impacted embayments, open ocean coastal and island locations. Our aim is to better characterize the community composition of local seagrass beds as well as understand the benefits and limitations of using eDNA compared to conventional survey methods in coastal ecology biodiversity monitoring.

Materials and Methods

2.1 Sample Collection

We conducted our study of Southern California *Zostera* beds off the coast of Malibu, CA, Catalina Island, CA and Newport Bay, CA seasonally during 2019-2020. We collected these samples seasonally in Summer (July/August 2019), Fall (November 2019), Winter (February 2020) and Spring (May 2020). No permits were required for this work.

We sampled five *Zostera sp.* beds around Southern California: Amarillo, Two Harbors, Big Geiger Cove, Inner DeAnza Peninsula, and Outer DeAnza Peninsula. We collected additional samples at a sandy bottom control site on Catalina: Cherry Cove to compare with the two seagrass sites on Catalina- Big Geiger Cove and Two Harbors (Table 1).

Table 1: Sampling design for the seagrass and control sites.

Site	Environment	Geographic Type	Depth	Coordinates	Location	Visual Survey
Amarillo	*Z. pacifica*	Open Coast-Mainland	12.5 m	34.02755, -118.700084	Malibu, CA	Yes-Fall
Two Harbors	*Z. marina*	Open Coast-Island	5.8 m	33.443405, -118.49843	Catalina Island, CA	Yes-Fall
Big Geiger	*Z. marina*	Open Coast-Island	7.9 m	33.459704, -118.517454	Catalina Island, CA	Yes-Summer
Inner DeAnza Peninsula	*Z. marina*	Embayment	2.42 m	33.619506, -117.90291	Newport Bay, CA	No
Outer DeAnza Peninsula	*Z. marina*	Embayment	2.07 m	33.619269, -117.901692	Newport Bay, CA	No
Cherry Cove	Bare sand	NA	7.2 m	33.45129, -118.50195	Catalina Island, CA	No

These five sites represent distinct geographic locations of seagrass habitat within Southern California. These sites are grouped by their geography including open coast seagrass beds (mainland- Amarillo, island- Big Geiger Cove and Two Harbors) and embayment seagrass beds (Inner DeAnza Peninsula and Outer DeAnza Peninsula) (Fig 1).

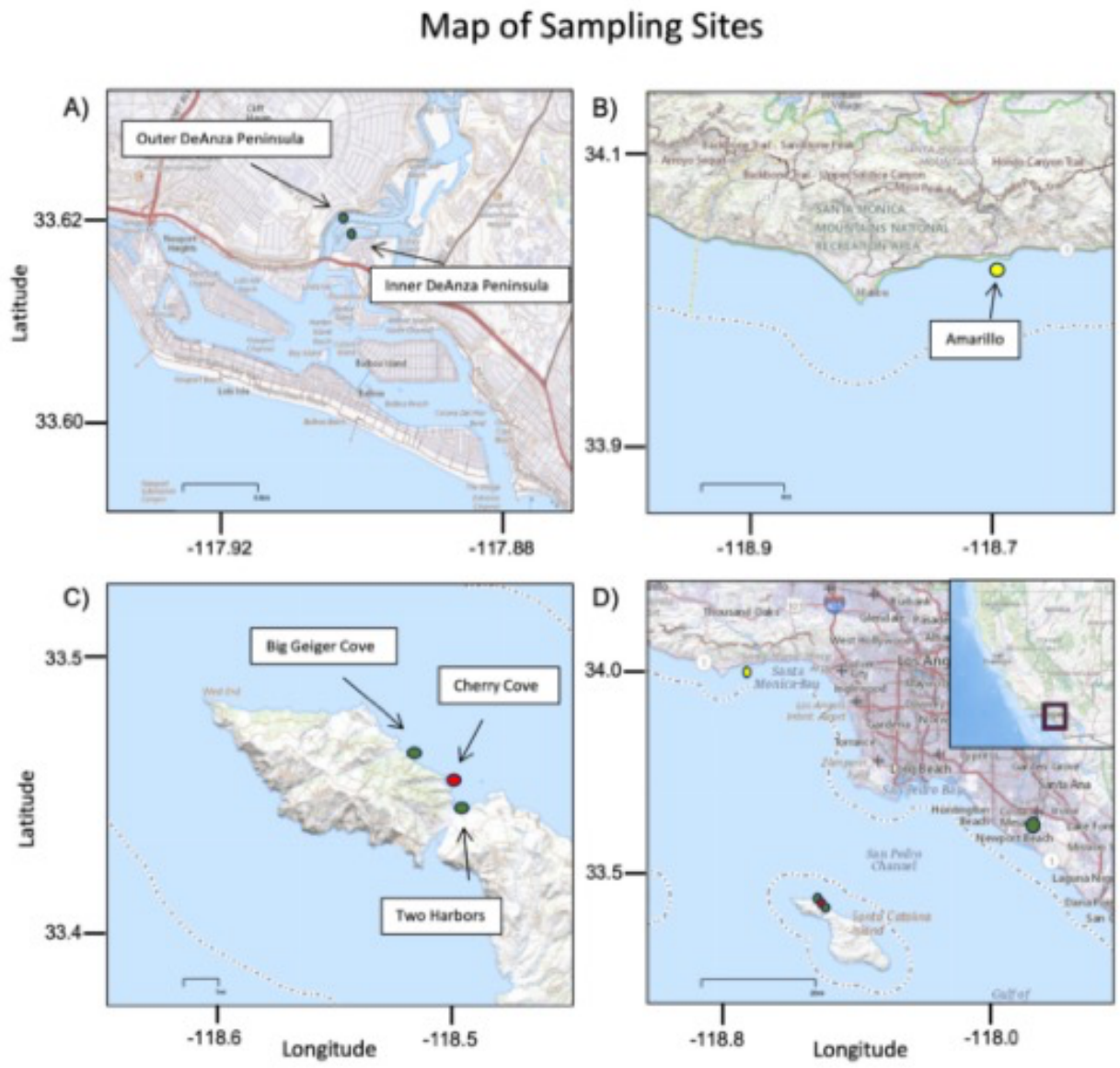

Fig 1. A) Map of embayment seagrass beds. B) Map of Open Coast Mainland seagrass beds. C) Map of Open Coast Island seagrass beds. D) Map of all sites. Yellow indicates *Zostera Pacifica,* green indicates *Zostera Marina*, and red indicates our no seagrass site. Maps from USGS National Map Viewer under a CC BY 4.0 license (2022): https://apps.nationalmap.gov/viewer/.

We employed the eDNA collection method of Curd et al. 2019 [45]. First, we collected seawater samples at depth directly above the seagrass beds using a 5L Niskin bottle. From the Niskin, we transferred one liter of seawater to a Kangaroo enteral feeding bag in triplicate. We immediately filtered the seawater through a sterile 0.22 μm Sterivex cartridge filter (MilliporeSigma, Burlington, MA, USA) using a peristaltic pump until the 1L was fully through our cartridge. We capped the filters and stored them on dry ice during sampling until we returned to the lab where they were stored at -20°C in the lab. During each day of sampling, we filtered one liter of Milli-Q water through the same process for a negative field control [46].

2.2 DNA extraction and library preparation

We extracted our eDNA from the Sterivex cartridge using a modified DNeasy Blood & Tissue Kit protocol (Qiagen Inc., Germantown, MD) optimized for increased eDNA yield [47]. Library preparation followed a modified protocol [35]. Using PCR, we amplified the extracted DNA using the Mifish Universal Telost 12S primer set [48]. We used a 25 μL reaction composed of 12.5 μL QIAGEN Multiplex Taq PCR 2x Master Mix,1.5 μL of molecular grade H2O, 5 μL of forward primer (2 mM), 5 μL of reverse primer (2 mM), and 1μL of sample DNA. Our cycling conditions consisted of a touchdown PCR profile with an initial denaturation at 95°C for 15 min, followed by 13 cycles of 95°C for 30s, beginning annealing at 69.5°C for 30 seconds which decreases in temperature 1.5°C per cycle until 50°C, extension at 72°C for 1 minute. After the 13 cycles we did 24 cycles of 95°C for 30s followed by annealing at 50°C and an extension of 72°C for 1 minute. We use a final extension for 10 min at 72°C. A negative PCR control of molecular grade water was added following the same protocol. We verified amplification success by checking product size on a 2% agarose gel electrophoresis stained with SybrSafe.

After amplification, we modified the samples by adding individual Nextera unique dual indices (Illumina, San Diego, CA, USA) . We used a 25 µL reaction composed of 12.5 µL Kapa Hifi MasterMix (Kapa Biosystems, Sigma Aldrich, St. Louis, MO, USA), 6.25 µL of molecular grade H2O, 1.25 µL of index and 5 µL of DNA from the PCR sample. Our PCR cycling parameters for indexing consisted of denaturation at 95°C for 5 min, followed by 8 cycles of denaturation at 98°C for 20s, annealing at 56°C for 30s, and extension at 72°C for 3 min, and then a final extension at 72°C for 5 min. We verified amplification success by checking product size on a 2% agarose gel electrophoresis stained with SybrSafe.

We cleaned the resulting libraries using Omega BioTek Mag-Bind RXNPure Plus beads (Omega Bio-tek, Inc., Norcross, GA, United States). We then measured DNA concentration of each sample with the Qubit dsDNA Broad Range DNA Quantification Assay (Thermofisher Scientific, Waltham, MA, USA). Samples were then pooled in equal copy number. The final library was sequenced at UCLA's Technology Center for Genomics and Bioinformatics (TCGB) on an Illumina NextSeq V2 PE 150 Cycles- Mid Output spiked with 12% PhiX.

2.3 Bioinformatics

We used the *Anacapa Toolkit* [45] for amplicon sequence variant parsing, taxonomic assignment, and quality control. The quality control step of the Anacapa Toolkit trims extraneous adapter sequences used to identify each unique sample, removes low quality reads, and sorts reads by metabarcode primer sequence. The amplicon sequence variant (ASV) parsing step uses DADA2 [49] to dereplicate our metabarcodes. Next the Anacapa toolkit module assigns taxonomy to

ASVs using Bowtie 2 [50] and a Bowtie 2-specific Bayesian Least Common Ancestor (BLCA) algorithm [51].

For the fish primer set, taxonomic assignment was conducted following benchmarking by Gold et al. (2021) using a taxonomic cutoff score of 60 and minimum alignment of 80% [43]. Taxonomy was first assigned using the curated regional database of California Current Large Marine Ecosystem fishes to identify native taxa. We then re-assigned the taxonomy using the global *CRUX* generated database to identify non-native and non-fish species. Taxonomic assignments of ASVs were synonymized between both methods by prioritizing higher resolution assignments (i.e. species level vs. genus level).

We then implemented a decontamination procedure to eliminate poorly sequenced samples and remove potential sources of contamination [44, 52-54]. Importantly, we applied a site occupancy modeling framework to retain only ASVs that occurred in high prevalence across locations and stations. For these analyses, we removed all non-fish taxa from the resulting data. All remaining ASV's had their read counts converted into the eDNA index [53]. The eDNA index transformation is conducted by first normalizing all reads for a particular sequence by the total number of reads in each sample, then scaling those proportions to the largest observed proportion for that sequence across all samples. This results in a sequence-specific (species-specific) scaling between 0 to 1, where 1 is the sample with the highest number of reads for a given species and 0 is the least.

2.4 eDNA data analysis

We tested if our sequencing depth reached species saturation for our samples using a rarefaction curve. In order to test if our eDNA field samples fully captured the species richness of the site, we used an iNext package [55] to model a site-specific species accumulation curve. We then ran a piecewise regression analysis to identify the breakpoint in the rate of species capture with the *R* package *segmented* [56]. Breakpoint analysis is the statistical method for showing the significant point in which the segmented regression changes slopes and thus where we begin to reach saturation for our sample's species discovery.

Next, we measured total species richness to compare alpha diversity between seagrass sites and sandy bottom and seasonally within seagrass sites. Total species richness was compared using a generalized linear model (GLM) with a Poisson regression and significant groups were determined using a tukey contrasts multiple comparisons of means test.

To test for differences in community composition (beta diversity), the eDNA indexes for the samples were converted into Bray-Curtis dissimilarity distances [52]. We tested for differences in community structure by site and season using an adonis PERMANOVA followed by a multivariate homogeneity of group dispersions test BETADISPER [57]. Community composition was visualized using non-metric multidimensional scaling (NMDS) [57]. Closer grouped data points indicate more closely related community composition in both species richness and diversity.

2.5 Visual fish surveys

We paired visual scuba fish surveys with our eDNA fish surveys. Visual surveys were taken at 1)Amarillo, 2) Big Geiger Cove and 3) Two Harbors. These three surveys occurred during the same month as our eDNA surveys but not on the same day. The timed roving visual surveys are described within Obaza et al., 2022 [18]. Briefly, we took six visual surveys at each site with three within the bed and three along the edge. We took each survey for 3-6 minutes each and recorded the fish species observed. We compared the presence or absence of species found by eDNA and visual surveys to identify strengths and limitations of both survey approaches.

Results

3.1 Species Richness

The NextSeq generated over 10 million reads that passed quality control. Of these reads, 9.8 million reads representing 95 samples, 76 field samples and 19 blanks, passed the quality control of the Anacapa Toolkit. After taxonomic assignment we were left with 6.8 million reads representing 324 ASVs across 76 field samples. These reads represented 41 families, 69 genera, and 81 species of fish of which 40 families, 67 genera and 79 species were found within the seagrass sites (Table S1 in S1 Table). The ASV read counts were then converted into an eDNA index (Table S2 in S1 Table). Species are listed per site and per geographic type (Table S4 in S1 Table) as well as broken up seasonally by site (Table S5 in S1 Table). Sequences that could not be identified down to species are listed as *Genus sp.* and ASV's that blasted to more than one species are listed as *Genus species/species*.

Sample rarefaction curves showed that for each sample sequencing depth was sufficient to capture all species diversity within that collected sample (Fig S1). Site-specific rarefaction

curves modeled using the *iNext* package shows that at each site, the number of field replicates that were taken did not capture the full diversity of that site (Fig S2). This analysis shows that for these sites roughly 12-19 samples were needed to reach the breakpoint in the rate of species diversity found per sample. (Amarillo: 14.1, Big Geiger Cove: 13.99, Cherry Cove: 12.39, Inner DeAnza Peninsula: 16.59, Outer DeAnza Peninsula: 19.01, Two Harbors: 15.22).

Comparisons between all sites found that the only significant difference in the mean number of species observed was between Outer DeAnza Peninsula and Amarillo (GLM $Pr(>|z|) = 0.03073$) and Outer DeAnza Peninsula and Cherry Cove (GLM $Pr(>|z|) = 0.00183$) (Table S6 in S1 Table, Fig 2). However seasonal variation in the number of species found at all combined seagrass sites was found to significantly differ with every season comparison except summer and fall (Table S6 in S1 Table, Fig 3). Species richness was highest during the spring and continued to decrease in the summer, fall and then winter.

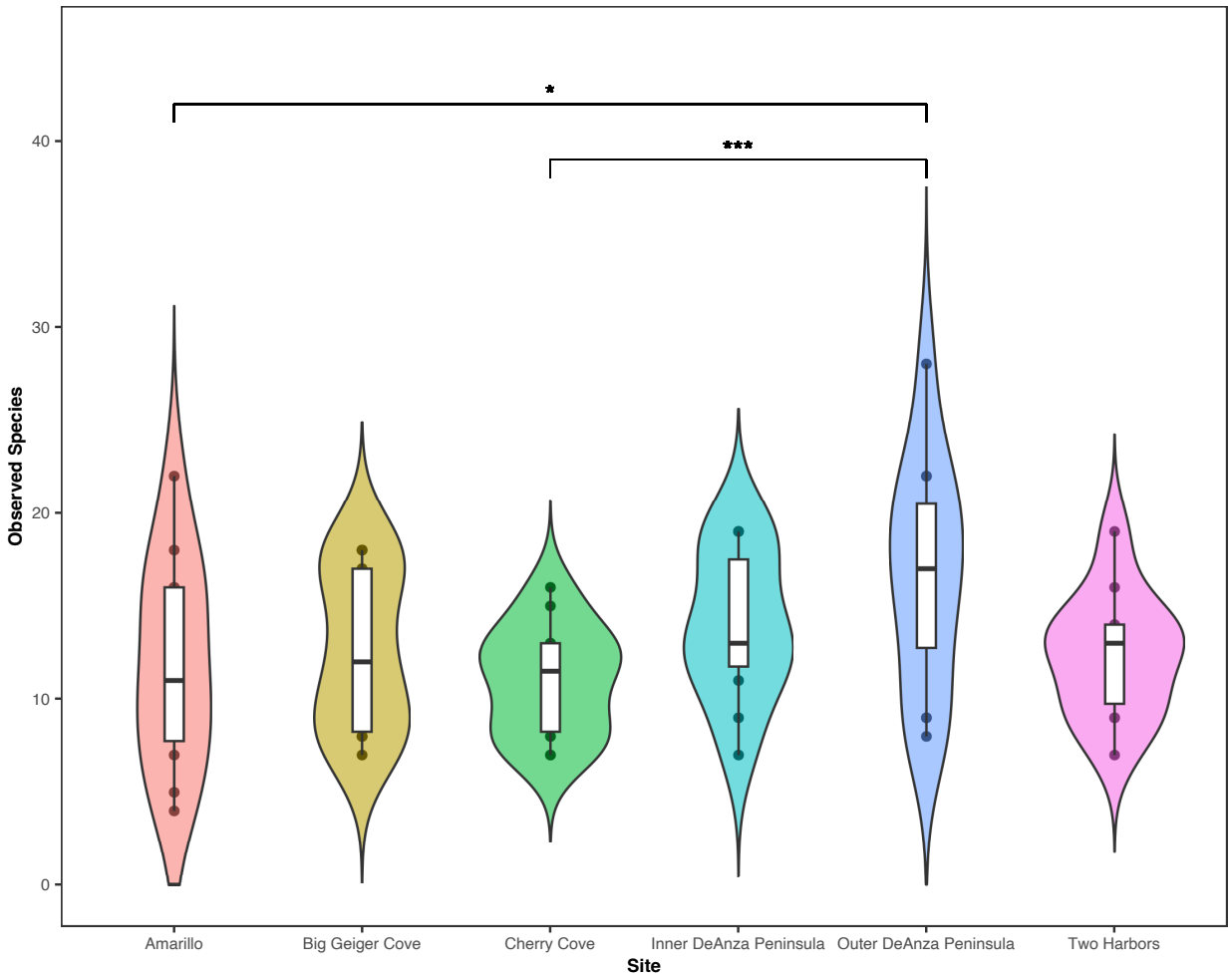

Fig 2: Violin plot of species richness by seagrass site across all seasons. A GLM and Tukey multiple comparison test shows the only significant difference between the number of species was found between Outer DeAnza Peninsula and Amarillo and Outer DeAnza Peninsula and Cherry Cove. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$

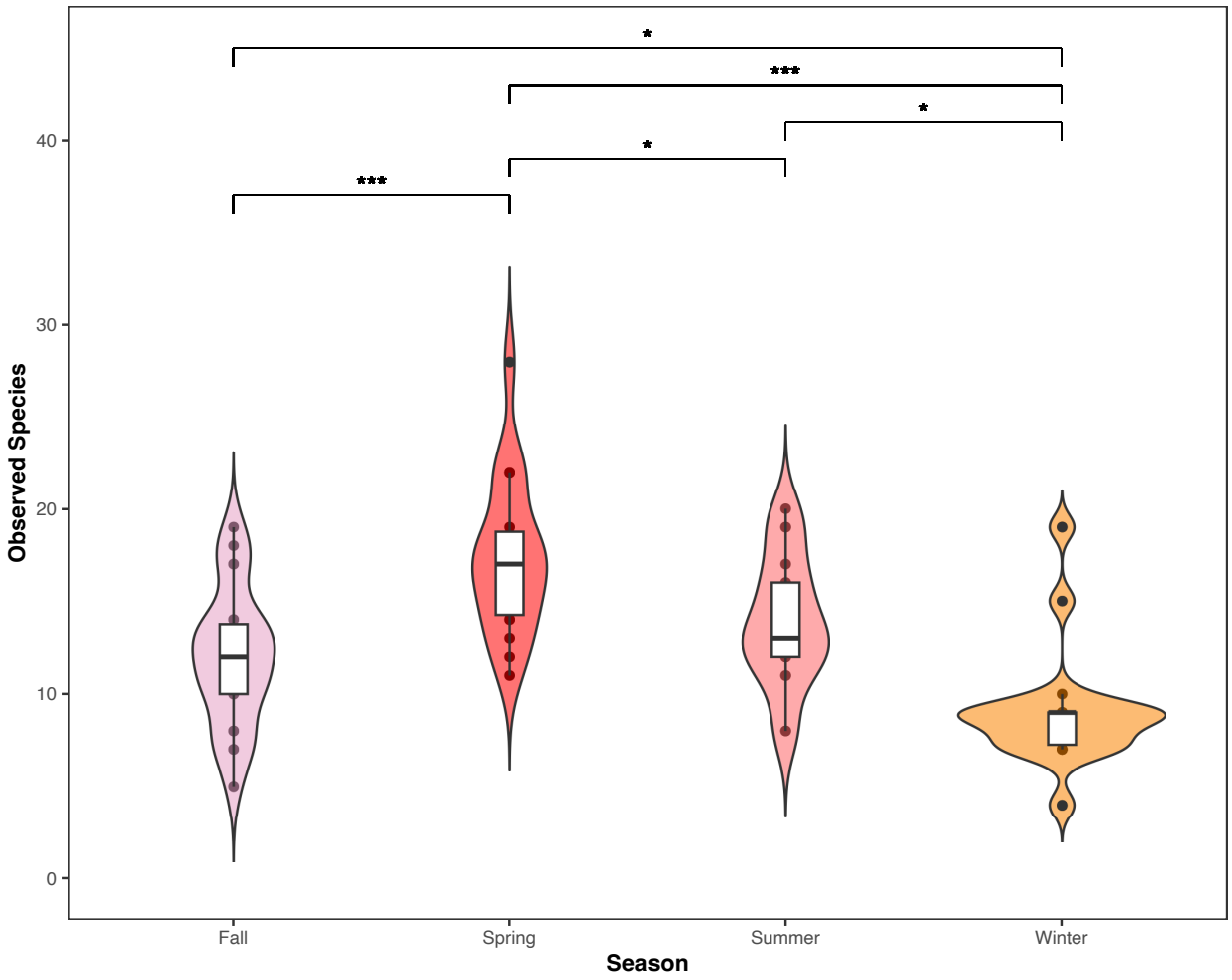

Fig 3: Violin plot of species richness at all five seagrass sites by season. The GLM shows there is a significant seasonal turnover in the number of species found at the beds throughout the seasons. * $P \leq 0.05$, ** $P \leq 0.01$, and *** $P \leq 0.001$

3.2 Community composition

We performed NMDS in order to compare community structure. The NMDS shows that embayment, open coast, and island seagrass beds are compositionally distinct from one another while sites of similar geographies show significant overlap. NMDS ordination showed good

clustering by both type (PERMANOVA $p<0.001$, $R^2 = 0.34393$, betadisper $p>0.05$) and season (PERMANOVA $p<0.001$, $R^2 = 0.09577$, betadisper $p>0.05$) (NMDS, Stress= 0.16, Fig 4A).

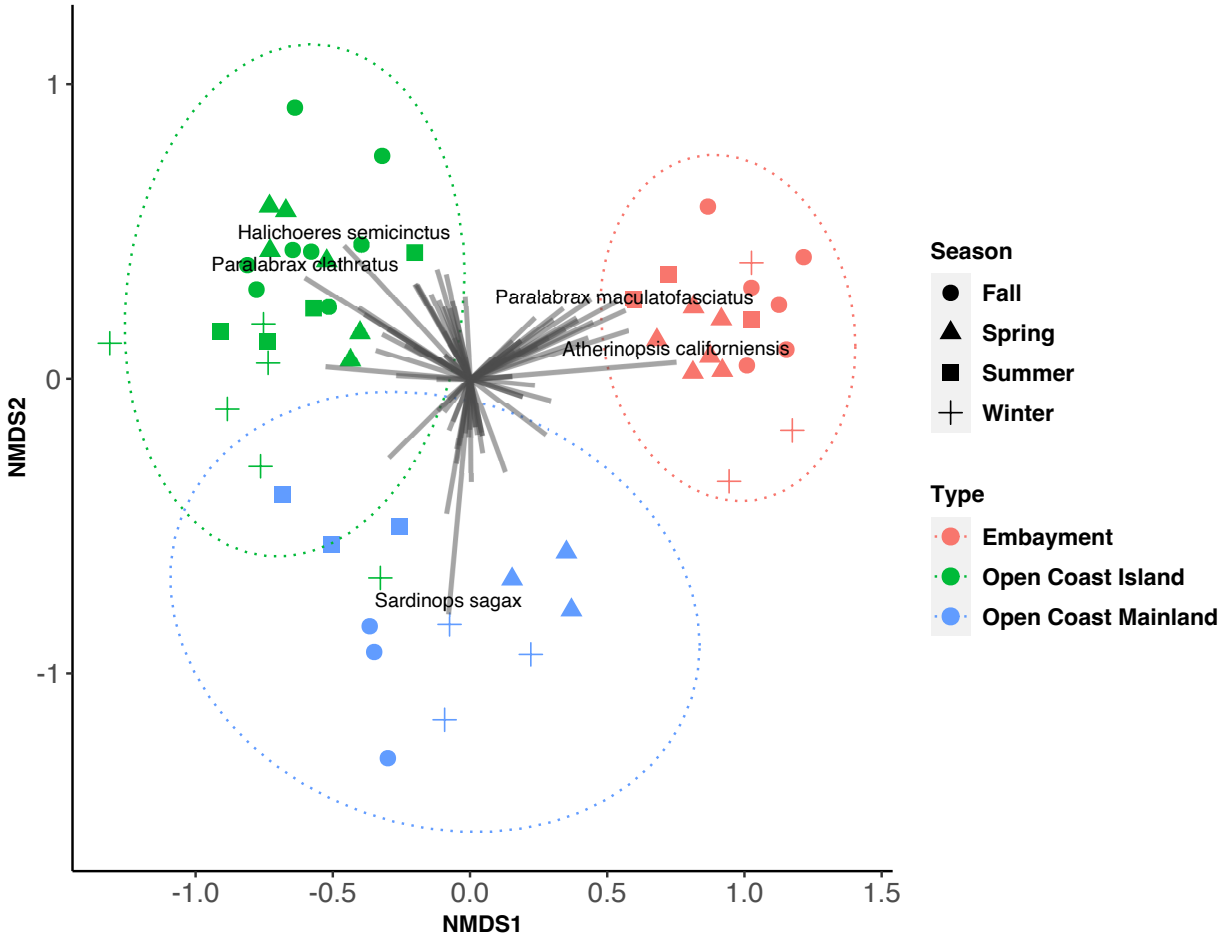

Fig 4: NMDS visualization of Bray-Curtis similarities between the geographic seagrass types. Types that are grouped closer to one another are more closely similar in both species richness and species count. NMDS shows that community composition of seagrass beds is more strongly dependent on their geographical location (embayment vs open coast mainland vs open coast island). Colors indicate type and shapes indicate season. Fish species were fit on the ordination where relative length indicates correlation between species and NMDS. The top five strongest associations are listed.

Seagrass sites were grouped by their geographic location - Open Coast (Mainland and Island) and Embayment. There were 35 fish found at both geographic locations with 23 fish unique to open coast beds and 19 unique fish found at embayment beds (Table 2). This supports our NMDS clustering, which showed that geographically distinct beds have different community composition than other types of beds. These beds remained distinct throughout the seasons.

Table 2: Fish found at the different geographic seagrass beds. [1]Species under 'Open Coast' with no symbol were found at both mainland beds and island beds, * represents species found only at open coast mainland beds and ^ represents species found only at open coast island beds. Sequences that could not be identified down to species are listed as *Genus sp.* and ASV's that blasted to more than one species are listed as *Species/Species*.

Every Geographic Location	Open Coast Only[1]	Embayment Only
Barred sand bass	*Amphistichus sp./Hyperprosopon sp.**	Albacore
Bat eagle ray	Blind goby*	American shadow goby
Bay blenny	California lizardfish*	Bay goby
Bay pipefish	California skate*	Bocaccio rockfish
Black perch	California tonguefish*	California grunion
California halibut	Hornyhead turbot*	California killifish
California kingcroaker/Corbina	Pacific pompano*	Californian needlefish
California pilchard/Pacific Sardine	Pacific/Longfin sanddab*	Diamond stingray
California sheephead	Thornback guitarfish*	Diamond turbot
Californian anchovy	White croaker*	Eastern Pacific bonito
Californian salema	Bennett's flying fish^	Gray smooth-hound
Chub mackerel	Blackeye goby^	Longjaw mudsucker
Fantail flounder	Blacksmith^	Shortfin weakfish
Flathead grey mullet	California scorpionfish^	Slough anchovy
Garibaldi	*Cheilopogon sp.* ^	Specklefin midshipman
Halfmoon	Horn shark^	Spotted turbot
Haller's round ray	Largemouth blenny^	*Thunnus sp.*
Jack silverside	Ocean whitefish^	White/Queen croaker

Kelp bass	Opaleye^	Yellowfin goby
Leopard shark	Spotted/Crevice/Striped kelpfish^	
Mussel blenny	California clingfish	
Pacific barracuda	Giant kelpfish	
Pacific jack mackerel	Señorita	
Pacific sanddab		
Reef finspot		
Rock wrasse		
Sebastes sp.		
Shiner perch		
Shovelnose guitarfish		
Speckled sanddab		
White seaperch		
Xantic sargo		
Yellowfin drum or croaker		
Yellowtail amberjack		
Zebra-perch sea chub		

3.4 Seagrass vs Sandy Bottom

Samples were taken at two Catalina seagrass beds and one nearby sandy bottom control site. Across the three sites, a total of 45 fish species were detected with eDNA. Of these 45 fish, 24 were shared between all three sites while 13 fish were only found in seagrass and 2 fish were only found at the sandy bottom control site (Fig 5; Table S7 in S1 Table).

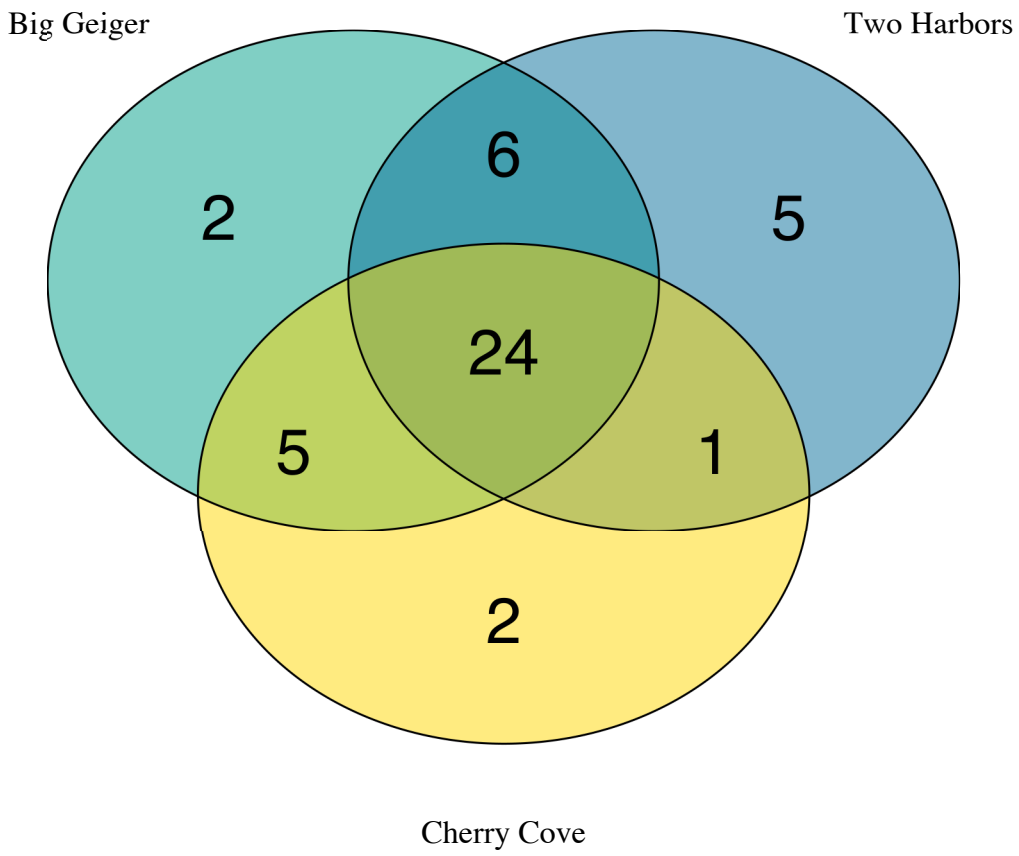

Fig 5: Venn diagram of fish species detected by eDNA between two seagrass sites (Big Geiger Cove and Two Harbors) and one sandy bottom site (Cherry Cove).

3.5 eDNA vs Visual Species Detections

For Big Geiger Cove, 8 species of fish were found by both methods during the summer time point. Environmental DNA detected an additional 7 unique species while scuba surveys found 1 unique species (Fig 6; Table S4 in S1 Table). At Two Harbors during our fall time point, both methods captured 11 species of fish with eDNA detecting an additional 6 unique species and scuba surveys with 3 unique species (Fig 6; Table S8 in S1 Table). Amarillo showed the least congruence between survey methods. Both captured 2 similar species of fish but eDNA had 16 unique fish species and scuba surveys had 2 unique fish species (Fig 6; Table S8 in S1 Table).

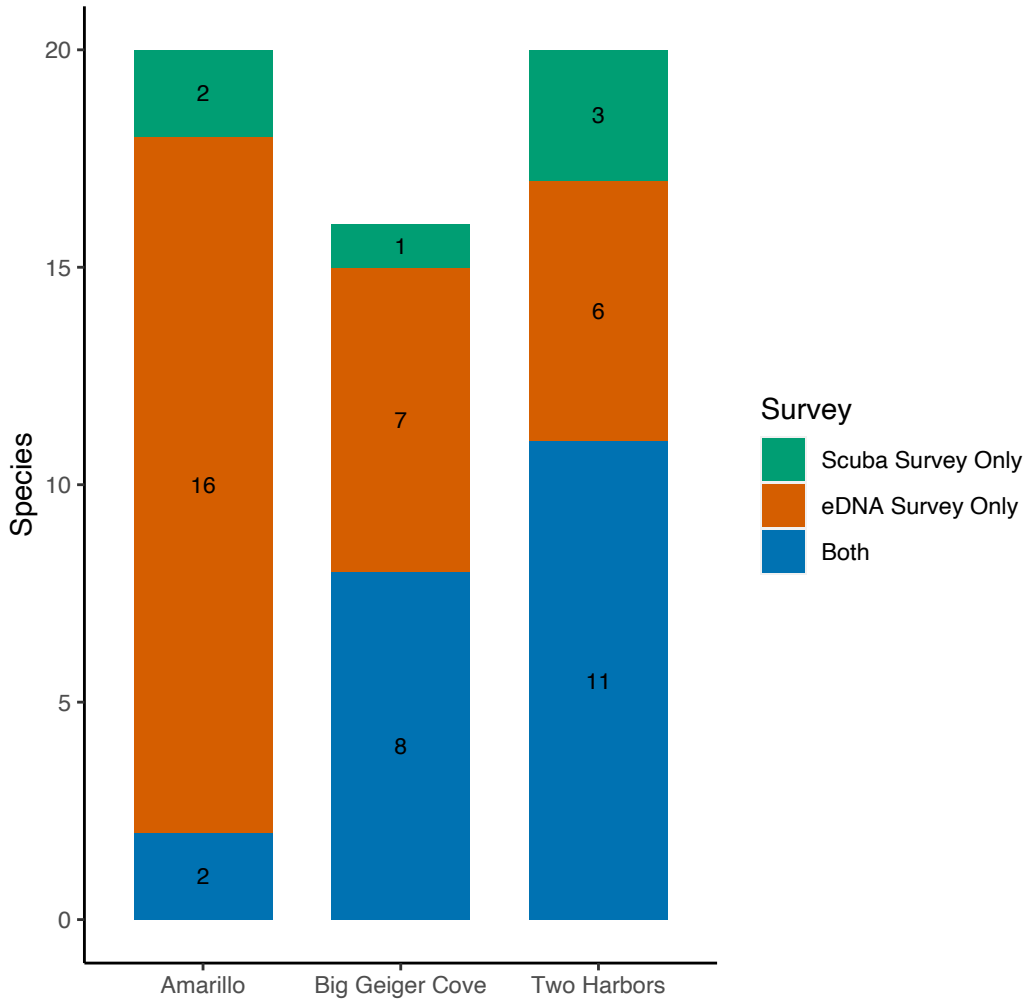

Fig 6: The number of species observed by eDNA and conventional methods. “Both” indicates species that were detected by both scuba surveys and eDNA surveys. “Scuba survey only” and “eDNA survey only” show the number of species that were uniquely detected by that method. Total number of species detected by scuba survey is “both” plus “scuba survey only” and total number of species detected by eDNA surveys is “both” plus “eDNA survey only”.

Discussion

We successfully demonstrate the ability of eDNA to monitor fish assemblages in Southern California seagrass habitats. Environmental DNA was shown to capture a suit of taxa known to

utilize these habitats based on previous surveys. We found distinct fish communities in embayment, mainland open coast and island open coast seagrass beds demonstrating the sensitivity of these approaches to characterize local biodiversity patterns. Environmental DNA was also able to largely recapitulate visual surveys while detecting a broader array of marine fishes, demonstrating the efficacy of these approaches for future seagrass monitoring efforts.

4.1 eDNA Captures Biogeographic Differences in Fish Assemblages

Our eDNA survey detected 78 unique species of fish within Southern California seagrass beds- 48 species off the mainland, 48 species off the island and 54 species in the embayment. The number of fish surveyed is on par with or greater than other previous surveys in the area. From 1987 to 2010, embayment seagrass beds in San Diego Bay and Mission Bay were found to have supported 50 species of fish [14]. Newport Bay, the site of our embayment seagrass beds, has been surveyed since 2003; the latest monitoring survey published in 2020 found 26 species of fish [15]. One survey of open coast and island seagrass beds around the Northern Channel Islands and Santa Barbara coastline found that open coast beds supported 20 species of fish while island beds supported 41 species of fish [16]. In 2018, island seagrass beds along Catalina Island were recorded to support 28 species of fish [17,18].

Our eDNA surveys detected distinct fish assemblages associated with open coast and embayment seagrass beds. The majority of fish species, 35, were shared between open coast and embayment beds and consisted of a mixture of rocky reef, soft bottom, and water column fish species. However, at geographically distinct sites, a noticeable pattern emerges.

For our embayment seagrass beds, 19 of its 54 fish species were only found at these two sites with the majority of them being soft bottom species. The embayment sites were the only sites to have fish associated with wetland species (California killifish and longjaw mudsucker) and also had the majority of detected estuary/bay associated species (Slough anchovy, diamond turbot, spotted turbot, American shadow goby, California needlefish, etc.). Notable at this site are two tuna species that were detected. To pick up signatures of its presence in a nearshore shallow environment in Southern California would be exceedingly rare. This is most likely an instance of fishers cleaning tuna catch in the back bay/harbor or an exogenous source of eDNA of this popular seafood in a highly urbanized area. On the other hand, our open coast seagrass beds had 23 unique species captured out of its 58 species total. In contrast to the embayment beds, the fish here were heavily linked to water column and rocky reef habitats. This distinction was further divided between beds located off the mainland and beds located off the island. Island sites had the highest proportion of what are typically rocky reef associated fish species (California scorpionfish, blacksmith, opaleye, blackeye goby, etc.) compared to open coast mainland beds, which had primarily soft bottom associated fishes. This difference in open coast vs embayment beds highlights the importance that other nearby coastal habitats play in the recruitment of fish to seagrass. Seagrass diversity and recruitment has been previously shown to be affected by distance from dispersal site [58], proximity to other habitats [59], and wind patterns [60]. Additional influences on fish diversity seagrass sites that may contribute to these differences beyond geographic setting of the meadow includes heterogeneity of environments surrounding the meadow [61], proximity to other seagrass sites [62,63], seagrass canopy height [64], and seagrass cover [65]. Although our in-situ design can’t account for all possible influences on

seagrass diversity, our surveys suggest that geographic location can impact up to half the found species at a given seagrass bed.

One specific group of interest that showed geographic differences was elasmobranchs. Worldwide, shark and ray populations have decreased 71% since 1970 [66]. Sharks assert a top-down control on their ecosystem through their predation of lower-level taxa which has a direct impact on the success of seagrass meadow [67, 68]. Elasmobranchs were well represented in the five beds surveyed, detecting one skate species, five ray species and three shark species. Similar to other fish in this survey, their habitat use was geographically varied. The bat ray, Haller's round ray, shovelnose guitarfish, and leopard shark were found at both open coast and embayment beds. The California skate, thornback guitarfish, and horn shark were only found in open coast sites while the diamond stingray and gray smoothhound were only found in embayment beds. Our results demonstrate the value of seagrass habitats to sharks and rays, encouraging continued conservation of this key marine habitat.

The differences in community composition of our seagrass sites emphasize the heterogeneity of seagrass associated fish assemblages which have been shown previously to be different between *Zostera* species [18] and now shown to be distinct across biogeographic regions. The significant differences of fish assemblages in Southern California underscores the importance of protecting multiple seagrass habitats across the region. Currently, only 5.2% of the eelgrass in Southern California is protected in a marine protected area [69]. Our results strongly suggest that California's Ocean Protection Council and Department of Fish and Wildlife management efforts

should consider both the quantity and biogeographic distribution of seagrass habitats in order to protect the greatest number of fish species.

4.2 Value of Southern California Seagrass

California's oceans are an important part of the state's economy, bringing in a gross state product of $84 billion dollars per year and supporting over 1 million jobs [70]. Nearly one quarter of the gross state product and jobs come out of Los Angeles County alone [70]. Marine vegetation, such as seagrass in Los Angeles, directly impacts the output of our oceans. Our eDNA recorded both commercially and recreationally important fish to California. Commercially, this includes California halibut which was the 7th largest commercial fishery in 2022 totaling 992,021 pounds valued at $5.4 million [71]. Recreationally, seagrass was home to the 5th (Flatfish, e.g. California halibut and Pacific sanddab), 8th (California scorpionfish), 9th (Sea bass) and 10th (Ocean whitefish) most caught fish categories by pounds in 2022 [71]. Seagrass meadows support these economically important species by acting as both nurseries for juvenile fish as well as habitat and food for adult fish. Four of these species was found at all seagrass bed types (California halibut, Pacific sanddab, kelp bass and barred sand bass) while the others were found in geographically distinct beds (ocean whitefish and California scorpionfish, open coast -island). Our results demonstrate the value of eDNA approaches for monitoring commercially important fish species and their utilization of key seagrass habitats, providing further evidence for the efficacy of eDNA approaches for routine marine biodiversity monitoring efforts.

4.3 Seasonality of Seagrass Fish Assemblages

Average seasonality for the sites followed a general pattern with the highest number of species being found in the spring followed by summer/fall and the least in winter. This follows conventional patterns of fish breeding in *Zostera* beds in the late spring to early summer periods which would increase the diversity present [72]. There were two sites that had notable exceptions to this. The first was Big Geiger Cove off Catalina Island, which saw the greatest number of fish species in the fall. Tanner et al., 2019 found that coastal seagrass use off Catalina attracted young of year kelp bass around the fall months with a significant amount of biomass being exported to other coastal habitats in the winter months [73]. Preferential nursery use based on geographic location could account for differences in number of species recorded compared to the other sites. The second site was Inner DeAnza Peninsula, which found the highest number of species in winter and subsequently decreased until fall, although it remained relatively constant during the year with a roughly one species difference per season. Inner deAnza's seagrass bed, being high within Newport Bay and protected by a sand bank, could provide a steady and safe environment for the fish in the embayment that the other sites could not.

4.4 Seagrass boosts higher diversity over sandy bottom site

The loss of seagrass has been shown to cause the rapid shift and subsequent decline in species richness in those areas experiencing decline [74-76]. In California, loss of seagrass has been linked to decreased epifaunal diversity [74] and shifts in fish assemblages [77]. This study aimed to evaluate the effect seagrass has on community composition by surveying three sites off Catalina Island- two of which were seagrass meadows found in Big Geiger Cove and Two Harbors, and one adjacent sandy bottom cove.

Between the three sites, there was substantial overlap in the majority of species found. Twenty-four species were found at both the seagrass sites and the sandy bottom site. eDNA found that there was higher diversity at the seagrass sites with 13 unique species captured and only 2 unique species found at the sandy bottom site. While NMDS shows that there is overlap between the sites, distinct communities were grouped together. Cherry cove was most similar to Two Harbors, a site of fragmented seagrass patches, in terms of community composition throughout the seasons. The sandy bottom site was even more dissimilar to Big Geiger, which is a cove with a continuous patch of seagrass. This suggests that seagrass beds density and size may play a part in their role as fish habitat. While it is unsurprising that a number of fish were found between both seagrass and sandy bottom coves, due to daily movement of fish in the ocean, seagrass is still important to these overlapped species as they rely on it for food and habitat. The species found only within the seagrass off Catalina included species known to use seagrass as nurseries (leopard shark and shiner perch), foraging grounds (shovelnose guitarfish), and habitat (Californian salema, bay blenny, and barred sand bass) while the two species found only at the sandy bottom site were common coastal pelagics (mackerel tuna) and known to hide under sand to attack pray (Pacific angelshark) [78].

One particular species of interest that was found in both seagrass meadows but not in the sandy site was the largemouth blenny, *Labrisomus xanti*. The largemouth blenny is a species native to Mexico with its previous range extending to the coast of Baja California [79]. The years of 2013-2015 brought an unusually warm ENSO event which caused a larger than normal distribution of warm water within the Pacific. Due to this, the first sighting of the largemouth blenny outside of its historical range was in La Jolla, California and Catalina Island in 2015 [79]. A recent study by

Stockton et al., 2021 evaluating their population off of Catalina Island found this species to be positively correlated with rocky habitat and negatively correlated with sandy habitats [80]. Their preference for structured habitats, along with known associations of other blenny species with seagrass, could point to seagrass playing a role in the future expansion of fish ranges with climate change.

4.5 Comparison of eDNA and visual fish sampling method

Previous literature has shown that environmental DNA often captures a larger number of species when compared directly to conventional methodologies [22]. This has been shown to be true for surf zone fish communities in Southern California [81]. The result of our comparison is concurrent with these previous findings by showing that eDNA captured the majority of fish the conventional method did and found a greater number of additional fish species that the conventional method wasn't able to do.

At Big Geiger Cove, eDNA captured 8 of the 9 (88.8%) species that scuba surveys captured plus 7 additional species. At Two Harbors, eDNA captured 11 of the 14 (78.5%) species that scuba surveys captured plus 6 additional species. The known habitat preference of the majority of fish eDNA captured support the conclusion that these are likely true positives. One possible reason for the discrepancy between survey methods was that they were taken within the same month but not at the same time, so the fish could have truly not been there during the other survey methods. Other possible explanations for being missed in the visual survey is that some species attach their eggs to seagrass (jack silverside), use seagrass at night (ocean whitefish and California scorpionfish) or engage in camouflage (fantail flounder and California flounder), which would

make it harder for visual surveys to observe them. The fish that were exclusively found in the visual surveys were only counted 1-2 times, which suggests eDNA surveys may have lower probabilities of detection for rarer taxa with presumably lower total DNA in the environment. These results align well with previous work comparing eDNA and manual methods [81-83].

When looking at the comparison between the two methods at Amarillo, eDNA captured 2 of the 4 (50%) species that scuba surveys captured plus 16 additional species. This example highlights eDNA as being less dependent on ambient conditions during sampling. Turbidity, low light, minimal visibility, and rough ocean conditions can all impact a scuba divers ability to see and properly identify fish species. Since eDNA relies on capturing DNA in the water column, these issues do not impact a researcher's ability to properly survey an area. While visual surveys provide additional information that eDNA surveys cannot, such as abundance or fish length, environmental DNA was able to detect a higher number of species at these three sites. By relying on solely conventional methods, environmental managers could possibly miss rare or ecologically and economically important fish species which could alter how they structure their conservation efforts. The use of eDNA is important for characterizing the full extent of a habitat's biodiversity.

4.6 Benefits and Drawbacks of eDNA

Environmental DNA is known to provide a number of benefits including differentiating morphologically similar species [20], detecting cryptic species [84], capturing a greater number of species compared to conventional methods [22], and being able to sample at greater spatial and temporal scales due to ease of use [20]. Our eDNA survey of seagrass beds around Southern

California was able to confirm these benefits. The study's sampling regime of five beds across Southern California took only 3 days per season due to the relative ease of eDNA field sampling. The survey results were able to differentiate between the juvenile fish species, that use seagrass as a nursery, which often look morphologically similar. Within this study, eDNA was also able to capture a number of rare and cryptic species. One rare species found within these seagrass meadows is the vulnerable diamond stingray (*Hypanus dipterurus*), which is of management concern due to its International Union for Conservation of Nature (IUCN) status. Environmental DNA was also able to detect cryptic species including those which might avoid conventional detection through camouflage (Pacific sanddab, speckled sanddab, California halibut, diamond turbot, and bay pipefish), burial (blind goby), and through their small size (California clingfish, American shadow goby, muscle blenny). By capturing free-floating DNA in the water column, researchers can circumvent some of the obstacles that visual identification has.

Despite existing literature supporting the use of eDNA for the surveying of marine ecosystems, there are limitations. One such limitation is the identification of false positives, i.e. fish that were detected in our eDNA sample without actually being in the seagrass. Fish that were not necessarily occupying the seagrass could have their DNA transported into the bed and captured by our surveys. Previous work has consistently demonstrated that within coastal marine ecosystems, fate and transport are less of a concern as marine eDNA signatures tend to vary at a scale of ~50-800m with the higher end of this range being in the Puget Sound which has a much higher tidal transport than Southern California [40, 43, 44, 85-88]. This range overlaps with the majority of the seagrass bed cover which ranges from 3,500m^2-31,000m^2 [17, 89] so sampling in the center of the bed should reduce outside DNA input. Additionally, marine vegetation is known

to slow hydrodynamic flow of water currents [90] which hypothetically shrinks the potential for DNA to be moved in or out of the system. The second limitation is not capturing the full species richness of the sites in our samples. Our analysis indicated that our sites needed roughly 12-19 samples to reach the breakpoint in the rate of species diversity found per sample and that our sampling would benefit from an additional 1-7 extra samples taken over the course of the entire year. This is in line with other Southern California eDNA studies which found similar values for their sampling to reach saturation of marine fish biodiversity [41,43]. A third limitation of eDNA is that, in its current state, it is an assessment tool of species richness which limits our understanding of the data and its ecosystem function that may otherwise be understood from additional data taken from conventional surveys including size frequency, sex ratio, and absolute abundance data. Despite this, the information from eDNA still provides a valuable insight into local biodiversity.

Conclusion

Seagrass ecosystems are crucial habitats for fish within Southern California. Over 78 fish were documented through metabarcoding in the seagrass beds around Southern California. Community composition was found to be spatially and seasonally distinct with different geographic locations and seasons impacting which fish were found to utilize the seagrass. Our results of the environmental DNA methodology supported its use as a biodiversity monitoring tool for coastal ecosystems as it was able to provide additional information in detecting species that visual surveys did not. Visual survey and eDNA may yet be best employed as complementary approaches with visual methods providing information on other parameters such as fish length and encounter rate.

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CHAPTER 2

Hidden in plain sight: the invasive macroalga Caulerpa prolifera evades detection by environmental DNA methods

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Abstract:

Environmental managers need a rapid and cost-effective monitoring tool for tracking the spread of invasive species, particularly at the onset of introduction. The macroalgae *Caulerpa prolifera* is considered an invasive species outside its native range, colonizing large patches of seafloor, reducing native species, and altering ecosystem functioning. Here we developed a droplet digital PCR assay for detection of *C. prolifera* from environmental DNA seawater samples using the internal transcribed spacer (ITS) region. While the assay itself was confirmed to be highly efficient we discovered concentrations of *C. prolifera* eDNA were present below detectable levels in the water column surrounding an outbreak. To understand why, we conducted tank-based experiments for two California invasive algae species, *Caulerpa prolifera* and *Sargassum horneri*. The steady state eDNA concentration (eDNA copies/ gram of biomass detected) of *C. prolifera* was found to be two orders of magnitude lower than *S. horneri*. A meta-analysis of

steady state concentrations reported in the literature showed a significant range from ~10^4-10^{11} (copies/g) and revealed *C. prolifera* to have the lowest recorded steady state concentrations of eDNA of any known species. We attribute *C. prolifera's* low steady state eDNA concentration to its unique biology as a unicellular macroscopic algae which reduces the possible modes of eDNA release compared to multicellular organisms. Critically our results demonstrate the potential limits of eDNA approaches, the influence of shedding rates in the reliability of species detection, and the importance of benchmarking and validating eDNA assays in both field and laboratory settings.

Introduction:

Invasive species are a threat to global marine biodiversity (Molnar et al., 2008). When these invasive species are introduced to a new environment, they can rapidly colonize the area because of their quick reproduction time, lack of natural predators, ability to outcompete native species, or a combination of all three (Havel et al., 2015). This causes both direct and indirect impacts to local ecosystems. Invasives can alter local biodiversity, impact ecosystem structure and reduce functional ecosystem services (Pimentel et al., 2000). Globally, marine invasive species have cost the economy an estimated $345 billion in damages (Cuthbert et al., 2021). This threat has only continued to rise in recent decades (Seebens et al., 2020) with the increase in globalized shipping, aquaculture, and accidental release (Bax et al., 2003; De Silva et al., 2009; Hulme, 2009). For these reasons, early detection and eradication before spread is a top priority for environmental managers (Larson et al., 2020).

Two invasive species of particular concern are *Caulerpa taxifolia and Caulerpa prolifera*. *C. taxifolia* is one of the top 100 worst invasive species (Global Invasive Species Database, 2023) and is named on the US Federal Noxious Weed List due to its history of overtaking marine ecosystems. It received this level of scrutiny because in the first 16 years since its introduction off the coast of Monaco in 1984, it grew to cover nearly 131 km^2 of Mediterranean coastline (Meinesz et al., 2001). The alga was first seen in 2000 in California in Carlsbad and Huntington Harbor, California (Jousson et al., 2000) with DNA barcoding of the tissue showing that it likely originated from an aquarium store (Jousson et al., 2000). It took nearly six years and seven million dollars (USD) to eradicate *C. taxifolia* from California (Merkel & Associates, 2006). In 2021, the first known case of *Caulerpa prolifera* was discovered off the West Coast of the United States in Newport Bay, CA (NOAA Fisheries). Species of the genus *Caulerpa* have been observed to stunt ecosystem services, reduce native biodiversity and significantly decrease species richness compared to native seagrass meadows (Parreira et al., 2021). These impacts and the species' relative ease in spreading made it a top priority for eradication efforts by local marine managers.

California has dealt with another invasive macroalgae for nearly 20 years, *Sargassum horneri*. *S. horneri* (Devil's weed) is a brown alga native to Eastern Asia and was introduced to the West Coast of North America in 2003. Since its introduction, its range has expanded from Baja California, Mexico to Point Conception, California including the Channel Islands (Marks et al., 2017). *S. horneri* often forms large mats off the coast that are anchored to rocky substrate. Researchers working on *S. horneri* have shown that removal techniques are most effective in culling population size and density if the scale of removal is sufficient to reduce propagule

supply (Marks et al., 2017). Thus, environmental DNA (eDNA) could be used for early detection of *S. horneri* prior to introduction via ballast water, for detection in areas that are difficult to survey, or for detection of small populations that can be successfully eradicated. Previous work has developed an eDNA assay for *S. horneri* (Hamaguchi et al., 2022), however, benchmarking an *S. horneri* assay and determining the species' shedding rates is needed to validate eDNA as an appropriate monitoring method for these applications.

Conventional survey techniques to identify *C. prolifera* involve divers visually scanning the seafloor. This presents an issue in Newport Bay, CA where the turbidity makes for poor visibility and difficulty identifying *C. prolifera* fragments. Survey efficacy through the use of artificial *C. prolifera* released in the bay found that nearly 20% of the fake fragments were never recovered, highlighting this challenge (Owens, 2021). Environmental DNA has the potential to offer an additional method to screen for Caulerpa invasions in aquatic systems, where early identification and removal are paramount to their eradication (Larson et al., 2020). Environmental DNA approaches have been shown to better detect rare and cryptic species and outcompete conventional survey techniques in the field, particularly in difficult to survey environments (Thomsen and Willerslev, 2015; Fediajevaite et al., 2021).

Our study evaluates the use of eDNA-based monitoring alongside conventional survey techniques for the tracking of *C. prolifera*. We developed a novel droplet digital PCR (ddPCR) assay for the *in-situ* identification of *C. prolifera* and characterize the first algal eDNA shedding rates in the literature for *C. prolifera* and *S. horneri* to benchmark this methodology as a monitoring tool for invasive algal species.

Materials and Methods:

Assay design

To create our eDNA assay, we downloaded reference sequences of *Caulerpa prolifera* from GenBank (Bensen et al., 2015) (https://www.ncbi.nlm.nih.gov/genbank/). Sequences were aligned in Geneious 2019.2.3 (https://www.geneious.com) and potential primer/probe sets were created using Geneious' design new primer/probes feature with guidelines based on Klymus et al., 2020. Our primers were created for the 'internal transcribed spacer' or ITS gene based on previous work which has used the ITS for *Caulerpa* sp. phylogenetics (Kazi et al., 2013; Stam et al., 2006). Primer specificity was tested *in-silico* using EcoPCR (Ficetola et al., 2010) and showed species-specific *Caulerpa prolifera* amplification. To validate the primers, we tested qPCR primer efficiency of our *C. prolifera* DNA from tank and field tissue samples using a dilution series from 5 ng/ul of genomic DNA to 0.00005ng/ul. Primer and probe sequences for the *C. prolifera* assay are given below (Table 1).

Table 1: Primer and probe details for the *C. prolifera* ddPCR assay.

Scientific Name	Primer/Probe	Sequence 5'- 3'	bp
Caulerpa prolifera	Caulerpa_ITS_F	TGGCGCTATGTAATGTTGATGTTG	106
Caulerpa prolifera	Caulerpa_ITS_R	GCAATTCGCAACACCTTTCGTA	

Caulerpa prolifera	Caulerpa_Probe	56-FAM-CGGTTCCCGTGTCGATGAAGGACG-3IABkFQ	

We ran an annealing temperature gradient to optimize amplification and identify the greatest difference between positive and negative droplet fluorescence amplitudes. Based on our optimization, cycling conditions for the ddPCR were 95ºC for 10 minutes, 40 cycles of 95ºC for 30 seconds and 58ºC for 60 seconds, 98ºC for 10 minutes and a 4ºC hold indefinitely. Mastermix concentrations were: 14.4 µl of 4x ddPCR Multiplex Supermix (Bio-Rad, Hercules, CA, USA), 0.5184 µl of 100uM forward primer, 0.5184 µl of 100uM reverse primer, 0.144µl of 100uM probe, 20.4192 µl of water and 12 µl of sample. This mix was partitioned into duplicate replicates of 22µl and added to a 96 well plate. The reaction mixture was combined with Bio-Rad Droplet Generation Oil (20 µl reaction mixture + 70 µl oil) and partitioned into nanodroplets via microfluidics in the Automated Droplet Generator (Bio-Rad). This resulted in a total nanodroplet volume of 40 µL, which was transferred to a standard 96-well PCR plate for amplification using a multichannel pipettor. The plate was heat sealed with pierceable foil using a PX1 PCR plate sealer (Bio-Rad) and PCR amplification was carried out in a S1000 thermal cycler (Bio-Rad, ramping speed at 2°C per second). After PCR, the plate was read by the Bio-Rad QX200 Droplet Reader and analyzed using the Bio-Rad QX Manager (v.1.2 or v.2.0) software.

Sargassum horneri primers were chosen from Hamaguchi et al., 2022. We used AkamokuITS2-F 5'-TCGCTATATGCAGGTTTA-3', AkamokuITS2-R 5'- GACTGCCTACCGTCAA-3' and AkamokuITS2-P 5'- HEX-AGCCTCTAGCAACGCTCCAA-BHQ1-3'. We ran an annealing temperature gradient on the primers which showed a 56ºC annealing temperature to be the optimum temperature. All other assay conditions were the same as described above for the *C. prolifera* assay.

Field testing

A *C. prolifera* patch was found in China Cove, Newport Bay in April 2021 via scuba diving surveys and was roughly one foot in diameter and contained ~20 fronds. We sampled seawater on June 30th, 2021, directly above the bed (33.596406, -117.879731), and then above the sea floor 5m, 10m, 50m 100m, and 500m bay-ward from the *C. prolifera* patch, employing the eDNA collection method of Curd et al. 2019, described as follows. First, we collected seawater samples directly above the patch using divers to collect 1L of water in a Kangaroo enteral feeding bag (Covidien, Minneapolis, MN, USA) as to not disturb the *C. prolifera*. Samples taken away from the patch were then collected using a 5L niskin bottle. From the niskin, we transferred one liter of seawater to a Kangaroo bag (Covidien, Minneapolis, MN, USA) in triplicate. We immediately gravity filtered 1L of seawater through a sterile 0.22 μm Sterivex cartridge filter (MilliporeSigma, Burlington, MA, USA) for all the samples simultaneously. We capped the filters and stored them on dry ice during sampling until we returned to the lab where they were stored at -20ºC. Additionally, we filtered one liter of Milli-Q water through the same process for a negative field control (Goldberg et al., 2016). Tissue samples from the patch were taken for species verification and preserved in 70% molecular grade ethanol.

Experimental design of shedding experiment

We tested the shedding rates of two California invasive macroalgae, *Caulerpa prolifera* and *Sargassum horneri*. We purchased the *Caulerpa prolifera* from an online aquarium store (ReefCleaners.org, Port St. Lucie, FL, USA) and divers from Cabrillo Aquarium identified and collected the *Sargassum horneri* off the coast of San Pedro, CA (33.774, -118.43). The algae were left to acclimate in tanks with artificial seawater for two days before the start of the experiment. We filled three replicate tanks per species with 20L of deionized (DI) water and 36 g/L of Instant Ocean sea salt for aquariums (Instant Ocean, Blacksburg, VA, USA). Wet weights of the algae were measured and recorded before they were added to the tanks. Once added, the algae were kept alive for the length of the experiment and were free-floating for this period. We kept the tank water at ambient room temperature in the lab (20 ± 1 °C) and exposed to natural, indirect sunlight through the window. An additional tank containing only artificial seawater was used as a control.

We took samples before the addition of the species (hour 0) and then subsequently at 1, 2, 4, 8, 12, 24, 48, 72 and 96 hours after they were added in the same manner as the field samples. We added 23.99g, 24.44g, and 23.39g of *C. prolifera* and 20.47g, 23.49g, and 22.36g *S. horneri* into their respective first, second and third tanks (Fig. 1). At each timepoint, we stirred the tank gently with a sterile stirrer for a well-mixed sample and then collected 1L of tank water into a Kangaroo enteral feeding bag (Covidien, Minneapolis, MN, USA). This 1L bagged sample was then filtered onto two sterile 0.22 μm Sterivex cartridge filters (MilliporeSigma, Burlington, MA, USA) running 500mL through each via gravity filtration to avoid filter clogging. We stored

the filters at -20ºC until they were extracted the following day. After each sample collection time point, we immediately refilled the tank with 1 L of sea water from a carboy so as to maintain consistent volume within the small tanks. At each timepoint, we collected water from the control tank and carboy in the same manner to test for contamination.

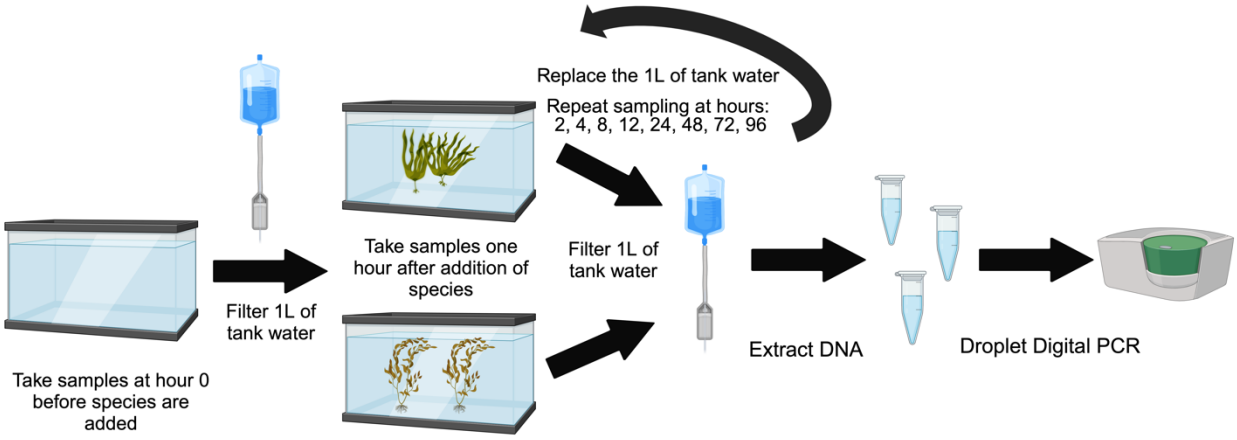

Fig. 1: A schematic of the tank experiment set up.

DNA Extraction

All eDNA and tissue samples we extracted from the Sterivex cartridge using a modified DNeasy Blood & Tissue Kit protocol (Qiagen Inc., Germantown, MD, USA) optimized for increased eDNA yield (Spens et al., 2017). Sterivex filters were incubated at 56ºC overnight with 720µl of ATL buffer and 80µl of proteinase K. After incubation, equal parts AL buffer and ice-cold molecular grade ethanol were added to the ATL buffer/ proteinase K mixture and spun through a spin column. AW1 and AW2 buffers were added to wash the columns. The DNA was eluted using 100µl of AE buffer and stored at -20ºC.

Data Analysis

Following recommendations in Cao et al. 2015 and Steele et al. 2018, a minimum of two reactions and a total of ≥10,000 droplets per reaction were generated per sample; samples that failed to meet the droplet requirement were reanalyzed. At least six no template control (NTC, RNA/DNA-free water; UltraPureTM, Life Technologies, Carlsbad, CA, USA) reactions were run per assay. NTC samples were required to contain less than 3 positive droplets. Two positive control reactions were included per assay. When samples exceeded the upper limit of quantification, these were diluted 1:100 with RNA/DNA-free water and reanalyzed.

Based on the concentrations from the ddPCR software, we back calculated the tank concentration of DNA. Specifically, the ddPCR output in copies/µL of reaction were converted to copies/µL in the filter and then converted to copies/µL of tank water (https://github.com/kylielanglois/SCCWRP/blob/main/ddPCR/ddPCR_autofill_clean.R). Since replicates came from the same 1L bag, they were averaged together to account for larger particles that were unevenly distributed between the two filters.

We then calculated the steady state concentration per gram of body mass using the equation from Sassoubre et al., 2016. Briefly, $V*dC/dt = S- kCV$ where V is the tank volume in liters, C is eDNA concentration, t is hours, S is the shedding rate and k is the first-order decay rate constant per hour. At steady state, dC/dt= 0 so the shedding rate/decay rate constant would equal the concentration of eDNA multiplied by the tank volume. Since our experiment did not measure the decay rate constant, we cannot directly solve for decay and shedding rates. However, we can

solve for the steady state concentration per gram of body mass by using the 96-hour concentration when our tanks reached steady state. We argue that this is still a meaningful metric as it reflects the total number of eDNA molecules per biomass (g) of an organism available to be captured in a given volume of water. We then compare steady state concentration per gram of body bass across other previously reported values for other species (Andruszkiewicz Allan et al., 2020; Kwong et al., 2021; Maruyama et al., 2014; Nevers et al., 2018; Plough et al., 2021; Sansom and Sassoubre, 2017; Sassoubre et al., 2016; Wilder et al., 2023). When shedding and decay rates for multiple conditions in a given study for a single species was reported, we report both the lowest and highest reported steady state to show the range. All values shown are from other tank-based, single species shedding experiments allowing for comparable results within the meta-analysis.

Results:

Assay Benchmarking

We successfully amplified extracted *C. prolifera* tissue DNA collected from the field invasion using our Caulerpa-specific primer set. We sequenced this tissue and the tank tissue using custom ITS primers on a Capillary Sequencer at Laragen Sequencing Facility (Culver City, CA). We evaluated chromatograms using Geneious. The consensus sequence is provided in the Supplement. We identified 2 mismatches between the field and tank consensus sequence in our forward primer and 2 deletions between our tank consensus sequence and reverse primer. No mismatches were found in the probe region. Our qPCR dilution series resulted in a 101.05% and 103.35% primer efficiency for the tank and field samples respectively. These results demonstrate

our primers to be highly efficient and robust to small mismatches discovered in the sanger sequences (S1 in Supplemental Table 1).

Field Sampling

Despite the efficiency of our assay, none of the field eDNA samples taken directly above or away from the *C. prolifera* patch detected *C. prolifera* eDNA. Additional replicate field samples taken directly above the *C. prolifera* patch were tested for inhibition using a serial dilution and a Qiagen DNeasy PowerClean Pro Cleanup Kit and similarly showed no ddPCR amplification of *Caulerpa prolifera* DNA.

Tank-based Experiment

The *Caulerpa prolifera* and *Sargassum horneri* in the tank experiments both yielded quantifiable eDNA in the water samples (Table S3 and Table 4 in Supplemental Table 1). *C. prolifera* was characterized by a sharp increase in initial eDNA concentration in the tank to ~$10^{5.75}$ copies/L followed by slight decline and then steady plateau at ~10^5 copies/L (Figure 2). *S. horneri* instead saw a general increase in eDNA tank concentration followed by a similar plateau at ~10^7 copies/L. Our sampling method of filtering 1L and replacing it with 1L of water would have diluted the concentrations by 5%, which would have no bearing on the final interpretation of the results given the orders of magnitude difference in steady state concentration observed. An ANOVA between the 48, 72 and 96hr concentrations show no statistical significance in the difference of means indicating that both tanks reached steady state by 96 hours (ANOVA, $p>0.05$). For the 20g samples of algae in each tank, this steady state equates to roughly $10^{4.5}$-10^5 copies of DNA/ L of tank water for *C. prolifera* and 10^7 copies/L for *S. horneri* (Fig. 2). This

equates to a nearly 100-315x greater amount of *S. horneri* eDNA concentration per gram of biomass compared to *C. prolifera*. All tank controls and PCR controls were negative.

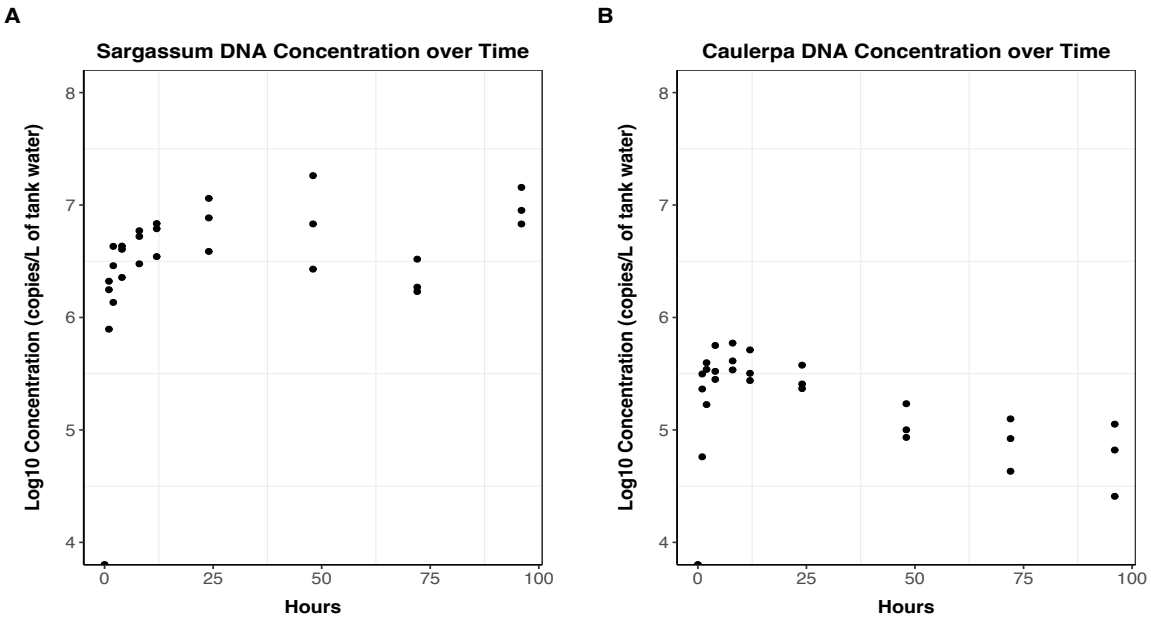

Fig. 2. Plots of the tank eDNA concentrations over time in log form. *S. horneri* shows an initial jump in eDNA copies/L and then a steady plateau after 24 hours. *C. prolifera* exhibits an initial spike in concentration before decreasing and leveling off after 48 hours. Steady state for both was reached at the 96-hour time point.

Steady State

Steady state copies of DNA per gram of biomass for available species in the literature spanned over 7 orders of magnitude (Table S2 in Supplemental Table 1). *Caulerpa prolifera* had the lowest steady state concentration while *Sargassum horneri* was one of the median reported values. (Table 2; Fig. 3)

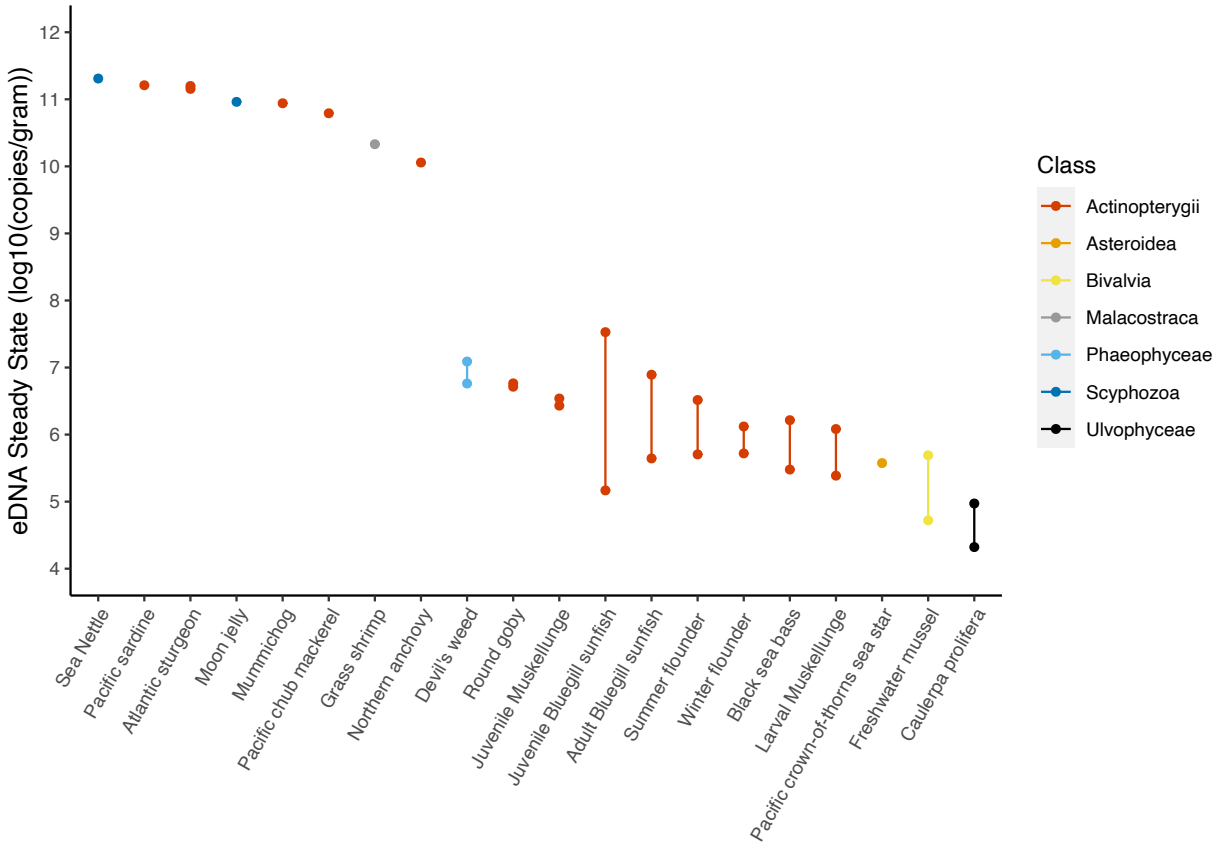

Fig. 3. Log10 conversion of the steady state concentration of eDNA by species and class. Where variable steady states were reported between experiments, we plot the lowest and highest rates reported and the range in values is indicated by the bar linking two points. Those with one steady state rate only reported one shedding and decay rate. Scientific names given in Table S2 in Supplemental Table 1.

Discussion:

Our results demonstrate the vital importance of lab and field validating eDNA assays prior to their adoption as a monitoring technique. Here, we created a novel eDNA assay that is able to amplify *C. prolifera* DNA in the lab and controlled mesocosms. Despite this, we were unsuccessful in identifying *C. prolifera in-situ* over a known patch of the algae. Tank-based experiments demonstrate that *C. prolifera* has the lowest observed steady state eDNA concentration of any reported species. In contrast, *Sargassum horneri* assay shows promise as an invasive monitoring tool given the higher observed eDNA steady state concentrations. Our results have implications on the use of eDNA in the field of invasive species monitoring and on our understanding of eDNA shedding mechanisms.

C. prolifera sheds negligible amounts of DNA

We demonstrate *C. prolifera* to have the lowest recorded eDNA steady state concentration of any currently reported species. We hypothesize that the low steady state eDNA concentration of *C. prolifera* can be attributed to the algae's unique organismal and cellular biology. The genus *Caulerpa* is home to some of the largest single celled organisms in the world (Jacobs, 1994). *C. prolifera* is a multinucleated single celled macroalgae which spreads primarily through asexual reproduction (Jacobs, 1994). Thus *C. prolifera* lacks conventional modes of eDNA release including shed cells via gametes, mucus, and other cellular debris etc., which account for a considerable amount of total eDNA release for other species (Klymus et al., 2015; Thomsen and Willerslev, 2015). *C. prolifera* is unique in that as a single-celled organism it may not shed small cell-like units into the environment but, at the same time, cannot be easily captured via water filtration as is the case for bacteria and phytoplankton.

This leaves only a few potential modes of eDNA shedding, namely cellular leakage of mitochondria or free-floating DNA. Previous work has demonstrated that cellular leakage accounts for only a small proportion of total environmental DNA and thus we would expect low *C. prolifera* shedding rates from this fact alone (Zhoa et al., 2021). However, given the unique physiology of *C. prolifera* there are two possible reasons for further reduced cellular leakage in this species. The first is the thick cell wall surrounding the algae which acts to prevents regular shedding of cellular material into the water column (Jacobs, 1994). The second is *C. prolifera's* unique cellular organization that allows for dramatic morphological differentiation within a single cell. Previous studies have shown dramatic differences in gene expression across the organism despite being a single cell with shared cytoplasm, helping explain the unique morphology of fronds, rhizomes, etc. of the organism (Arimoto et al., 2019; Ranjan et al., 2015). This dramatic differentiation of distinct parts of the single cell, suggest additional cellular mechanisms to limit DNA and RNA activity and transport within the shared cytoplasm. It is currently unknown what mechanisms allow for such differentiation within the organism despite shared cytoplasm. However, one potential explanation may be a high degree of RNAse and DNAse activity which would limit the spread of transcription and translation to specific regions of the cell, allowing for the substantial phenotypic differentiation observed across the organism. Such a mechanism may also act to reduce the amount of free-floating DNA available within the cytoplasm, and thus reduce the amount of eDNA shed via cellular leakage.

Together, these factors strongly suggest that the unique physiology and morphology of *C. prolifera* contribute to low shedding rates. Unfortunately, our results indicate that this invasive species is uniquely equipped to evade detection through eDNA surveying, indicating the need for alternative detection methods in low visibility and difficult to survey environments.

We note that the other reported values from previously reported shedding and decay experiments also underestimate the amount of eDNA release that occurs *in-situ*. During tank-based trials, animal species are restricted from food before and during the experiment to minimize the amount of eDNA introduction to the tanks from sources such as feces. This means in the wild, when species have access to food and likely higher metabolic rates, eDNA release rates are expected to be higher. Previous work has demonstrated that sea stars in tank experiments that were given food released roughly 7x more eDNA than when not given food, strongly supporting this hypothesis (Kwong et al., 2021). Thus, given that the majority of steady state concentrations were generated from heterotrophic species, we expect their relative eDNA shedding rates to be even higher than the photosynthetic *C. prolifera*, providing further evidence of distinctly lower steady state eDNA concentrations of this invasive species.

Furthermore, a large number of aquatic plant and animal species also introduce eDNA through the release of gametes during spawning events. However, *C. prolifera's* predominantly asexual mode of reproduction limits shedding rates compared to broadcast spawning organisms (Smith and Walters, 1999). Thus, we are confident that values presented for *C. prolifera* in this study appropriately capture the expected steady state concentration this species would exhibit in the wild.

We note that *C. prolifera* steady state concentrations at 96 hours were 5-20% of the maximum concentration (Fig. 2). However, previous tank experiments results show an initial spike in DNA concentration as a result of stress to the organism, leading to greater cellular degradation and thus more free-floating cells and materials (Klymus et al., 2015; Nevers et al., 2018) followed by a decline in production. All values used in our comparisons of steady state

concentrations were reached within 24-48 hours into their respective experiments so as to avoid any differential physiological effects of initial stress influencing our comparisons.

Additionally, the steady state values calculated were normalized by grams of biomass so that the values were comparable across taxa. This metric undervalues the difficulty in detecting *C. prolifera in-situ* compared to other species in this list. The next lowest mean steady state concentrations are from the freshwater mussel and Pacific crown-of-thorns sea star (Sansom and Sassoubre, 2017; Kwong et al., 2021). These species weigh roughly 100g and 3000g, respectively, whereas a single *C. prolifera* frond weighs just a fraction of a gram. A colony of *C. prolifera* that consists of 100-3000g of biomass would make environmental DNA as a tool unnecessary because the patch, likely to be multiple square meters in size depending on its density, would be easily visible to conventional visual surveys.

eDNA as a tool to track *Sargassum horneri*

Previous *S. horneri* research found that removal efforts have considerable challenges to success once the alga has been established (Marks et al., 2017). This emphasizes the importance of early detection to the protection of our coastal ecosystems. This study calculated *Sargassum horneri*'s steady state concentration to be roughly 10^7 which placed it within the middle range compared to recorded fish and invertebrates. We demonstrate here that because of its relatively high steady state concentration, and large biomass in the wild, *S. horneri* is an ideal invasive candidate for environmental DNA detection as demonstrated in previous studies via metabarcoding (Ely et al. 2021 and Gold et al 2022). Specifically, the use of this ddPCR assay in areas with low abundance before species establishment, environments of high turbidity and low visibility, locations that are difficult to dive in, and in ballast water of ships would allow for higher

sensitivity monitoring and earlier intervention. State and federal agencies such as CDFW, NOAA, USGS, and USFWS that are tasked with monitoring and stopping invasive species would particularly benefit from the use of eDNA to monitor *Sargassum horneri* populations.

Implications for environmental DNA studies

The results of this study show a multiple order of magnitude difference in species' eDNA steady state concentrations. The differential steady state values highlight the influence various eDNA release modes play in detection probabilities and the difficulty in ascribing quantitative metrics to eDNA data between species. Notably, fish species show a significant range of steady state concentrations from 10^6-10^{11} copies/gram. Fish with a higher steady state concentration are expected to have higher probabilities of being detected in the wild. Furthermore, we expect that all else being equal, species with 5 orders of magnitude higher steady state concentrations will be overrepresented in environmental DNA surveys. As the field of eDNA moves to be more quantitative, accounting for differences in such biases will be critical (Harrison et al. 2019). Importantly, we find that relative shedding rates operate on similar orders of magnitude as amplification efficiencies and thus controlling for both biases will likely be critical for deriving quantitative metabarcoding approaches (Shelton et al. 2023). Our meta-analysis also highlights the limited number of non-fish species in eDNA shedding and decay experiments. As eDNA aims to become a holistic monitoring tool for biodiversity, characterizing shedding and decay relationships across a broad diversity of taxa and not just those that are commercially important, will be crucial to understanding the applicability of this methodology for biodiversity monitoring efforts. Thus additional studies to characterize shedding and decay rates, particularly for species of interest like invasives are clearly warranted.

Previous work has highlighted the utility of eDNA as a complement to conventional survey techniques (Bohmann et al 2014; Kelly et al. 2017). In the case of invasive species, eDNA can aid in early detection of areas of concern given the sensitivity of qPCR/ddPCR assays; however, there is always value for "boots on the ground" confirmation, especially when there are significant management implications (Gold et al. 2022). A strong advantage of eDNA is the ability to reduce the complexity of the field logistics by narrowing the range of visual surveys and the time it would take to complete them. Our study demonstrates than an eDNA approach is not equally effective for all species and was particularly ineffective in capturing the *C. prolifera* signal in the field using standard protocols. We demonstrate the value in benchmarking eDNA assays both in the lab and in the field prior to its deployment as a monitoring tool. Best practices in method validation should be adopted for all eDNA assays to ensure that results in the field, such as the negative results obtained in this study, are properly scrutinized and validated. These practices are summarized below (Table 2)

Table 2: Summary of best practices for eDNA ddPCR invasive species assays before field deployment.

Stage	Recommendation	Explanation
Primer/probe creation	Sanger sequence invasive tissue	The reference sequences available on NCBI might not be the same sequence as your target invasive's sequence. Sanger sequencing ensures the researchers primers and probe have no mismatches.

Primer/probe creation	Follow eDNA primer/probe guidelines	For high efficiency and high sensitivity assays, follow established guidelines for primer and probe creation, such as Klymus et al., 2020.
Primer/probe creation	qPCR for primer efficiency	When working with ddPCR, testing primers using traditional qPCR is important for measuring primer efficiency (Ramon-Laca et al. 2021).
Pre-field deployment	Tank experiment	Running a tank experiment allows researchers to establish a species eDNA shedding signature to better contextualize field results (Thalinger et al. 2021).

With any new methodology it is important to understand its strengths and limitations. Understanding the value and shorting comings of eDNA is especially important so that researcher and managers can maintain reasonable expectations when deploying novel molecular assays. As demonstrated here, there may be applications where eDNA-based approaches cannot adequately replace traditional methods. Researchers must use caution and conduct rigorous validation of eDNA assays in the field and lab to understand the efficacy of this tool within a given system. Our study here presents a cautionary tale for eDNA applications and we expect there to be dozens if not hundreds of more taxa that similarly cannot be readily detected because they exhibit low steady state eDNA concentrations. Future limitations for the detection of *C. prolifera* eDNA may be ameliorated with the development of a more sensitive assay (perhaps targeting a chloroplast or mitochondrial marker gene which may be more robust than ribosomal target) or the filtration of larger water volumes using tangential flow filtration to acquire hundreds of liters of water. Ultimately, we demonstrate the limitations of eDNA as a survey tool

as it relates to the invasive algae *Caulerpa prolifera* and demonstrate the importance of contextualization and validation of eDNA assays for biomonitoring applications.

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CHAPTER 3

A Community Science Approach to Assessing the Impact of Los Angeles' Marine Protected Area on Biodiversity through Environmental DNA

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Abstract:

Marine protected areas (MPAs) are critical tools for preserving marine biodiversity from the threat of overharvesting. Understanding their impact is important in justifying the areas that have

been protected and expanding additional protections. This study evaluates the impact that marine protection status has on the community composition of both fish and metazoan species off the coast of Malibu, CA at Point Dume State Marine Reserve and Point Dume State Marine Conservation Area. We integrate community scientists from Heal The Bay's volunteer network into the sampling scheme of this project to increase the scope and scale of the eDNA project We sample at two MPA sites and two nonMPA sites for two years to evaluate 12s Mifish and 16s metazoan diversity. We find that environmental DNA was able to survey critical species of concern to MPA managers detecting 60 fish species and 92 metazoan species. Our results found that while there were little differences between fish communities, MPA's produced more diverse and more stable metazoan communities compared to their nonprotected controls. Our results also demonstrate the power that partnering with community scientists has in bettering marine biodiversity surveying.

Introduction:

The Southern California Bight (SCB) is home to highly productive reef formations and kelp forests. Within the SCB, more than 500 species of fish (Dailey et al., 1993) and 5000 invertebrate species (Schiff et al., 2016) can be found. These ecosystems are vital to the environmental, economic, and social health of Southern California. Unfortunately, these resources are threatened due to human-led activity (Halpern et al., 2014). Overfishing, in particular, poses one of the largest threats to fish populations and diversity (Jackson et al., 2001; Jennings and Kaiser, 1998). Fish populations have decreased by 72% in overall abundance in California in the last four decades (Koslow et al., 2015).

In order to combat this, California became the first state to establish a marine protected areas (MPAs) network under the California Marine Life Protection Act (MLPA). When originally passed in 1999, there were 63 MPAs that protected ~2.7% of California's oceans (Gleason et al., 2013; Kirlin et al., 2013; Murray and Hee, 2019). Five years later the MLPA Initiative was created to help redesign California's MPAs through public and private partnership. From 2004-2011, the group planned and designed a new network of parks accounting for stakeholder needs and goals. This system was implemented in 2012 with the creation of 124 MPAs in California's waters in order to protect the diversity and abundance of marine life, protect and sustain marine populations, and protect our marine natural heritage. In Southern California, there are currently 50 MPAs that cover roughly 15% of State waters with three types of marine protected area designations: State Marine Reserves, State Marine Parks and State Marine Conservation Areas. State Marine Reserve (SMR) is the strictest of the classifications and prohibits all take of living organisms except by permit for research. State Marine Park (SMP) allows for some recreational take of living organisms but prohibits commercial take. State Marine Conservation Areas (SMCA) are the least strict and allow for some recreational and commercial take. The take allowed in SMPs and SMCAs is specific to each MPA. During this time the two sites of this study, the Point Dume SMR and Point Dume SMCA, were created. The Point Dume SMR restricts all fishing while the SMCA allows for recreational fishing of white seabass and pelagic finfish.

A recent meta-analysis of 277 marine protected areas globally found that 71% of the MPAs found a positive association between fish populations and MPA designation with fish biomass being 1.6x higher in MPAs than nonMPAs on average (Gill et al., 2017). Similarly, in a study of

124 marine protected areas it was found that average biomass of organisms within the MPA increased by 446%, average density increased by 166%, average size increased by 28% and species richness increased by 21% (Lester et al., 2009). This effect of MPAs on fish populations has been shown in a number of studies (Babcock et al., 2009; Claudet et al., 2008; Lester et al., 2009; Miller et al., 2012; Rojo et al., 2019). This is also true for California's local ecosystem where the Channel Island MPA's have increased fish diversity and biomass by 1.7x compared to nonMPA sites (Airamé and Ugoretz, 2008). Recreationally and commercially targeted fish in California have also shown an increased mean density of 150%, size of 30%, biomass of 440% and egg production of 730% inside marine protected areas compared to nonMPAs (Tetreault and Ambrose, 2007). More recently it has been shown that inside MPAs around the Channel Islands, biomass increased 4x faster than outside the MPAs (Caselle et al., 2015).

The conventional method to monitor fish and invertebrate populations in MPAs is based on scuba surveys. Scuba surveys are logistically complex in the field and are conditions-dependent, which limit the scope and scale of MPA monitoring efforts (Ruppert et al., 2019). This study employs a community science led environmental DNA (eDNA) approach to sample fish and metazoan diversity in two protected and two unprotected areas in Malibu, CA. eDNA metabarcoding methods offer a cost effective and comprehensive tool for standardized and conditions-independent monitoring of species occurrences in MPAs (Gold et al. 2021). Environmental DNA has been shown to match and outperform traditional survey's ability to identify species presence/absence (Hänfling et al., 2016; Nakagawa et al., 2018; Shaw et al., 2016; Yamamoto et al., 2017; Fediajevaite et al., 2021). This study also partners with a local nonprofit, Heal the Bay, to engage their vast volunteer base to train and collect environmental

DNA samples. Community science allows for a higher spatially and temporally resolved sampling schema by leveraging the time and effort of trained volunteers while also improving participants attitudes and perceptions of marine conservation. (Carcia-Soto and van der Meeren, 2017).

This study tests the utility of eDNA methods to provide seasonally resolved fish and metazoan survey information on the impact of two marine protected areas. Our aim is to also demonstrate eDNA's ability to be adopted through community science to greatly expand the scope and scale of biodiversity monitoring projects while reducing field-based complexity of surveying.

Materials and Methods

2.1 Sample Collection

We conducted our study of Southern California marine protected areas off the coast of Malibu, CA. These sites included the Point Dume State Marine Reserve, the Point Dume State Marine Conservation Area, and two nearby unprotected control sites (Table 1; Fig 1).

Table 1: Sampling design for MPA sites

Site	GPS Coordinates	Designation
Point Dume (SMR)	34.00239, -118.804997	State Marine Reserve
Latigo (SMR Control)	34.02604, -118.756081	State Marine Reserve Control
Lechuza (SMCA)	34.0349, -118.860703	State Marine Conservation Area
Piedra (SMCA Control)	34.03827, -118.88354	State Marine Conservation Area Control

Scientists, alongside our community nonprofit partner Heal the Bay, held quarterly training sessions for volunteers on MPA science, visual surveying techniques, and eDNA methodologies. Community scientists collected samples from April 2019 to March 2020. One month of data, April 2020, is missing from the dataset due to COVID-19 shutting down the beaches in LA County and making our sites inaccessible. From May 2020-April 2021, researchers alone took the monthly samples as COVID-19 guidelines limited field work by the community scientists. This chapter specifically looks at a subset of seasonal samples for the two years. We examine May 2019, August 2019, November 2019, February 2020, May 2020, August 2020, November 2020, and February 2021 which have been split up into four seasons.

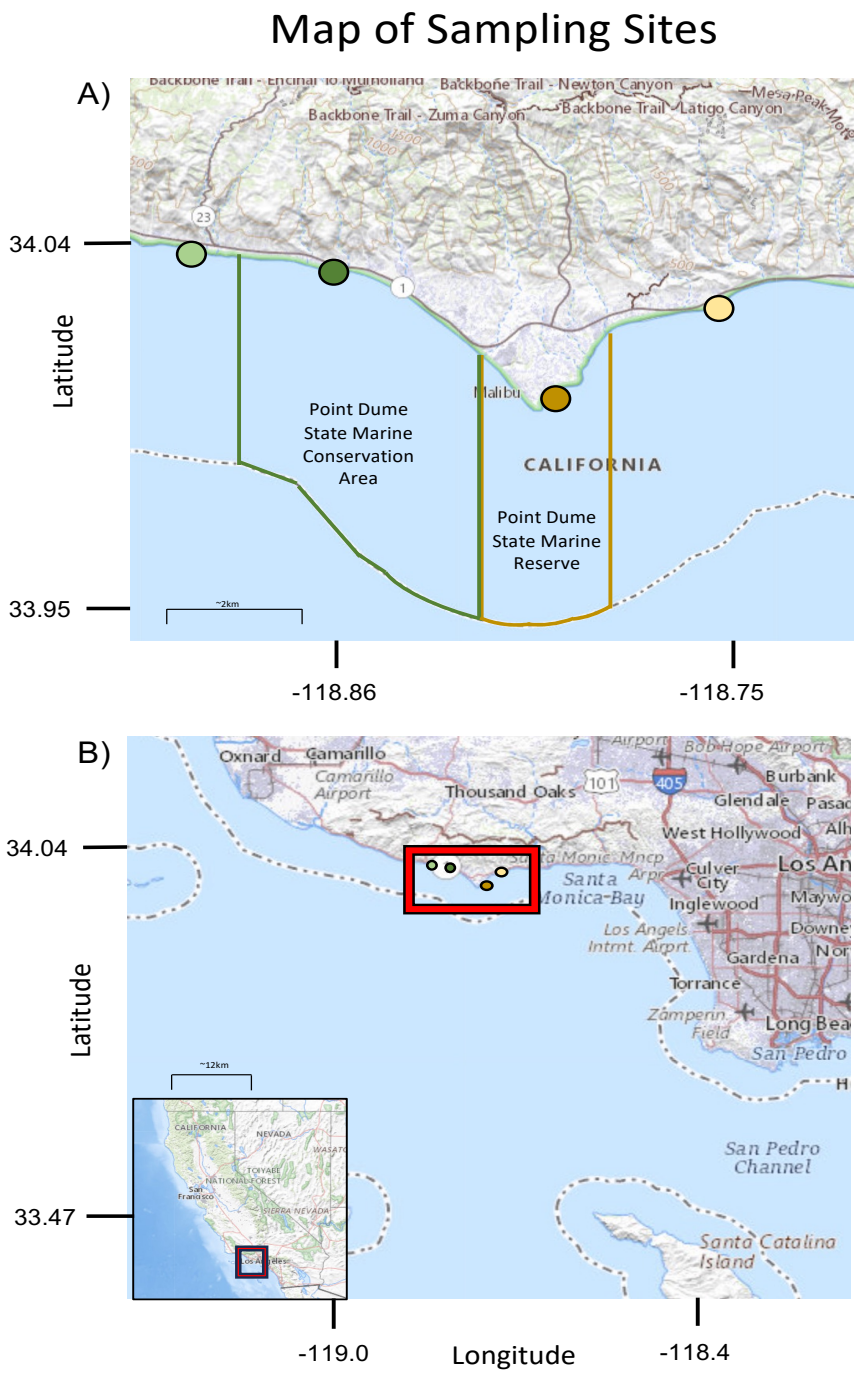

Fig 1. A) Map of sampling sites. Light green indicates Piedra (SMCA Control), dark green indicates Lechuza (SMCA), light yellow indicates Latigo (SMR Control) and dark yellow indicates Point Dume (SMR). B) Map of sites within Southern California Bight. Maps from

USGS National Map Viewer under a CC BY 4.0 license (2022): https://apps.nationalmap.gov/viewer/.

We employed the eDNA collection method of Curd et al. 2019. The first Saturday of each month UCLA researchers met a central location to the four sites and set up a congregation and filtration center. Heal the Bay volunteers arrived at 9am and were given a refresher training of how to collect environmental DNA samples. From here, volunteers were paired up and given a cooler filled with supplies such as ice packs, gloves, kangaroo bags, and markers for sample collection. Community scientists and researchers collected seawater samples at knee-depth off the beach at each site. We captured one liter of seawater in a Kangaroo enteral feeding bag in triplicate. We kept samples on ice for the roughly 20-30 minutes until we returned to filtration tent on the beach where the water was filtered through a sterile 0.22 µm Sterivex cartridge filter (MilliporeSigma, Burlington, MA, USA) using gravity filtering. While we filtered, we explained the process to the volunteers and answered any conservation or marine questions they had. We capped the filters and stored them on ice until we returned to the lab where they were stored at -20ºC in the lab. During each day of sampling, we filtered one liter of Milli-Q water through the same process for a negative field control.

2.2 DNA extraction and library preparation

We extracted our eDNA from the Sterivex cartridge using a modified DNeasy Blood & Tissue Kit protocol (Qiagen Inc., Germantown, MD) optimized for increased eDNA yield (Spens et al., 2017). Using PCR, we amplified the extracted DNA using the Mifish Universal Telost 12s primer set and the Metazoan 16s primer set. We used a 25 µL reaction for both primer sets

composed of 12.5 µL QIAGEN Multiplex Taq PCR 2x Master Mix,1.5 µL of molecular grade H2O, 5 µL of forward primer (2 mM), 5 µL of reverse primer (2 mM), and 1µL of sample DNA. Our cycling conditions for both primer sets consisted of a touchdown PCR profile with an initial denaturation at 95°C for 15 min, followed by 13 cycles of 95°C for 30s, beginning annealing at 69.5°C for 30 seconds and decreases in temperature of 1.5°C per cycle until 50°C, extension at 72°C for 1 minute. After the 13 cycles we did 24 cycles of 95°C for 30s followed by annealing at 50°C and an extension of 72°C for 1 minute. We use a final extension for 10 min at 72°C. A negative PCR control of molecular grade water was added following the same protocol. We verified amplification success by checking product size on a 2% agarose gel electrophoresis stained with SybrSafe.

After amplification, we modified the samples by adding individual Nextera unique dual indices (Illumina, San Diego, CA, USA) . We used a 25 µL reaction composed of 12.5 µL Kapa Hifi MasterMix (Kapa Biosystems, Sigma Aldrich, St. Louis, MO, USA), 6.25 µL of molecular grade H_2O, 1.25 µL of index and 5 µL of DNA from the PCR sample. Our PCR cycling parameters for indexing consisted of denaturation at 95°C for 5 min, followed by 8 cycles of denaturation at 98°C for 20s, annealing at 56°C for 30s, and extension at 72°C for 3 min, and then a final extension at 72°C for 5 min. We verified amplification success by checking product size on a 2% agarose gel electrophoresis stained with SybrSafe.

We then measured DNA concentration of each sample with the Qubit dsDNA Broad Range DNA Quantification Assay (Thermofisher Scientific, Waltham, MA, USA). Samples were then pooled in equal copy number across two pools. Biological replicates 1 was put on Run 1,

biological replicates 2 were put on Run 2 and biological replicates 3 were randomized and split between both runs to minimize any sequence batch effects. We cleaned the resulting library using a Qiagen gel extraction to remove both small and large DNA fragments that are the improper size. The final library was sequenced at UC Davis' Genome Center on two Illumina NovaSeq SP PE250 spiked with 15% PhiX. Not enough PhiX limited the number of sequences we received.

2.3 Bioinformatics

We used the *Anacapa Toolkit* for amplicon sequence variant parsing, taxonomic assignment, and quality control. The quality control step of the Anacapa Toolkit trims extraneous adapter sequences used to identify each unique sample, removes low quality reads, and sorts reads by metabarcode primer sequence. The amplicon sequence variant (ASV) parsing step uses DADA2 to dereplicate our metabarcodes. Next the Anacapa toolkit module assigns taxonomy to ASVs using Bowtie 2 and a Bowtie 2-specific Bayesian Least Common Ancestor (BLCA) algorithm.

For the fish primer set, taxonomic assignment was conducted following benchmarking by Gold et al. (2021) using a taxonomic cutoff score of 60 and minimum alignment of 80%. Taxonomy was first assigned using the curated regional database of California Current Large Marine Ecosystem fishes to identify native taxa. We then re-assigned the taxonomy using the global *CRUX* generated database to identify non-native and non-fish species. Taxonomic assignments of ASVs were synonymized between both methods by prioritizing higher resolution assignments (i.e. species level vs. genus level). For the metazoan primer set, taxonomic assignment was conducted following benchmarking by Gold et al. (2021) using a taxonomic cutoff score of 60 and minimum alignment of 90% (Gold et al., 2023).

We then implemented a decontamination procedure to eliminate poorly sequenced samples and remove potential sources of contamination. Importantly, we applied a site occupancy modeling framework to retain only ASVs that occurred in high prevalence across locations and stations. All remaining ASV's had their read counts converted into the eDNA index. The eDNA index transformation is conducted by first normalizing all reads for a particular species by the total number of reads in each sample, then normalizing those proportions to the largest observed proportion for that species across all samples. This results in a species-specific scaling between 0 to 1, where 1 is the sample with the highest occurrence for a given species in all the samples and 0 is the least. Based on computational modeling this metric was shown to have the highest correlation with biomass, with a median Spearman correlation of .87 with biomass (Kelly et al., 2019). The index assumes that biases originate from DNA-primer interactions, which would remain consistent across samples, and thus allow us to infer relative abundance changes of a single taxa between samples (Kelly et al., 2019).

2.4 eDNA data analysis

We tested if our sequencing depth reached species saturation for our samples using a rarefaction curve (Fig S1; Fig S2). Total species richness was compared using a generalized linear model (GLM) with a Poisson link function. The data was subset into fish species with commercial or recreational value based on those listed as commercially important on fishbase.com (Froese, R. and D. Pauly, 2023) and their eDNA indexes were compared between MPA and controls. Comparison between species' eDNA index was completed using a binomial GLM to compare relative abundances between sites. We tested differences in distribution of species' eDNA index using the Mann-Whitney U test.

To test for differences in community composition (beta diversity), the eDNA indexes for the samples were converted into Bray-Curtis dissimilarity distances. We tested for differences in community structure by site and season using an PERMANOVA followed by a multivariate homogeneity of group dispersions test BETADISPER. Community composition was visualized using Principal Coordinate Analysis (PCoA). Closer grouped data points indicate more closely related community composition in both species' richness and diversity. Zeta decay was measured using the *zetadiv* package (Latombe et al., 2017) between MPAs and nonMPAs to measure overlap in the observed communities (Hui and McGeoch, 2014).

Results:

3.1 Species richness

After removing sequences and samples through taxonomic assignment and quality control in the *Anacapa Toolkit*, we generated ~4.1 million fish reads across 85 samples and ~4.0 million metazoan reads across 93 samples. The fish reads represented 1 phylum, 2 classes, 20 orders, 33 families, 55 genera, and 60 species (Supplemental Table 1). The metazoan reads represented 11 phyla, 21 classes, 37 orders, 75 families, 87 genera, and 92 species (Supplemental Table 3). The species read counts were then converted into an eDNA index (Supplemental Table 2; Supplemental Table 4). Sample rarefaction curves showed that for each sample sequencing depth was sufficient to capture all species diversity within that collected sample (Fig S1).

Across all time points, our eDNA surveys captured 60 unique fish species with 37 species found at Point Dume, 48 at the Latigo, 51 at the Lechuza, and 42 at the Piedra and 92 unique metazoan

species with 61 species found at Point Dume, 74 at Latigo, 73 at Lechuza, and 74 at Latigo. Across the replicates, sites had a mean value of 10-20 fish species (Fig 2) and a mean value of 12-30 metazoan species depending on season (Fig 3). These species are listed seasonally by site (Supplemental Table 6; Supplemental Table 7)

Comparisons between the SMR and its control and the SMCA and its control found no significant difference in the mean number of fish species observed overall (GLM $Pr(>|z|) = 0.369$ and 0.98) or within each season (GLM $Pr(>|z|) > .05$).

Similarly, there was no significant difference in the mean number of metazoan species observed overall (GLM $Pr(>|z|) = 0.505$ and 0.966) but the SMCA did have significantly more metazoan species in summer compared to its control (GLM $Pr(>|z|) < .001$) and the SMCA's control had more metazoan species in the spring compared to the SMCA (GLM $Pr(>|z|) = 0.0028$).

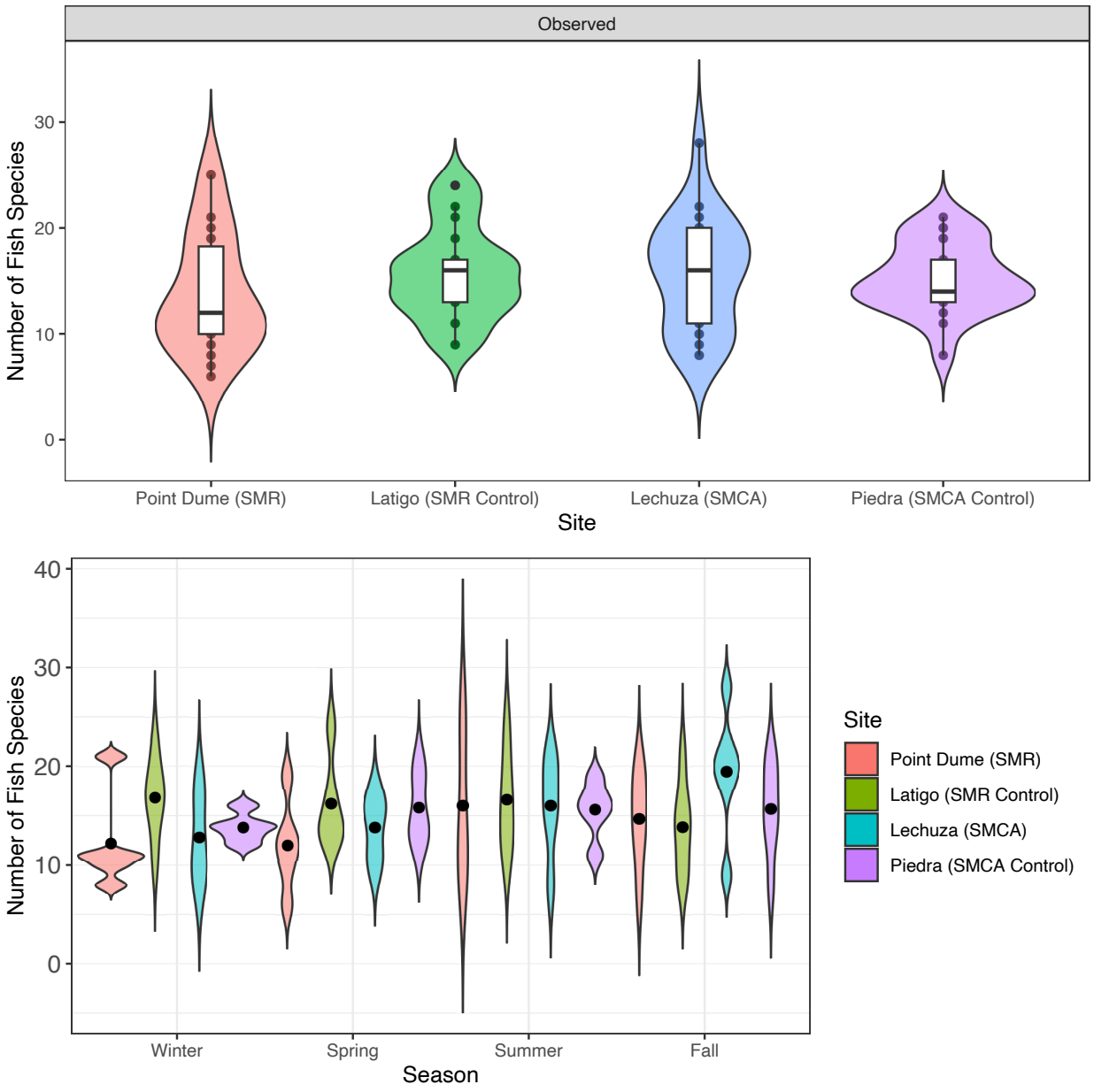

Fig 2. Violin plot of fish species richness by site across all seasons.

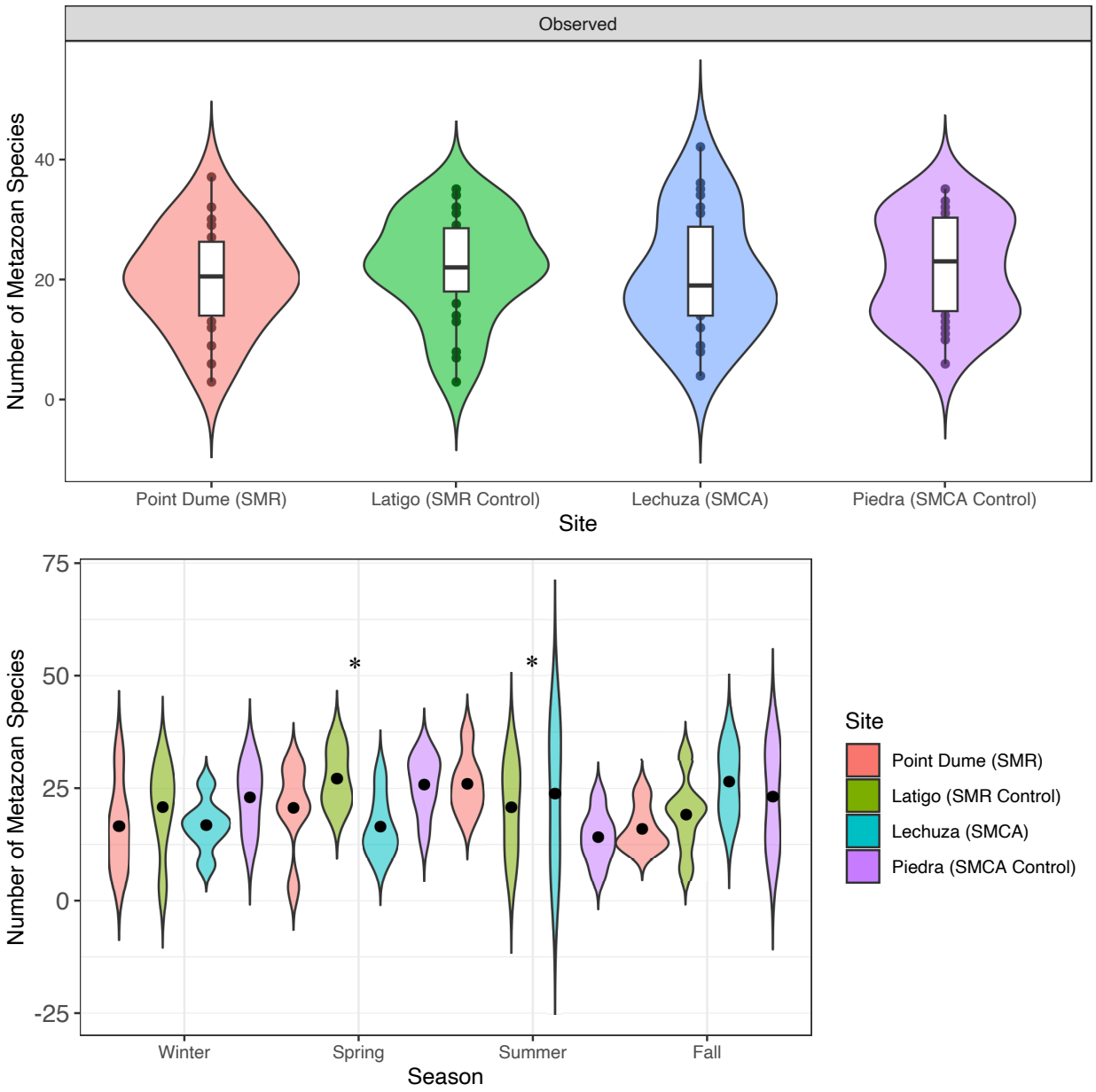

Fig 3. Violin plot of metazoan species richness by site across all seasons. * indicates significance between the two sites on either side.

3.2 Community composition

The PCoA shows that our samples cluster with one another based on site and MPA status and that protected vs unprotected areas, while overlapping in parts, cluster distinctly from one

another (Fig 4). This is especially true for metazoan communities which share less points within each ellipse than the fish communities do (Fig 4).

The data showed significant differences in fish community composition by site (PERMANOVA $p<0.001$, $R2 = 0.1134$, betadisper $p>0.05$), season (PERMANOVA $p<0.001$, $R2 = 0.0988$, betadisper $p>0.05$), and MPA status (PERMANOVA $p<0.001$, $R2 = 0.045$, betadisper $p>0.05$). Fish show little seasonality within nonMPAs and show more differentiation in MPAs with fall and winter more similarly related as compared to spring and summer (Fig 5). When comparing fish communities between MPA and nonMPAs, we see significant overlap in grouping during fall and winter and the greatest difference in spring followed by summer (Fig 7).

The data also showed significant differences in metazoan community composition by site (PERMANOVA $p<0.001$, $R2 = 0.1046$, betadisper $p>0.05$), season (PERMANOVA $p<0.001$, $R2 = 0.099$, betadisper $p>0.05$), and MPA status (PERMANOVA $p<0.001$, $R2 =0.072$, betadisper $p>0.05$). Like fish, metazoan communities show greater overlap within nonMPAs. MPAs sites were more similar in winter and spring as compared to summer and fall (Fig 6). When comparing metazoan communities between MPA and nonMPAs, we see that they remain fairly separated throughout all four seasons (Fig 7).

Zeta diversity plots show near-complete overlap between MPAs and nonMPAs for fish communities with a core-community of 5 species for MPAs and 4 species for nonMPAs (Fig 8). Zeta diversity for metazoan communities show MPAs having a higher zeta diversity over zeta orders indicating more diverse communities and have a higher asymptote than the nonMPAs indicating larger core community and thus more stable metazoan community (Fig 8)

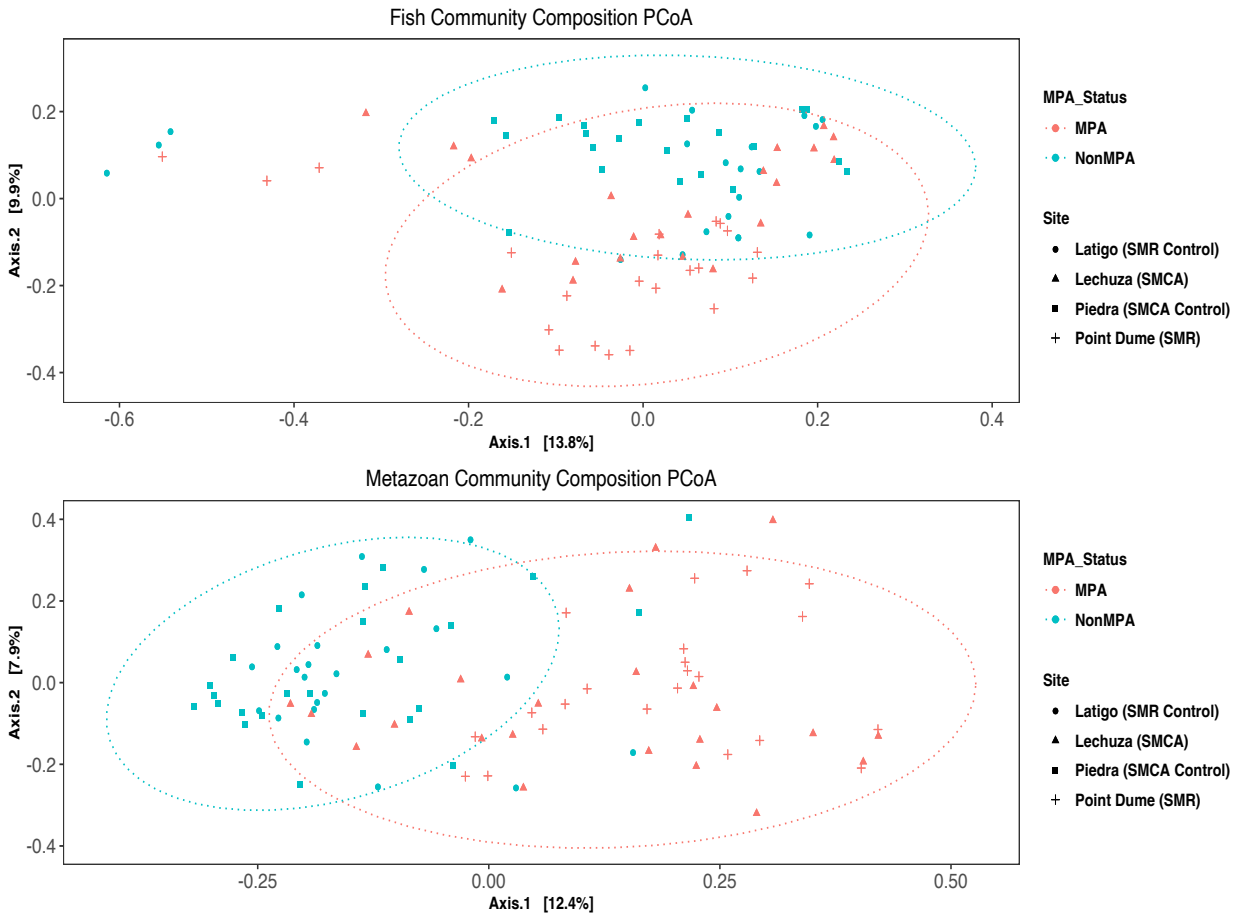

Fig 4. PCoA visualization of Bray-Curtis dissimilarities between the MPA and nonMPA sites by site.

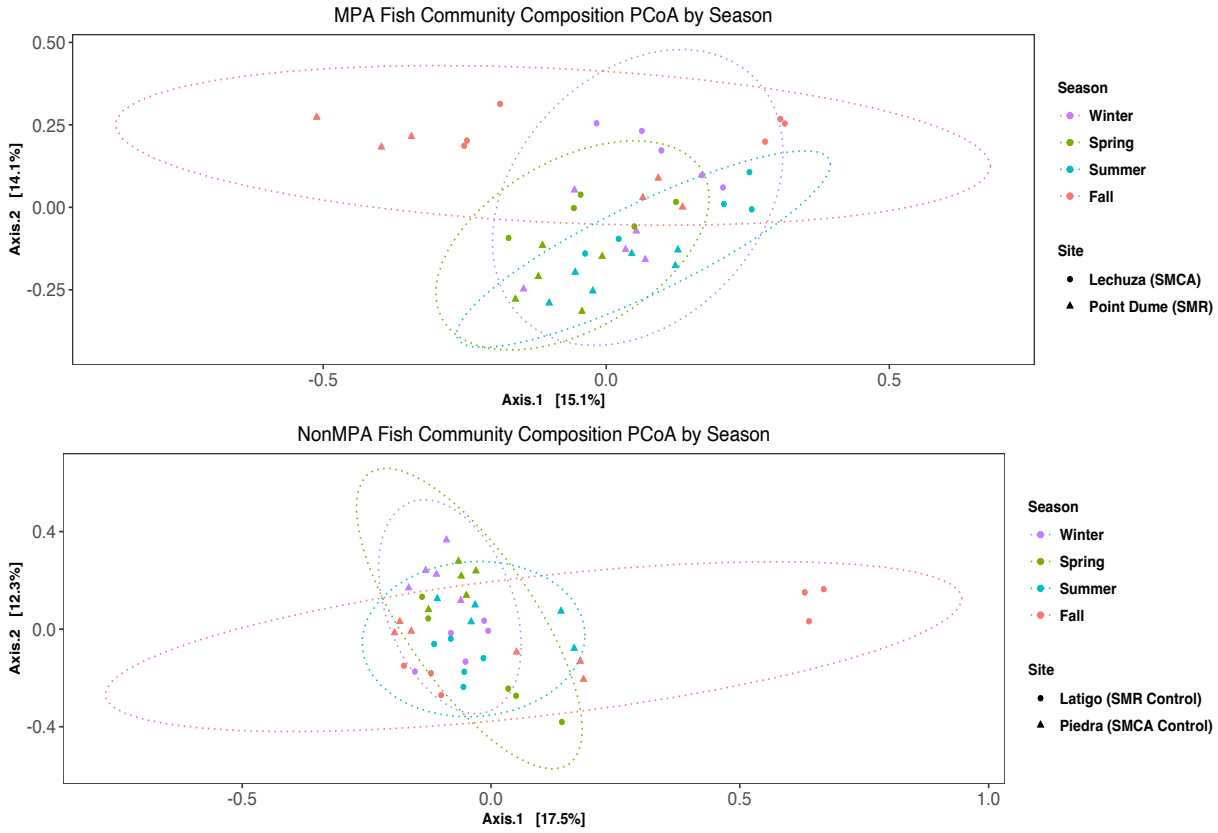

Fig 5. PCoA visualization of seasonal fish Bray-Curtis dissimilarities at the MPA sites and at the nonMPA sites.

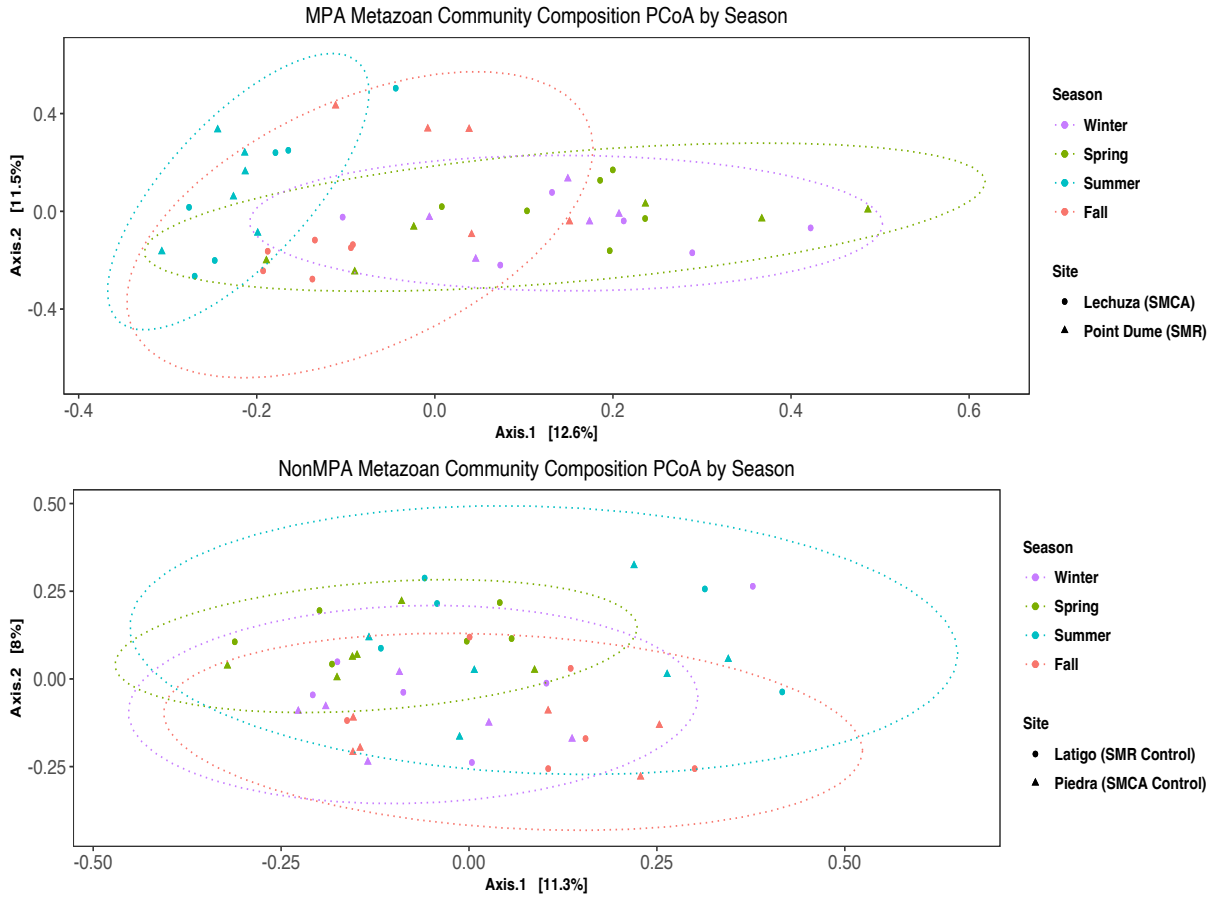

Fig 6. PCoA visualization of seasonal metazoan Bray-Curtis dissimilarities at the MPA sites and at the nonMPA sites.

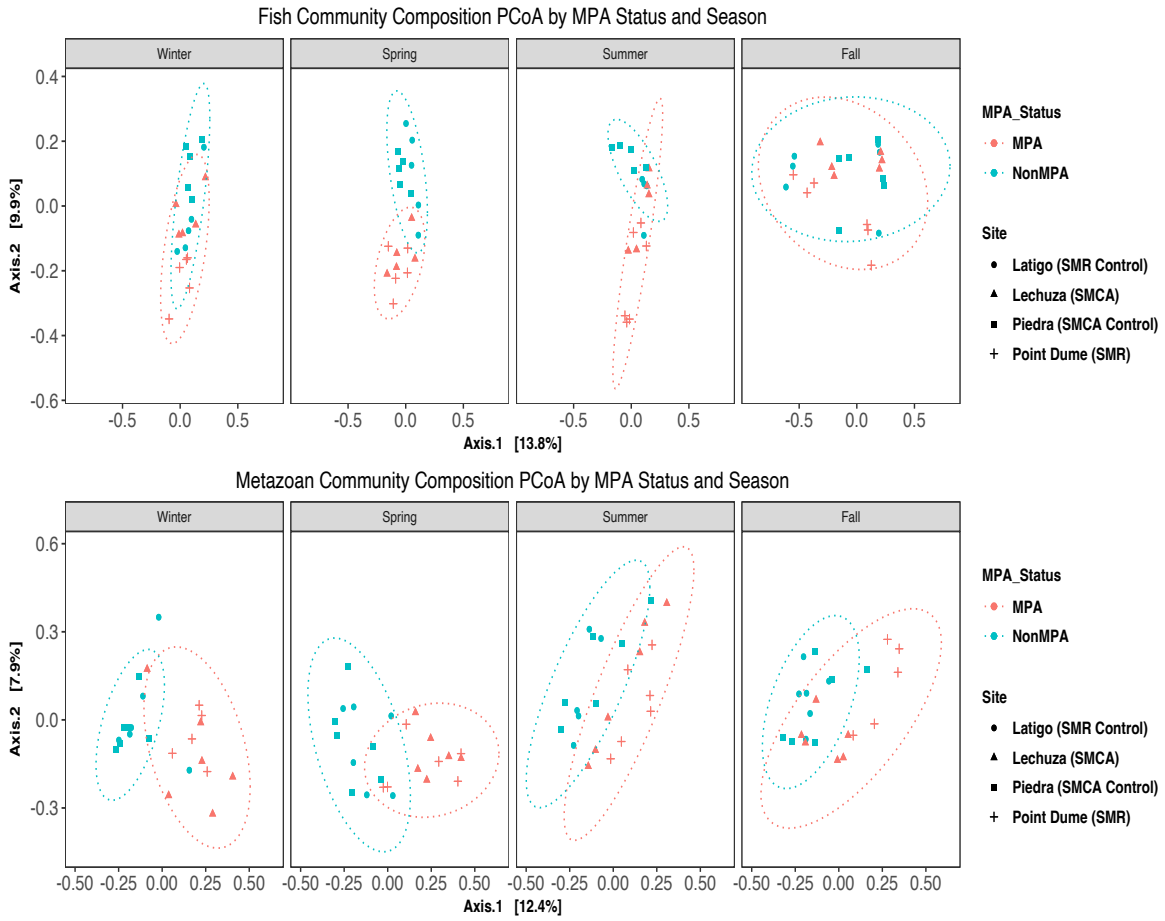

Fig 7. PCoA visualization of seasonal fish and metazoan Bray-Curtis dissimilarities by MPA status.

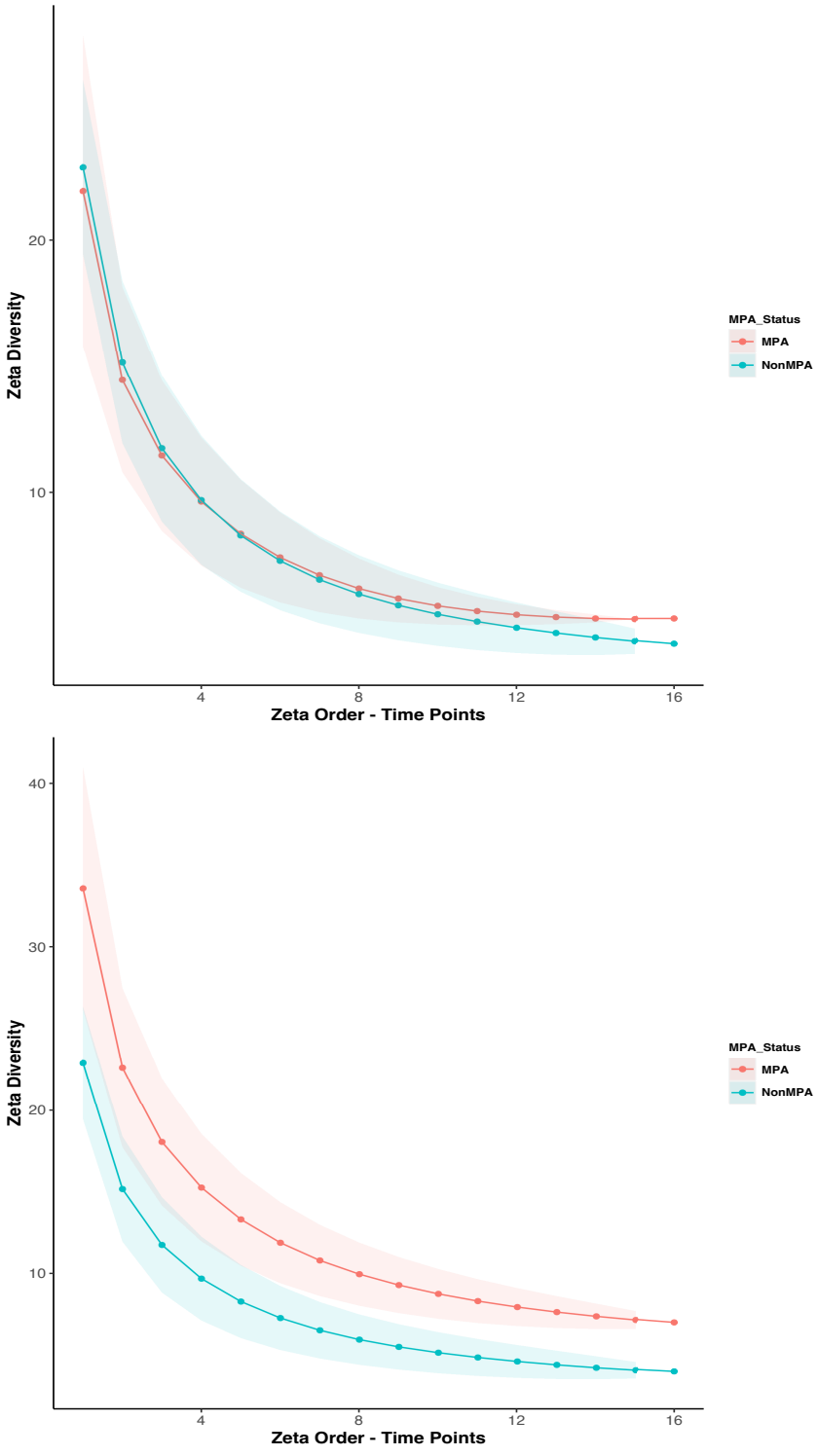

Fig 8. Zeta diversity decay graphs for fish communities (top graph) and metazoan communities (bottom graph) for MPAs vs nonMPAs.

3.3 Relative Abundance

There are a number of recreationally and commercially important species that showed high eDNA indexes throughout the sites including jack silverside (*Atherinopsis californiensis*), opaleye (*Girella nigricans*), California corbina (*Menticirrhus undulatus*), kelp bass (*Paralabrax clathratus*), Pacific sardine (*Sardinops sagax*) and yellowfin croaker (*Umbrina roncador*). For each of the commercially/recreationally important fish species, there was no difference in their relative abundance between the protected and unprotected areas ($Pr(>|z|) > 0.05$ for each species)(Fig 9). For *Atherinopsis californiensis* (Jack silverside) and *Scorpaenichthys marmoratus* (Cabezon), their eDNA index had a significant difference in distribution with higher distribution of values for MPA vs nonMPAs ($p = .011$ and $p = .0026$ respectively). For *Menticirrhus undulatus* (California corbina), their eDNA index had a significantly higher distribution of values in nonMPAs ($p < .001$).

For metazoans species, the only commercially or recreationally caught species that showed a significant difference in abundance was California mussels (*Mytilus californianus*) which were in greater abundance in MPAs vs nonMPAs ($Pr(>|z|) < .001$) (Fig 10).

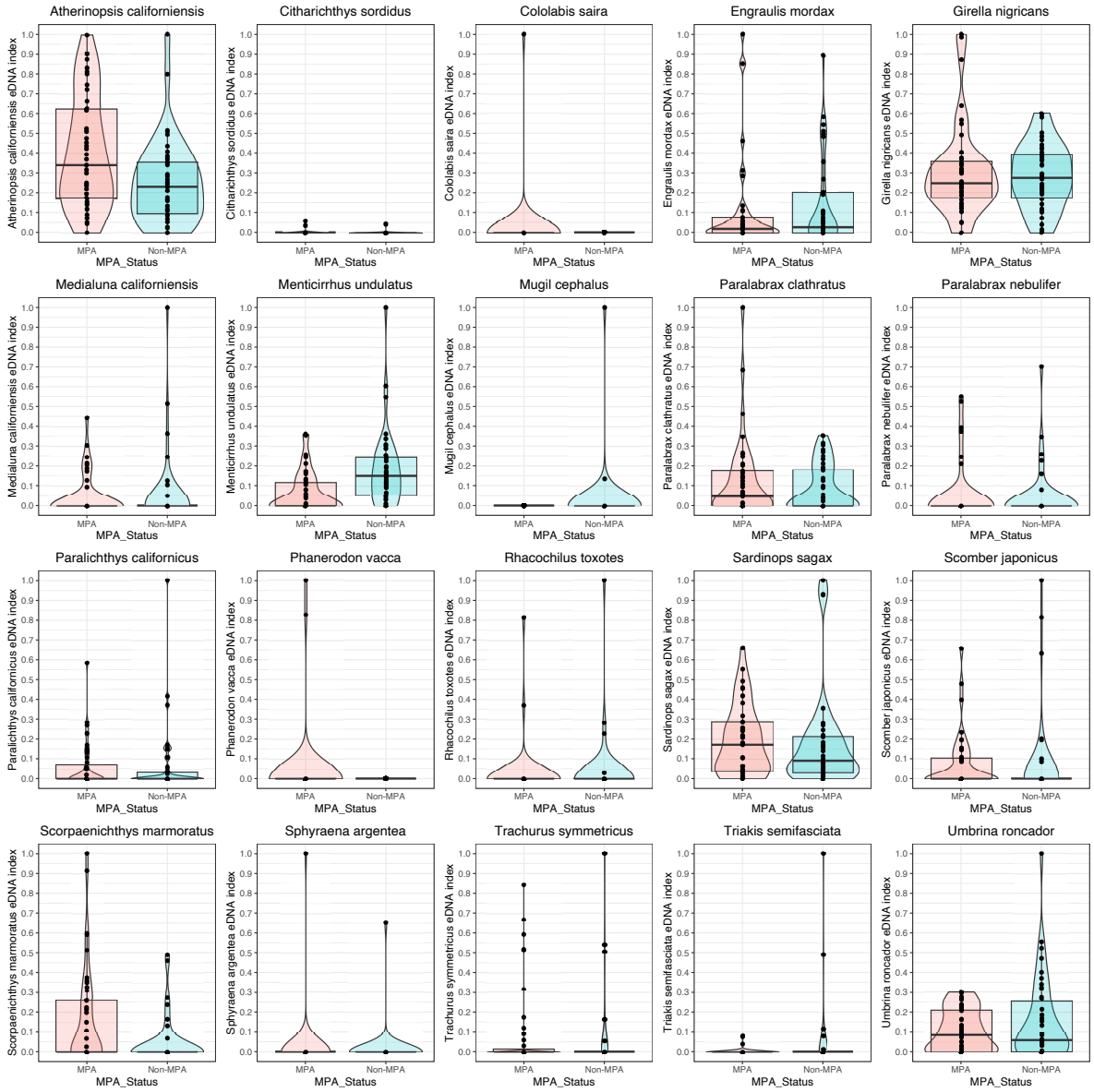

Fig 9. Relative abundances of commercially and recreationally important fish species in MPA sites (red) and nonMPA sites (blue).

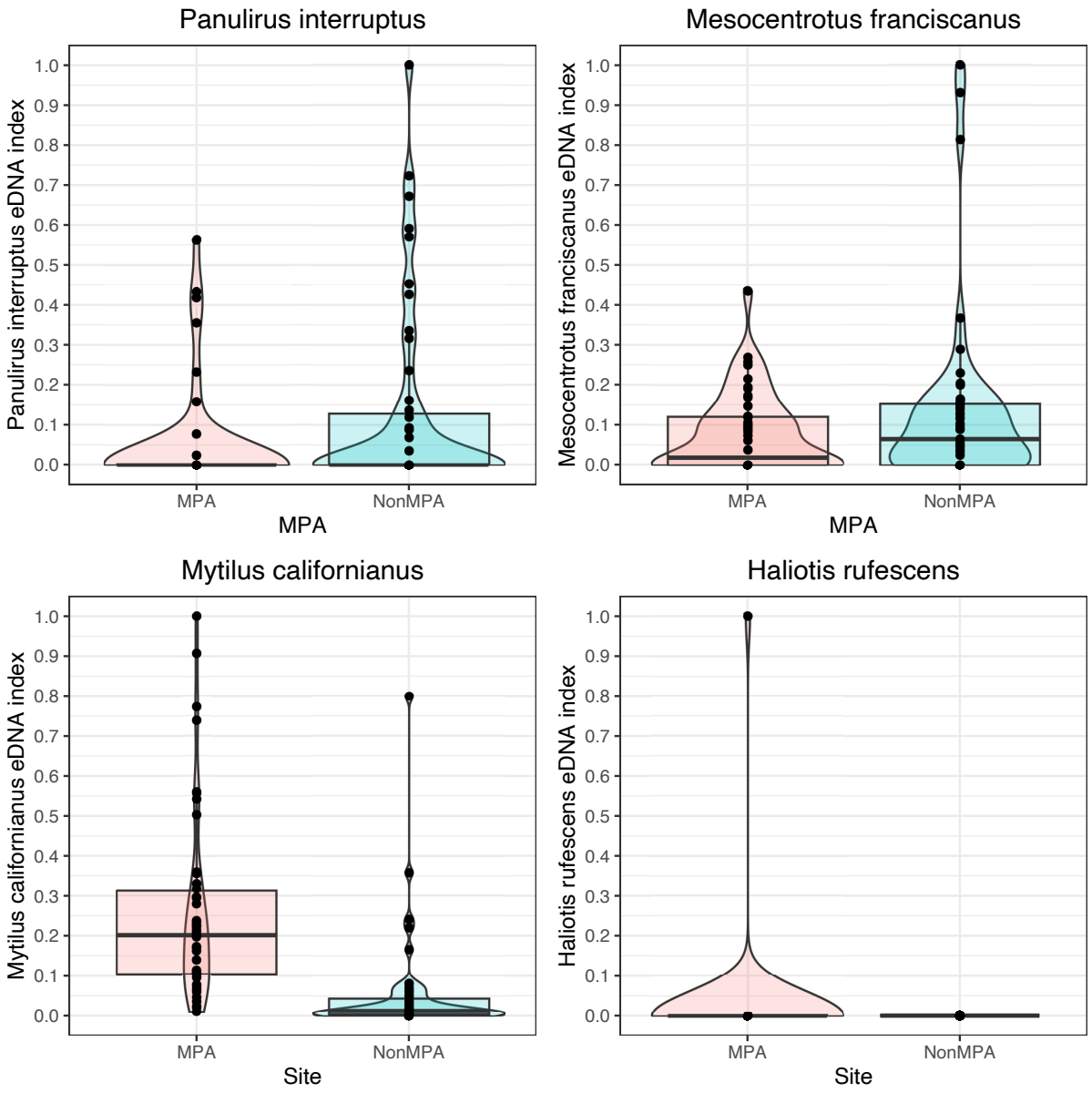

Fig 10. Relative abundances of commercially and recreationally important metazoan species in MPA sites and nonMPA sites.

Discussion:

4.1 Environmental DNA offers a low barrier yet effective means to field sampling of biodiversity monitoring via community science

Environmental DNA surveying was highly effective at detecting both fish and broader metazoan species at our two protected and unprotected sites. This is true during both the sampling periods completed by community scientists and by UCLA researchers. In the first year of the project we were able to successfully engage ~70 participants and collect 144 eDNA samples. Through the participation of community members in biodiversity monitoring, we are able to increase both the scope and scale of samples that could be collected in a considerably reduced amount of time and effort.

Conventional survey methods for fish and metazoans include scuba surveys, baited remote underwater videos (BRUV) and net sampling. These techniques require specialized equipment and knowledge to complete. There are community science based scuba survey programs that take important biodiversity data, such as ReefCheck (www.reefcheck.org), but unfortunately the barrier to participation is high because it requires costly scuba diving certification and intimate taxonomic knowledge of the species in order for proper identification (Falk et al., 2019; Gardiner et al., 2012). This limits the scale at which community-based biodiversity monitoring can occur by restricting the potential pool of non-researchers to those with the resources and expertise to engage in science. Scuba surveys also limit the number of sites that can be sampled due to the time and energy intensive nature of scuba diving. Field collection of environmental DNA is logistically less complex which allows for increased participation in sampling. A number of previous eDNA studies has shown community sciences ability to be incorporated into their

projects, such as CALeDNA (Meyer et al., 2021). Our sampling design made it so that the only requirements to participate were that the volunteer could 1) get to the site and 2) could collect a liter of water in a bag from knee-depth off the beach. This allowed people from various age groups, demographics and previous exposure to science to participate in our research. Aside from the increased quantity of samples that could be taken, eDNA is also more cost-effective. A previous study of eDNA MPA monitoring in Southern California has shown that eDNA surveys were nearly 60% less costly per site (Gold et al., 2021). Thus, by using both eDNA and community scientists, we were able to get a greater number of samples from a wider range of sites with less effort and cost than traditional sampling.

Community science based projects don't just benefit the research they also benefit those who engage in it. Community participation in science has been shown to enhance scientific literacy (Bonney et al., 2015), lead to more informed conservation advocacy (Toomey and Domroese, 2013), create a sense of ownership/connection with the natural environment (Pocock et al., 2023), build networks of like-minded individuals (Fraisl et al., 2022), incorporate local knowledge into scientific expertise (Albagli and Iwama, 2022) and encourage engagement in local decision making processes (Bonney et al., 2015). Programs like MPA Watch, facilitated by LA Baykeeper and Heal the Bay, and other efforts to develop recommendations for long-term sustainable monitoring of California's MPAs are already underway (Freiwald et al., 2018). The incorporation of eDNA into these pre-established networks of volunteers could provide an easy yet powerful long-term monitoring program for California MPAs.

4.2 MPAs support more diverse and stable metazoan communities compared to non-protected areas but show little differentiation in fish communities.

Our environmental DNA data showed that it was able to capture species of interest for environmental managers. MPAs are designed to protect key commercially and recreationally important fish and invertebrate species. We note that among the many species of concern, we found California spiny lobster (*Panulirus interruptus*), red sea urchin (*Mesocentrotus franciscanus*), red abalone (*Haliotis rufescens*), sea stars (ex: *Pisaster ochraceus*), the giant California sea cucumber (*Parastichopus californicus*), crabs (ex: *Pugettia richii*) and many others. For fish we detected sharks (ex: *Heterodontus francisci*), flat fish (ex: *Paralichthys californicus*), bass (ex: *Paralabrax clathratus*), groundfish (ex: *Scorpaenichthys marmoratus*) and the California sheepshead (*Semicossyphus pulcher*) among other important fish.

At the species level, our data shows little differences between MPA's and nonMPAs. The first comparison shows the same overall mean number of fish and metazoans species found at the MPAs vs their controls. When these species were analyzed for their relative abundance, we again see no difference in key commercially targeted species between MPAs and nonMPAs. The only species which was found significantly more frequently in MPAs was the recreationally important California mussel. Despite this similarity in the number of species and overall relative abundance across seasons, we do find a difference in the composition of the communities found at each site. When comparing beta diversity, marine protected areas cluster with one another more than they do with either of their control sites. The unprotected sites are compositionally more similar to one another even though they are at a distance of 11.8 km away than, for example, the SMCA and its control site which are only 2.1 km away from one another. Based on surveys of

microhabitats offshore, we know the SMCA and its control are compositionally more similar than the controls are to each other or the MPAs are to each other. Since the MPAs cluster with one another better than with their controls, it supports the idea that protection status is imparting effects on the organisms within their bounds.

Fish communities show seasonal differences in their relationship within MPAs and nonMPAs. Fish show the greatest divergence in spring and summer, which are the seasons most marine fish spawn in Southern California (Harada et al., 2015). By fall and winter, these differences disappear. Fish may be using MPAs as a nursery to protect against harvesting pressures during these seasons but, because they're highly mobile, once they grow out of the nursery they travel to nearby sites. Metazoan communities remain distinct between MPAs and nonMPAs throughout all the seasons. The PCoAs highlight that MPA status does confer community level changes to both fish and metazoan composition.

Zeta diversity plots follow a similar narrative to the other analyses. We see no difference in diversity and community turnover of the fish communities between MPAs and nonMPAs. For metazoan communities, zeta diversity shows that we have more diverse communities and less turnover in MPAs vs nonMPAs. Less turnover implies more stable communities across time. Stability is important for resilience and indicates that metazoan communities in MPAs might be more resilient to pressures such as climate change than nonMPAs.

While we see community level differences, MPAs having no impact on the relative abundances of the species is not consistent with the majority of MPA literature, which shows that MPAs

increase diversity and size of species living within them. There are two main explanations for what we saw. The first is that MPAs haven't had a significant enough impact on the fish community composition yet. This explanation is consistent with previous California MPA work such as Starr et al., 2015. In that study, researchers monitored Central California MPAs for seven years after their creation and found no differences in changes between the MPAs and the reference sites in those seven years. This is because of the timescale at which targeted fish are recruited to the sites and how fast these species mature. Due to this, the researchers stated they'd expect to see differences on the 1-2 decade scale within the MPA (Starr et al., 2015). Similarly they state that California's strict fishing regulations means that there wouldn't be an expected rapid drop in fish populations in unprotected areas after the establishment of an MPA. Our two Southern California MPAs were created 7-9 years prior to our sampling and might not be materializing individual species impacts because of the timeframe at which these species' life histories operate on. One advantage of using multivariate analysis is being able to discover trends that when picked apart individually aren't evident or don't have enough statistical power behind them to be significant but combined across all taxa can illustrate differences. We see this not to be true in the metazoan community which does show significant differences compared to their non protected controls. This could be because a large number of these species are smaller, sessile organisms with shorter generation times. This would mean that species are more directly impacted by the MPAs and whether through direct, or most likely indirect channels, the protections of the MPAs are materializing differences within the metazoan community.

The second explanation is that MPAs are significantly altering community composition but that that is masked within our limited spatial data set. The minimal observed differences between

protected and unprotected areas could be because of the spillover effect. The spillover effect suggests that since there's no physical boundary confining species within marine protected areas that they end up having a net-positive effect on adjacent areas through their migration between protected and unprotected areas (Medoff et al., 2022). This effect has been seen in numerous MPAs (Di Lorenzo et al., 2020) as well as in Southern California specifically (Lenihan et al., 2021). Another reason could be that the metric we're using, comparisons between eDNA indexes, while the best tool we have at comparing biomass differences, may not be the best proxy for abundance. There are limitations in the eDNA index in collapsing all species to the same scale due to differences in shedding rates, DNA recovery, amplification efficiency in PCR and other post collection impacts.

One positive aspect of this longitudinal study was that the PCoA data shows our samples cluster by protection status and by site rather than by season. This suggests that for future research, taking samples during just one or two seasons may be sufficient to tease-apart MPA's impact. Previous California MPA studies on fish populations have sampled a fraction of the year, typically 2 - 5 months which can be due to visibility issues while conducting transects or constraints from sea conditions (Caselle et al., 2015; Starr et al., 2015). Our multi-year study highlights that, while there is a seasonal effect, reduced temporal frequency may still be enough to evaluate the impact of MPAs. There is still utility in taking monthly samples which can uncover patterns that might not be seen when taking a coarser seasonal analysis. This would include things like detailed species migration patterns or capturing short climatic anomalies like heatwaves.

4.3 eDNA limitations

Environmental DNA has proven itself to be an effective biodiversity monitoring tool. As any tool, its efficacy depends on the application to which it is applied. There are three main limitations that hinder eDNA's applicability for MPA monitoring. The first limitation is the gap in genetic reference databases needed for species identification (Marques et al., 2021). Identification is reliant on which species have already been barcoded so the databases are often skewed towards charismatic and commercially relevant species (Marques et al., 2021). For incomplete databases, this can bias the composition of the results. In the case of the metazoans, less than 15% of metazoan species have been estimated to have been barcoded (deWaard et al., 2019). We are therefore minimizing the potential to see differences in the community composition by reducing the number of possible species that are included.

The second limitation is primer selection. This study uses the universal 12s Mifish primers for fish identification. While the 12S database for California fish species is largely completed (Gold et al., 2020), the 12s barcode is unable to resolve fish in the rockfish genus *Sebastes* (Min et al., 2021). Rockfish are commercially important species in California and targets of MPA protection. Being unable to evaluate the impact of MPAs on rockfish without the additional time and energy to add the rockfish primers (Min et al., 2021) means that we might be missing effects of MPAs on key species.

The third limitation is that eDNA metabarcoding as implemented in this has limited capacity to differentiate abundances. Marine protected areas are known to increase the length, biomass, egg production, and other physical characteristics of the taxa inside (Lester et al., 2009). While

changes in these characteristics may lead to increased eDNA shedding and thus a relatively higher eDNA index in the samples- researchers are unable to know if a higher eDNA index is due to bigger/longer fish or due to greater number of fish in the area. There is also a number of things that impact abundance values in the lab like differential PCR amplification efficiencies. Mock communities or variable PCR cycles can be used to overcome some of these quantitative issues (Shelton et al. 2023) but require additional work. Conventional surveys, in this instance, are able to provide information on species traits that eDNA can't.

Future steps for this project will include analyzing the full monthly samples that were collected at the sites. Additional work that would further the value of the dataset collected would be to add additional primers that could target things such as rockfish and phytoplankton to continue to resolve a more holistic look at MPAs. This project is also continuing into its third and fourth year with Heal The Bay which will allow us to further study the impact that MPAs are having in Southern California.

Conclusion

MPAs are an important conservation tool for marine managers. Our project shows the importance that MPAs play in protecting species and the value that eDNA holds in measuring biodiversity. Specifically, we find 60 fish species and 92 metazoan species between our protected and control sites- many of which are species of concern for MPA managers. Our seasonal surveys for two years highlight that MPAs are imparting community level differences in the metazoan community leading to more diverse and stable communities. We were unable to find significant differences within the fish community except within spawning months. Our project also highlights the value that incorporating community scientists has in eDNA research.

We show that community scientists can effectively sample for biodiversity monitoring and can increase both the spatial and temporal scale of an eDNA project.

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Conclusions

In the past decade, studies employing environmental DNA for biodiversity monitoring, invasive species tracking, and fisheries management has increased exponentially. These studies continually show the utility that eDNA has as a methodology to survey our oceans, lands and air. The results of this dissertation not only continue to highlight the power of eDNA and refine its potential use cases but also provide evidence for the impact of various conservation efforts in Southern California.

Chapter 1

In this chapter, we illustrate two main findings. The first is that seagrass beds within Southern California are biological distinct in the fish communities that they support. We find that while half of the fish found at each site are consistent between embayment beds, mainland beds and island beds, the other half of fish are unique to these certain geographic seagrass types. These other half of fish for embayment seagrass beds consisted of mainly estuary/bay associated species, for island seagrass beds consisted of mainly rocky reef associated species and for mainland beds consisted of mainly soft-bottom associated species. This finding is crucial to environmental managers in Southern California as they continue to conserve and restore these ecosystems because conserving/restoring an acre of seagrass will have disparate impacts on fish depending on the seagrass' geographic type.

The second finding in this study is that eDNA can provide critical biodiversity monitoring of Southern California ecosystems. We were able to use eDNA to discern distinct fish communities in seagrass beds. Our study also compared conventional scuba based surveys to environmental

DNA at three of our seagrass sites. Our results agree well and outperform the number of species found using scuba surveys. Previous studies have shown eDNA's use in other ecosystem types, such as kelp forests and surf zones, and this study adds to the building literature of eDNA's applicability for monitoring fish communities. These findings support the adopted use of eDNA to monitor seagrass communities in Southern California.

Chapter 2

In this chapter, we identify limitations in the use of environmental DNA for invasive species tracking and layout recommendations for benchmarking and validating eDNA assays in both field and laboratory settings for future researchers. Our study creates novel eDNA primers for the tracking of the invasive *Caulerpa prolifera* and publishes the first instance of algal eDNA steady state in the literature. Despite the high efficacy of our assay, we couldn't detect any *Caulerpa* eDNA in our field samples. The results of our tank experiment show that the unique biology of the single-celled algae means that its eDNA signature in the field is negligible. This finding is crucial to the field of environmental DNA because it demonstrates that eDNA isn't the solution for every type of monitoring application. We further highlight that this isn't an issue for all algae as our results identify *Sargassum horneri*, another invasive macroalgae in Southern California, is an ideal candidate for tracking with eDNA.

Chapter 3

In the final chapter of the dissertation, we evaluate the effectiveness of marine protected areas in Southern California and test the utility of community science to the environmental DNA methodology. The findings of this chapter show that MPA status impacts the fish and metazoan

community composition. While we argue that the MPAs are too young to see impacts in individual species abundances due to fish recruitment and life histories, we do see multivariate differences in communities. Additionally, using zeta diversity metrics we find metazoan communities in MPAs to be more diverse and more stable than nonMPAs. The two years of monthly eDNA samples we collected also created one of the richest eDNA datasets for this area. While our study shows that we may be minimizing some of the impacts that MPAs generally seen, due to eDNA's inability to collect data on physical characteristics such as longer, bigger and old organisms found within the MPA, our results still highlight eDNA's strength and ease of sampling of various taxa within surf zone communities. This information is important to environmental managers, specifically to CDFW and OPC who are tasked with leading the MPA management program, because it points towards the positive impact that MPAs have on our coastal waters while cautioning the rapidity in which these impacts are to be expected. We believe that as our sampling continues into its third and fourth year, the differences in these communities will further differentiate.

This study also evaluates the integration of community science into eDNA sampling. Through our community partner Heal The Bay, we were able to engage dozens of community members in scientific monitoring and ocean conservation. Our study highlights the ease of field sampling with eDNA by training non-scientists to complete a year of monthly sample collection that successfully resulted in the identification of hundreds of fish and metazoan species. We conclude that eDNA researchers should leverage this aspect of eDNA to increase the spatial and temporal scale at which monitoring projects can be completed. Furthermore, we note the benefits, not just

to researchers and biodiversity monitoring as a whole, but to the community and the individuals who are able to be integrated into locally based marine conservation efforts.

Overall

This dissertation sits squarely in the field of environmental DNA and helps to further understand and refine the methodology as a whole. We demonstrate its capabilities as a biodiversity monitoring technique by showing that eDNA has significant utility in various marine ecosystems across Southern California (seagrass, rocky reef, kelp), has the potential to track specific invasive species (*Sargassum horneri)*, and is able to detect critical species of interest to marine managers. We also highlight its limitations as a technique by showing that eDNA is unable to detect species with low eDNA steady states under standard sampling conditions and can't report traits important to MPA monitoring, such as species length and weight, which might minimize evaluation of conservation efforts. By properly characterizing what eDNA can and cannot do, we can best apply this methodology to biodiversity monitoring.

In order for eDNA to garner more widespread adoption, parametrizing the nature of eDNA and expanding the knowledge that we can extract from the samples we take will be crucial. The first step of the eDNA process for future research will need to be understanding how eDNA is created. The literature shows that species produce DNA at different rates and in different forms. These variable rates lead to varied probabilities of detection and thus produce community data that are not fully representative of the true community. To control for this, tank-based shedding experiments for species of concern and modeling of trait-based identifiers (i.e. species size, morphology, reproduction style, etc.) that affect shedding rates will need to be done to

contextualize the species' proportion of reads in each sample. The type of eDNA that is created also varies and will have impacts on species detection and abundance values. eDNA researchers will need to better understand the numerous types and sizes of eDNA (i.e. free-floating DNA, cells, feces, etc.) that can be captured through size-based distribution studies.

The second step of the eDNA process after its creation is when it exists in the environment before capture. During this time, our understanding of spatial extent and temporal extent of the signal is still limited. This information will be location specific for where the researchers are collecting the data based on current direction, current speed, ocean temperature, microbial activity, and other factors. To better understand eDNA transport, modeling of eDNA in physical models will allow location-based contextualization of signal extent. Future research into the decay of eDNA can be done through tank based experiments where eDNA is subjected to various conditions to understand its approximate decay rate order. Understanding how far eDNA can travel before it degrades and is no longer detectable will allow for researchers to better characterize the environments they're aiming to monitor. Another avenue of future work for this field will be a greater emphasis on environmental RNA. RNA is a less stable molecule which would have a shorter lifespan within the environment and could be more representative of the population of species there. Since the laboratory analysis is nearly the same, other than the conversion to cDNA, eRNA could provide an exciting addition to the field of eDNA.

Within the laboratory work, a standardization of protocols would be helpful to allow for easier comparisons between different projects from different research groups. There are a number of extraction kits, PCR kits and barcodes used which may affect what DNA is recovered, amplified

and then sequenced. These will all lead to differences in results that arise from processing of the eDNA rather than differences in the actual environment. In order to better derive quantitative estimates of species and communities, PCR amplification efficiencies between species also need to be characterized. Using one set of primers for PCR is what allows us to perform metabarcoding but we must recognize the efficiency at which these primers amplify different species DNA is variable. Thus by doing the PCR for metabarcoding we're changing the proportion of species' DNA to each other. Future research can create mock communities of these sequences or perform variable PCR cycles to quantify amplification efficiency and allow for post-sequencing correction of barcode proportions.

Bioinformatically, eDNA must continue to expand its reference databases through the individual barcoding of species. Environmental DNA is only as good as the most complete reference database for the taxa of interest. At its core eDNA is used to identify which species are in the environment and if we don't have barcodes then we can't identify those species. Future steps in expanding these databases will be working with museums and other collections of already identified species to sanger-sequence a core set of primers for reference database creation.

Finally, future research will also need to look into other signatures beyond just DNA sequences for information about the species it's monitoring. One such example is measuring methylation levels to give age estimates of the population. Environmental DNA will need to continue past a presence-absence methodology in order to be an increased value to researchers and agencies.

These recommendations would improve the utility of eDNA, but we note that eDNA's ease of sampling, ability to collect highly resolved spatial and temporal samples, and repeated better performance compared to conventional surveys makes it an effective biodiversity monitoring tools. This dissertation shows that eDNA provides high resolution biodiversity data critical to both marine mangers and can measure the impact of conservation efforts that are being implemented to combat these problems.

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