Environmental DNA

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Evaluating the Potential for Citizen Science Divers to Monitor Fish Biodiversity Through Passive eDNA Collection

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ABSTRACT

Environmental DNA (eDNA) marker gene sequencing, or metabarcoding, can be a powerful tool for monitoring marine biodiversity. Traditional eDNA sampling methods involve manual water collection and active filtration to collect DNA. This process can be laborious and require expensive filtration equipment. To explore the possibility of making eDNA a more accessible tool, we investigated the efficacy of passive eDNA capture during citizen science SCUBA diving in the Gulf of California, Mexico. Rather than actively collecting and filtering water samples, filters that passively collected eDNA from seawater were attached to citizen scientist divers as they carried out visual surveys on fish species. eDNA metabarcoding from these filters was amplified using MiFish primers (12S rRNA gene) and sequenced to assess fish community composition and compare to visual surveys. We also investigated marine mammal detection with mitochondrial D-loop metabarcoding primers on some of the samples. Sequencing results initially yielded 64 fish species, compared to 183 fish species observed by the divers, and shared similar biogeographic assemblage patterns. However, 95 visually observed species lacked publicly available reference sequences. To address this, we generated reference sequences for 64 fish species vouchers obtained from the Scripps Institution of Oceanography Marine Vertebrate Collection. Dedicated barcoding efforts yielded eDNA detections of 15 additional species. We discuss the relative advantages and disadvantages of this passive eDNA collection method for marine fish detection. Marine mammal sequencing results only yielded 3 species; however, one was the Guadalupe fur seal, a threatened species, which highlights the potential of eDNA for rare or threatened marine mammal detection. Our results suggest that passive capture of eDNA can be used as a tool for citizen scientists to supplement visual surveys for marine species detection and is particularly useful for species that are rare or visually inaccessible to divers.

1 | Introduction

Marine biodiversity is linked to ecosystem stability and efficiency (Cardinale et al. 2012; Taberlet et al. 2018a; Duffy et al. 2007; Loreau and de Mazancourt 2013; Pennekamp et al. 2018). Monitoring biodiversity is therefore an important component of conservation and management practices. Traditional monitoring schemes include observation-based techniques, such as fishing, trapping, or catching species, deploying underwater cameras, or conducting underwater visual or acoustic surveys. These methods can be restricted by time, cost, visual or acoustic conditions, sampling inefficiencies, and invasiveness (Zhang et al. 2024; Bessey et al. 2020). Notably, all rely on taxonomic identification based on human sensory interpretation (Bessey et al. 2020).

Over the past decade, environmental DNA (eDNA) metabarcoding has emerged as a non-invasive and efficient alternative

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or supplemental method for aquatic biodiversity monitoring (Zhang et al. 2024; Foote et al. 2012; Thomsen et al. 2012). eDNA encompasses both intra- and extracellular DNA that organisms shed into their surrounding environment and can be part of feces, sloughed cells, and gametes (Bessey et al. 2021; Taberlet et al. 2018b). Researchers extract, amplify, and sequence eDNA from seawater samples using primer sets for particular groups of organisms. For eDNA metabarcoding, these sequences are then compared to known sequences in reference databases to identify the species from which the eDNA originated (Bessey et al. 2021; Bessey et al. 2022). eDNA has demonstrated high efficacy for detection of the presence and diversity of many marine species, including fishes (Zhang et al. 2024; Bessey et al. 2020; Dalongeville et al. 2022). The use of eDNA metabarcoding for species abundance is more complex, as there are a myriad of factors in the environment and laboratory processing that affect the relationship between species' biomass and observed sequences, including shedding and decay rates, allometry, fate and transport of eDNA, and PCR amplification biases (Bessey et al. 2021; Veron et al. 2023). Despite this limitation, eDNA metabarcoding offers several advantages over traditional biodiversity inventorying approaches in marine ecosystems. First, by applying molecular genetics for taxonomic assignment, eDNA enables the identification of tens of thousands of species, beyond the taxonomic expertise of most scientists (Bessey et al. 2020; Veron et al. 2023; Chen et al. 2022). Second, eDNA metabarcoding can detect species that can elude other monitoring methods, such as rare, cryptic, nocturnal, small, and delicate species (Dalongeville et al. 2022; Veron et al. 2023; Nester et al. 2020). Finally, eDNA provides a minimally-invasive form of species monitoring that bypasses potentially harmful capture processes (Chen et al. 2022).

The most common method for marine eDNA collection is filtering seawater through a membrane using a mechanized pump (active filtration). However, because concentrations of eDNA can be low or show small-scale heterogeneity in aquatic environments, previous studies have recommended that active filtration eDNA studies maximize sampled water volume in order to achieve sampling sufficiency (Zhang et al. 2024; Bessey et al. 2020; Bessey et al. 2022). Collecting and actively filtering seawater can be a labor- and time-intensive process that itself mandates specialized equipment, storage space, and often water transportation prior to filtration. These longer processing times can lead to more eDNA degradation and opportunities for error. Additionally, because samples come from one area of water at one time, active filtration represents only a spatiotemporal snapshot of the ecosystem (Chen et al. 2022).

Recently, passive sampling of eDNA has emerged as a potential alternative to active filtration, and features the direct collection of eDNA from the marine environment through submersion of eDNA-retentive materials (Bessey et al. 2021; Bessey et al. 2022; Chen et al. 2022; Kirtane et al. 2020). By removing the active filtration step, passive eDNA collection offers a faster, cheaper, and simpler way to directly collect DNA in situ (Bessey et al. 2022; Kirtane et al. 2020). Recent studies have demonstrated that passive eDNA collection can be as effective as active filtration with as few as 5 min of submersion (Bessey et al. 2022). The downstream effects of these benefits include expanded access to eDNA collection and potentially increased sample replication, since the method does not require specialized water filtration equipment or expertise in such equipment (Bessey et al. 2021). In turn, these advantages greatly expand the possible ecological questions that can be addressed through marine eDNA collection (Bessey et al. 2021).

As passive eDNA collection remains a relatively new method, few studies have investigated its efficacy in marine environments (Zhang et al. 2024; Bessey et al. 2021; Chen et al. 2022; Kirtane et al. 2020; Nichols and Marko 2025). Those that have can be separated into studies investigating biotic substrates or artificial membranes for passive eDNA collection. Among the biotic substances group (Mariani et al. 2019), (Turon et al. 2020), and more recently (Jeunen et al. 2024), established that natural substrates like sponges can act as effective passive samplers, capturing representative eDNA signals of nearby fish communities. Other studies have also established the efficacy of aquatic biofilms (Rivera et al. 2022) for indirectly surveying environmental fauna. However, the performances of these substances depend on the environments in which they are deployed and can be difficult to standardize (Zhang et al. 2024; Chen et al. 2022).

Alternatively, artificial membranes offer increased application and standardization potential. A few studies have tested various "passive eDNA sampler" (PEDS) adsorbents, including montmorillonite clay and granular activated carbon (Kirtane et al. 2020), as well as filter membranes made of nylon (Zhang et al. 2024; Bessey et al. 2021), cellulose ester (Bessey et al. 2021), and mixed cellulose acetate and nitrate (Zhang et al. 2024). Recent field studies have also demonstrated the efficacy of natural fiber membranes such as cotton (Bessey et al. 2022; Nichols and Marko 2025; Nichols et al. 2025). Chen et al. (2022) and Bessey et al. (2022) compared 12 and 9 sorbent materials, respectively, for passive eDNA collection (Bessey et al. 2022; Chen et al. 2022). These latter two studies illustrated that several of these inexpensive materials can be very effective for passive eDNA collection, with Chen et al. (2022) finding that glass fiber filters (GFF) outperformed all other materials in both laboratory and field settings (Chen et al. 2022).

One potential method for deploying PEDS is to engage citizen scientists, leveraging volunteer members of the public to assist with scientific endeavors (Lin Hunter et al. 2023). There has been significant debate over this term, with some institutions favoring "community science" or "community-based participatory research" over "citizen science" because the former two are more inclusive than the latter (Eitzel et al. 2017; Woolley et al. 2016). This paper will use citizen science because community science has historically held a different meaning (one in which community members lead the science and project goals are primarily social rather than scientific), and its substitution in this context would be an appropriation of community-led scientific endeavors (Lin Hunter et al. 2023; Cooper et al. 2021). In this paper, we define "citizen scientist" in its broadest sense: as referring to all members of the public, regardless of background, who are interested in contributing to scientific knowledge.

Citizen science has proven valuable for biodiversity monitoring in many ecosystems (Pocock et al. 2018; Bonney

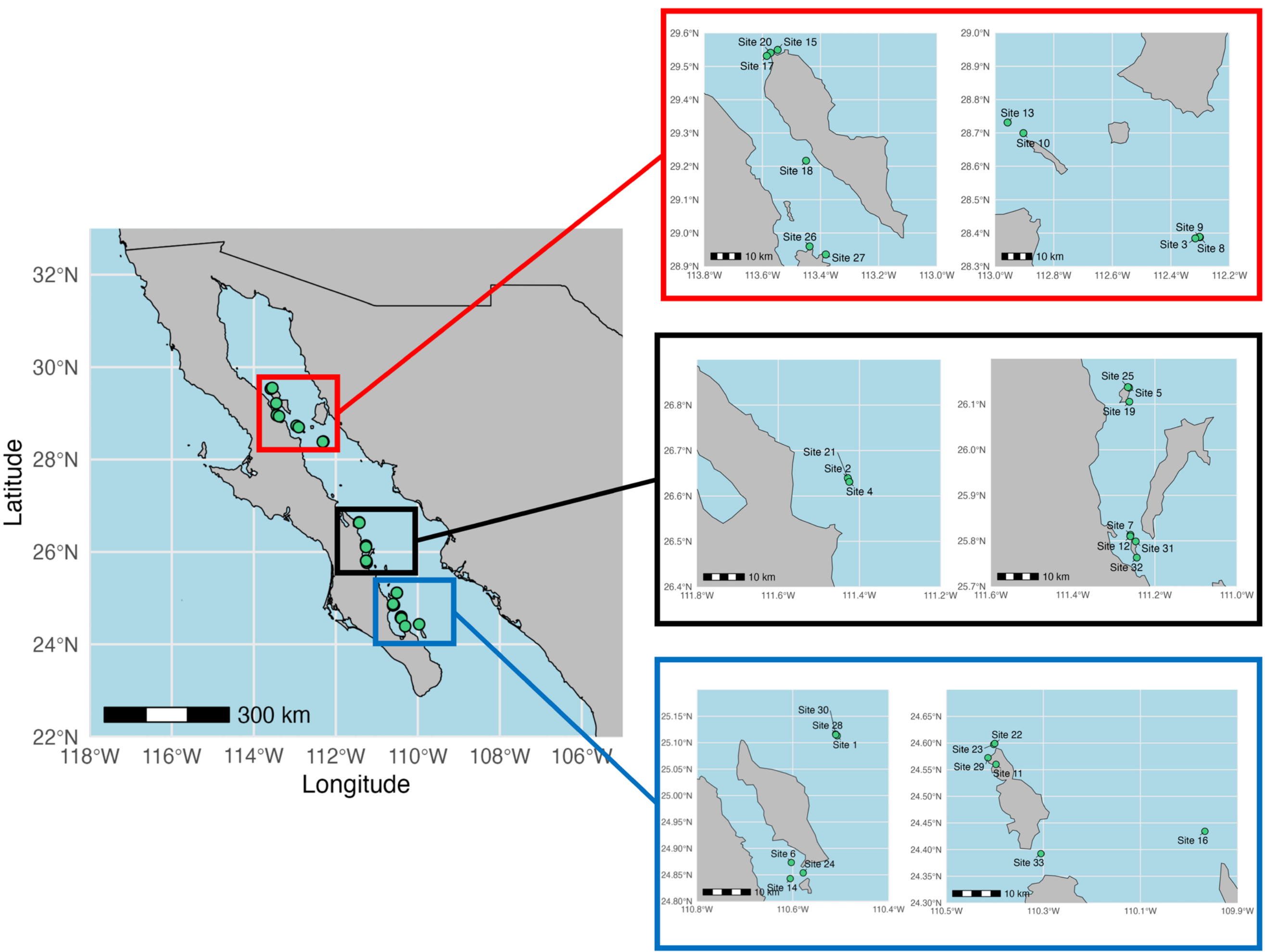


FIGURE 1 | Map of sampling sites in the Gulf of California. The left panel shows all sites, while the paired panels on the right present zoomed views of sites within each latitude group: North (red; 11 sites), Middle (black; 10 sites), and South (blue; 12 sites).

et al. 2009; Chandler et al. 2017; Amano et al. 2016). It offers a reliable, cost-effective mechanism for biodiversity monitoring while fostering environmental stewardship among the public (Branchini et al. 2015). In marine ecosystems, recreational SCUBA divers can act as citizen scientists by conducting visual surveys of marine species, and data from these surveys have been invaluable for biodiversity monitoring efforts and subsequent conservation management decisions (Branchini et al. 2015; Lucrezi et al. 2018; Cerrano et al. 2017; Ben Lamine et al. 2018). Citizen scientists have also assisted with aquatic eDNA collection and filtering (Valsecchi et al. 2023; Agersnap et al. 2022; Miya et al. 2022), and citizen scientist and SCUBA diver visual observations have been used to supplement eDNA analyses (Feng and Lougheed 2023; Bessell et al. 2023). Research divers have also collected seawater samples for active eDNA filtration (Acharya-Patel et al. 2024). A recent study demonstrated the potential for citizen scientist divers to sample marine communities using passive eDNA methods (Neave et al. 2025); however, this approach has not yet been compared to traditional forms of biodiversity monitoring, such as visual surveys.

In the present study, we use eDNA metabarcoding to evaluate the effectiveness of passive eDNA collection by citizen science SCUBA divers in the Gulf of California for capturing fish biodiversity, particularly when compared to concurrent visual surveys.

2 | Methods

2.1 | Sampling Location

Divers collected samples from 33 dive sites ranging from 24.392°N, 110.305°W at the southern end to 29.550°N, 113.547°W at the northern end in the Gulf of California (Figure 1). Widely recognized as a hotspot for marine biodiversity, the Gulf of California is one of the most important fishing regions in Mexico, as well as a marine system of great importance to the international conservation community (Lluch-Cota et al. 2007; Lluch-Cota et al. 2010; Munguia-Vega et al. 2018). This sea supports both temperate and tropical habitats, with over 300 teleost species. Previous fish community ecology studies have divided the Gulf into north, central, and south faunistic zones due to a notable environmental gradient based on latitude (Valdivia-Carrillo et al. 2021). In particular: the northern Gulf of California is less tropical, subject to more annual fluctuations in productivity, and has a different Temperature-Salinity profile than the central and southern regions (Valdivia-Carrillo et al. 2021; Fernández-Rivera Melo et al. 2018). We

expected to find similar trends in our community ecology data and investigated the effects latitude in our analyses by dividing our sites into three latitude groups: North (28°–30°N), Middle (25.7°N–27.9°N), and South (24°N–25.6°N).

2.2 | Sampling Materials (PEDS)

We used GFF membranes with nominal pore sizes of 0.7 μm for the passive eDNA filters based on evidence that this material outperforms other common materials for passive aquatic eDNA collection (Chen et al. 2022). These filters were fitted into 3D printed plastic "honeycomb" puck-shaped housings inspired by open source designs (the STL files used for 3D printing are publicly available in the GitHub repository listed in the Data Availability Statement). Together, these materials will henceforth be referred to as the PEDS.

2.3 | eDNA Collection

Passive eDNA collection was facilitated by trained volunteer divers on a diving trip organized by Reef Environmental Education Foundation (REEF). The dives took place between September 25 and October 5, 2022. REEF staff attached PEDS to divers' first stage regulators (Figure 2), and covered PEDS with unused medical gloves or plastic bags before divers entered the water to prevent contamination. After completion of the dives, REEF staff removed the PEDS from diver regulators and placed them in new plastic bags. All staff wore clean gloves when handling PEDS. PEDS were then transported back to the live-aboard dive boat from which all dives were staged, where REEF staff unscrewed the honeycomb pucks, used clean tweezers to remove the GFFs, and placed them into individually marked vials filled with Longmire's buffer (100 mM Tris, 100 mM EDTA, 10 mM NaCl, 0.5% SDS, 0.2% sodium azide) for DNA preservation (Longmire et al. 1997). Transportation time before PEDS filters were stored in vials with Longmire buffer ranged from 30 to 150 min. Four samples and one field blank (in which the PEDS was brought into the field and then transferred directly from its box to the vial) were collected from each dive. These vials were transported and stored in the dark at room temperature (Renshaw et al. 2015) until extractions began in March 2023.

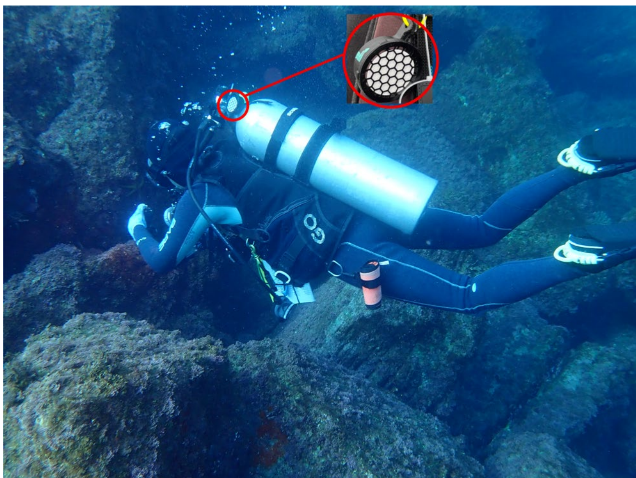


FIGURE 2 | Photo showing a PEDS attached to a citizen science diver's first stage regulator (PEDS circled in red, with enlarged view of the PEDS inserted in the photo). The PEDS consists of a black honeycomb plastic puck-shaped housing with a GFF filter inside. Photo courtesy of Reef Environmental Education Foundation.

2.4 | Visual Surveys (RVS)

Divers conducted visual surveys (REEF Visual Surveys, henceforth RVS) using the Roving Diver Technique, in which they freely swam through dive sites with slates, preprinted data sheets, and pencils, and recorded all observed fish species throughout the water column (Schmitt and Sullivan 1996; Pattengill-Semmens and Semmens 1999; Ward-Paige et al. 2010). Abundance values were scored between 1 and 4, with 1 for "Single" (1 fish), 2 for "Few" (2–10 fish), 3 for "Many" (11–100 fish), and 4 for "Abundant" ($>$100 fish). At the end of each dive, volunteers added metadata such as dive bottom time, site, habitat type, and maximum depth, and entered their data from these sheets into REEF's database via an online data entry form. Maximum depth was represented categorically with 1 for snorkel, 2 for $<$3.0 m, 3 for 3.0–5.8 m, up to 13 for 33.5–36.3 m. For analyses, we used the maximum value of each category for RVS maximum depth. All volunteer divers received extensive training in fish species identification, and the efficacy and value of RVS are well documented and have been applied to marine species monitoring, management, and conservation for decades (Pattengill-Semmens and Semmens 1999; Greenberg et al. 2024; Pattengill-Semmens and Semmens 2003; Pattengill-Semmens and Semmens 1998). Five species labeled with "sp." (indicating that species-level taxonomy could not be visually resolved) were removed from analyses concerning the number of detected species.

2.5 | eDNA Extraction

We extracted eDNA in a molecular biology laboratory and regularly cleaned and sterilized equipment and benches prior to and during extractions and sequencing to prevent contamination. Using the *Quick*-DNA Microprep Kit (Zymo Research, Irvine, CA, USA), we extracted total DNA from all samples following the manufacturer's instructions for "Cell Suspensions and Proteinase K Digested Samples," with the following modifications: we centrifuged samples for 1 min at ≥10,000g after step 1, added 400 μL of DNA Pre-Wash Buffer in step 3, and added 200 μL of gDNA Wash buffer after step 4 with an additional centrifuge for 1 min at ≥10,000g. DNA was eluted into 50 μL of DNA Elution Buffer. To assess potential contamination, two field negative controls were included and processed alongside biological samples through DNA extraction. Following reviewer feedback, all remaining negative controls were extracted using identical protocols. All negative controls (n = 32) were first quantified using the Qubit HS DNA assay (Thermo Fisher Scientific) and were below the assay detection limit. All 32 field negative samples were then amplified using MiFish primers without Nextera adapter sequences, resulting in nine field negative samples amplifying faint bands. These nine samples were then amplified using MiFish primers containing Nextera adapter sequences, resulting in no observed bands in a 2% agarose gel for any field blanks or PCR no-template control. We nevertheless included

TABLE 1 | Forward and reverse primers used to amplify fish (MiFish-U) and marine mammal (D-loop) eDNA.

Primer name	Forward sequence	Reverse sequence	References
MiFish-U (with reverse modification)	5′-GCCGGTAAAACTCGTGCCAGC-3′	5′-CATAGTGGGGTATCT AATCCCAGTTTG-3′	Sales et al. (2019)
D-loop	5′-TCACCCAAAGCTGRARTTCTA-3′	5′-GCGGGTTGCTGGTTTCACG-3′	Baker et al. (2018)

these field negative controls and the no-template control in the sequencing library for precaution. These results were used to assess the likelihood of contamination in the field samples.

2.6 | PCR Amplification and Sequencing

We amplified each sample, as well as positive controls (*Thunnus thynnus* DNA, isolated from fish larvae in the Gulf of Mexico, extracted in a separate lab space) and no-template controls (PCR reagents with molecular grade water instead of template DNA) for each reaction, with two primer sets in separate reactions (Table 1). PCR positive controls consistently amplified, and no amplification was observed in PCR negative controls. The first amplification was conducted using MiFish-U (Universal) primers, which target a hypervariable region in the 12S rRNA gene (163–185 bp) and was developed for metabarcoding eDNA of fishes (Miya et al. 2015). We used a modified version of the MiFish-U forward primer used, from Sales et al. (2019). The second amplification was conducted using primers targeting the D-loop from the mitochondrial control region of marine mammals (Baker et al. 2018).

PCR reagents included 2X Phusion Green Hot Start II High-Fidelity PCR Master Mix (Thermo Scientific, 7.5 μL per reaction), 10 μM forward and reverse primers (0.75 μL of each per reaction), Dimethyl sulfoxide (DMSO, 0.45 μL per reaction), nuclease-free water (3.3 μL per reaction), Recombinant Albumin (rAlbumin, 0.75 μL per reaction), and 1.5 μL of template DNA from each sample for a 15 μL volume per reaction. We performed PCR using the following conditions: initial denaturation at 98°C for 30 s, followed by 35 cycles (45 for D-loop) of 10 s at 98°C for denaturation, 30 s at 60°C for primer annealing, 45 s at 72°C, and a final extension for 10 min at 72°C. PCR products were purified using Exonuclease I Reaction Buffer (New England Biolabs) and Shrimp Alkaline Phosphatase (rSAP) (New England Biolabs). We then ran an additional PCR on all samples with the addition of Illumina tag primers unique to each sample, as well as an additional purification using AxyPrep Mag PCR Clean-Up Protocol (Axygen Biosciences). The sequencing library was created by quantifying all PCR products using a Qubit fluorometer (Thermo Scientific) and then pooling samples in equimolar ratios (range: 0.814–44.4 μg/mL). Pooled samples were submitted to the UC Davis sequencing core (https://dnatech.genomecenter.ucdavis.edu/illumina-high-throughput-sequencing/) for 2 × 250 bp paired-end Illumina MiSeq sequencing.

2.7 | Data Processing

Following Illumina sequencing, we filtered the data through quality control workflows. Primers were trimmed from sequences using Cutadapt (Martin 2011) and processed with DADA2 (Callahan et al. 2016) in R. Code is publicly available in the GitHub repository listed in the Data Availability Statement. Briefly, DADA2 steps included quality filtering, denoising, merging, chimera detection and exclusion, and length filtering. Reads longer than 110 bp were excluded due to known off-target amplification of longer bacterial sequences by MiFish-U primers (Kawato et al. 2021; Stoeckle et al. 2022; Baidouri et al. 2025). Additionally, to improve the detection of rare taxa, we leveraged the "priors" parameter in DADA2, which allows for the inclusion of known ASV sequences to adjust model parameters. We chose ASVs assigned to *Diodon hystrix*, *Sphoeroides annulatus*, and *Paralabrax auroguttatus*, which were the rarest fish species detected in our eDNA data (by both total number of reads and number of filters detected) but were observed in RVS. Samples with zero processed reads were excluded from further analysis. Taxonomic assignment was conducted with DADA2, which uses a naïve Bayes classifier rather than an alignment-based method like BLAST, and the rCRUX Generated Universal MiFish Expanded reference database (Gold et al. 2023; Curd et al. 2024). We have deposited all sequences associated with this study under the BioProject ID: PRJNA1234680. Following taxonomic assignment and processing, we manually removed 5 terrestrial contaminant species from the results: *Homo sapiens*, *Gorilla gorilla*, *Sus scrofa*, *Bos taurus*, and *Felis catus*.

2.8 | Fish Tissue DNA Extraction, PCR, and Data Processing

To add missing reference sequences to the rCRUX database, we first searched NCBI GenBank (GenBank 1982) to identify existing publicly available sequences for fish species observed in the RVS. Briefly, we identified sequences using keyword searches, then extracted the MiFish region and quality-filtered them using QIIME2 (Bolyen et al. 2019) and RESCRIPt (Robeson et al. 2021). This process provided 19 additional references from NCBI GenBank to the rCRUX reference database.

To generate new reference sequences, the Scripps Institution of Oceanography Marine Vertebrate Collection (https://scripps.ucsd.edu/marine-vertebrate-collection) generously made voucher specimens available for tissue sampling. We extracted DNA from the right pectoral fin using the DNeasy Blood and Tissue Kit (QIAGEN, Germantown, MD, USA) following the manufacturer's instructions. PCR was conducted using the same MiFish protocol as described above for eDNA samples with slight modifications (first reaction only, primers without Illumina overhangs). Fish tissue PCR products were then sequenced using capillary ("Sanger") sequencing (Eton Bioscience, San Diego, CA, USA; https://www.etonbio.com/).

After sequencing data were returned as raw .fastq and .ab1 files, 64 sequences were trimmed at the forward and reverse ends to remove low quality bases, converted to FASTA format, and added to our reference database file. Sequence chromatograms were evaluated for quality and two sequences were excluded from analysis because a large proportion of nucleotides were ambiguous. All tissue voucher sequences have been deposited in NCBI PV263179-PV263242 (Table S1).

In total, we added 83 fish 12S rRNA marker gene sequences to our rCRUX reference database: 19 from publicly available NCBI GenBank records and 64 from newly generated fish tissue samples.

2.9 | Patterns of Diversity and Statistical Analysis

All statistics and graphics were conducted or produced using R (version 4.3.2; R Core Team 2023).

To investigate fish biodiversity, we first examined patterns of species accumulation, turnover, and retention using iNEXT (version 3.0.1) (Hsieh et al. 2016) and zetadiv (version 1.2.1) (Latombe et al. 2017) packages in R, at both the replicate level (for each PEDS and RVS) and after aggregating by site. Zeta diversity is a measure of the overlap of species across multiple levels of sampling (e.g., eDNA samples, sites, regions) and has been used to understand patterns of fish biodiversity from eDNA in other studies (Marwayana et al. 2022; Hui and McGeoch 2014). For beta diversity analyses, PEDS data were standardized using the Hellinger transformation and ordinated using Euclidean distance (Legendre and Gallagher 2001; Legendre et al. 2005), and NMDS values for the RVS were calculated using the Gower distance for categorical abundance data (Fong et al. 2023). PERMANOVAs were run for each metadata variable and corresponding NMDS values, as well as a full model with all five metadata variables. Site maps were created and annotated using the following packages in R: ggplot2 (Version 3.5.2) (Wickham 2016), rnaturalearth (Version 1.0.1) (Massicotte and South 2026), and ggrepel (Version 0.9.5) (Slowikowski 2024). Phyloseq (version 1.46.0) (McMurdie and Holmes 2013) and vegan (version 2.6.8) (Oksanen et al. 2025) packages in R were used for diversity analyses.

To examine the similarity in presence/absence data between PEDS and RVS, we created a map that plots the similarity by site between PEDS and RVS using the number of species that were either detected by both methods (present in both) or not detected by both methods (absent in both). Finally, to evaluate whether the agreement between PEDS and RVS varied along known latitudinal gradients in environmental conditions in the Gulf of California, we conducted a linear regression testing for a relationship between latitude and the similarity of species detection between the two survey types.

3 | Results

129 of 136 PEDS (95%) were fully sequenced and processed. A total of 359 RVS were included in analyses, with 14,137 total species-by-site observations.

3.1 | Metadata

The PEDS were almost always attached to the same four divers (D1, D2, D3, D4), with 1 PEDS dive by D5, 2 by D6, and 1 by D7, while fifteen total divers conducted RVS (D1—D15). Maximum depth in PEDS data ranged from 6.1 to 34.4 m, and in RVS ranged from 5.8–36.3 m. Bottom time on dives with PEDS ranged from 18–75 min (mean = 65.1 min), and with RVS ranged from 35–78 min (mean = 67.7 min).

3.2 | Initial Species Detection Counts Prior to Expanded Reference Database Construction

Initially, we recovered 66 unique marine species from the PEDS using the MiFish-U primers (Figure 3; Table S2), including 64 unique fish species, one marine mammal, and one sea star. 17 of these species are not known to inhabit the Gulf of California (Table S2) (Love et al. 2021). RVS yielded a total of 183 unique marine species, 177 of which are marine fish species, and 6 additional fishes identified only up to the genus level (e.g., “Atheriniformes sp./Clupeiformes sp.”) (Figure 3; Table S3). RVS detected 50 Actinopteri families total. The PEDS and RVS had 30 species of overlap (Figure 3; Table S4), representing 45% of PEDS species and 16% of RVS species.

3.3 | Field Negative Controls

The field negative controls yielded 24 unique taxa, including 17 fish species (Table S5). Fifteen of these taxa were also in the PCR no-template control. Twelve of these species were not observed in any eDNA samples, suggesting that the majority of the contamination in the field and no-template controls is at very low levels, and if present at all in the field samples, was overwhelmed by the eDNA signal captured from the environment.

Four species in the field negative samples were observed eDNA samples: *Engraulis mordax* (Northern anchovy), *Brachygenys californiensis* (California salema), *Synodus lucioceps* (California lizardfish), and *Anisotremus davidsonii* (Xantic sargo). *E. mordax* and *B. californiensis* were also detected in the no-template control. Each of the four species was detected in only one eDNA sample each, representing 0.1%–8.7% of those respective samples' total reads, and none of those samples were associated with the respective field negative that amplified (i.e., samples were not collected on the same day as the field blank was generated). Moreover, all four species are known to occur in the Gulf of California, and three of the field negative species were also observed in visual surveys (also not associated with the dives on which the respective field blanks detected those species). Taken together, these results strongly suggest there was very slight cross-contamination among field samples, but represent a small fraction of the total species richness and occurrences generated in this study.

3.4 | Construction of Expanded Reference Database

Over half of all detected RVS species, or 95 species, were not in our initial reference database. We generated MiFish 12S

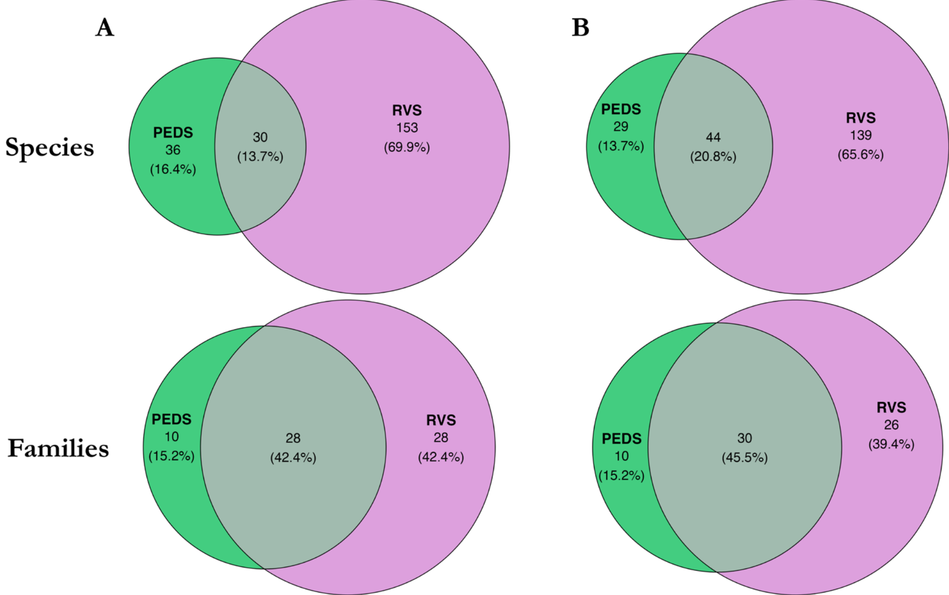


FIGURE 3 | Venn diagrams showing species overlap (top) and family overlap (bottom) between PEDS and RVS for (A) original PEDS species and (B) updated PEDS species. Green corresponds to PEDS and purple to RVS.

reference barcode sequences for 64 Gulf of California fishes and were able to find an additional 19 publicly available sequences from Gulf of California Fishes published to NCBI GenBank that were made available since the initiation of the project to construct an expanded reference database.

Using this expanded reference database, we detected 15 additional fish species in the PEDS data, increasing the total number of PEDS detected species to 73 species (Table S6), with 44 species of overlap with RVS species (Figure 3; Table S7) and an average of 8.9 species detected per PEDS. We generated a total of 295 ASVs and were able to assign 256 to Class Actinopteri. This represented 37 unique Actinopteri families and 73 genera. We detected a total of 73 species, including 70 marine fishes, 2 marine mammals, and one sea star.

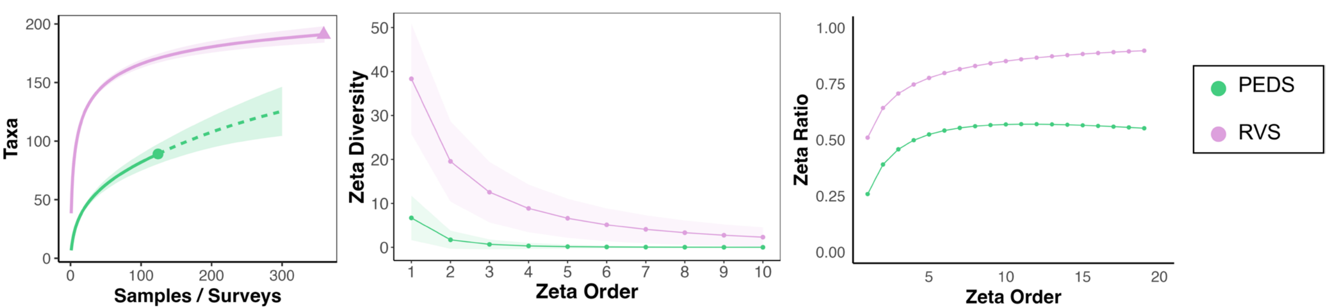
Using the expanded reference database resulted in updated taxonomic assignment for 8 species. Four species were reclassified as Gulf of California congeners from non-endemic sister species, representing improved classification from the inclusion of additional reference sequences (*Caranx crysos* to *Caranx caballus*, *Cirrhitus pinnulatus* to *Cirrhitus rivulatus*, *Entomacrodus caudofasciatus* to *Entomacrodus chiostictus*, and *Malacoctenus triangulatus* to *Malacoctenus hubbsi*). Likewise, one species was reclassified from one Gulf of California species to another endemic species with higher percent identity (*Hypsoblennius jenkinsi* to *Hypsoblennius brevipinnis*). In addition, three species were reassigned to the genus level as newly added reference sequences were identical to existing sister species reference sequences. Together, the expanded reference database resulted in improved taxonomic assignments to biogeographically relevant species, with only 11 of the 73 observed species undocumented in the Gulf of California as opposed to the initial 17 (Table S6) (Love et al. 2021).

3.5 | Species Accumulation, Turnover, and Retention

We plotted species accumulation, turnover, and retention for both PEDS and RVS data by individual samples and by aggregating by site (Figure 4). Species accumulation curves across replicate samples illustrate that RVS approaches saturation while PEDS does not. PEDS and RVS species accumulation curves after aggregating by site both show greater richness, but RVS still approaches saturation with fewer sites than PEDS. Species turnover (zeta decay) plots demonstrate higher turnover in PEDS than in RVS, indicating that a greater number of species were observed and shared across replicate RVS than PEDS. We observe similar turnover patterns at the site level. Species retention plots depict a high overall retention among RVS (which approaches 1 in both replicate and site-based plots), suggesting that there is a core set of species shared across surveys and sites. In contrast, we observed a decline in species retention for PEDS with fewer species shared across increasing samples. At the site level, species retention markedly increased for PEDS, although it remains lower than RVS species retention. In total, RVS had both a higher total number of species observed and a greater number of shared species observed across replicate surveys as compared to PEDS.

3.6 | Beta Diversity

We calculated beta diversity metrics for both PEDS and RVS (Figures 5 and 6). PERMANOVAs revealed statistically significant relationships ($p < 0.05$) between NMDS values and both latitude group and maximum depth for both PEDS and RVS (Table 2). Additionally, RVS PERMANOVA showed significant relationships between NMDS values and diver identity as well as dive bottom time. Notably, the group differences were substantially larger for RVS across all variables, with pseudo-F values



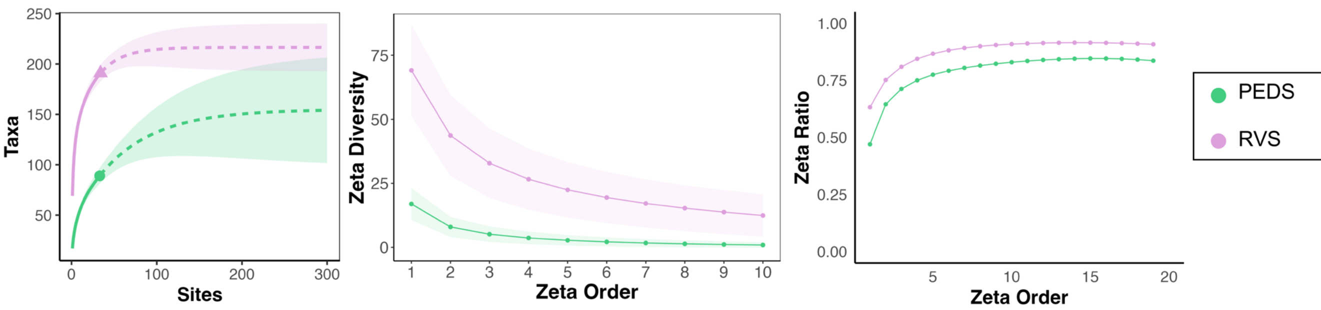


FIGURE 4 | Species accumulation, turnover, and retention plots for PEDS and RVS, for each PEDS and RVS (top) and after aggregating by site (bottom). Zeta order is the number of sites considered in calculating zeta diversity. Zeta ratio refers to the probability that a shared species across n sites will be shared with an additional site and is a proxy for species retention. Dashed lines in the accumulation curves indicate extrapolation.

that were 5–35 times greater than PEDS values for the same variables.

3.7 | Method Comparison

For site-wise comparisons, similarity scores for PEDS and RVS, representing the proportion of species detected or not detected by both methods, ranged from 0.2 to 0.67 (Figure 7). In total, 39% of sites had equal to or over 50% similarity in presence/absence/data for the 44 overlapping species. The number of overlapping species for each site and their proportion to the total number of overlapping species can be found in Table S8. To determine whether higher latitudes are associated with greater overlap between the two methods, we plotted similarity scores against latitude and ran a linear regression, which yielded a p value of 0.007 (Figure 8).

3.8 | Marine Mammals

We detected three species of marine mammal using D-loop primers: *Tursiops truncatus* (Common bottlenose dolphin), *Zalophus californianus* (California sea lion), and *Arctocephalus townsendi* (Guadalupe fur seal). *T. truncatus* was detected in two samples, and the other two species were each detected in one sample. These species were represented by 6 ASVs which are provided in the D-loop ASV table in the GitHub repository in the Data Availability Statement. Other ASVs (20 total) included 1 *S. scrofa* (pig) with the remainder annotated as bacteria or having no significant alignment similarity using BLAST against the NCBI core_nt database.

Additionally, we detected *Zalophus californianus* (California sea lion) and *Tursiops truncatus* (Common bottlenose dolphin) using the MiFish primers.

4 | Discussion

Monitoring marine biodiversity is critical for understanding ecosystem health and guiding conservation efforts, yet collecting reliable species data across broad spatial and temporal scales remains a major challenge. Citizen science programs, including those involving SCUBA divers, have potential to expand data collection efforts, but are often limited by participants' varying levels of taxonomic expertise and the logistical demands of traditional survey methods. eDNA approaches offer a promising solution by enabling species detection without requiring direct observation or identification skills. Our results demonstrate that GFF PEDS (Passive Environmental DNA Samplers) can be effectively used by citizen science SCUBA divers to collect eDNA samples. In our study, 95% of all samples yielded amplifiable eDNA using MiFish-U

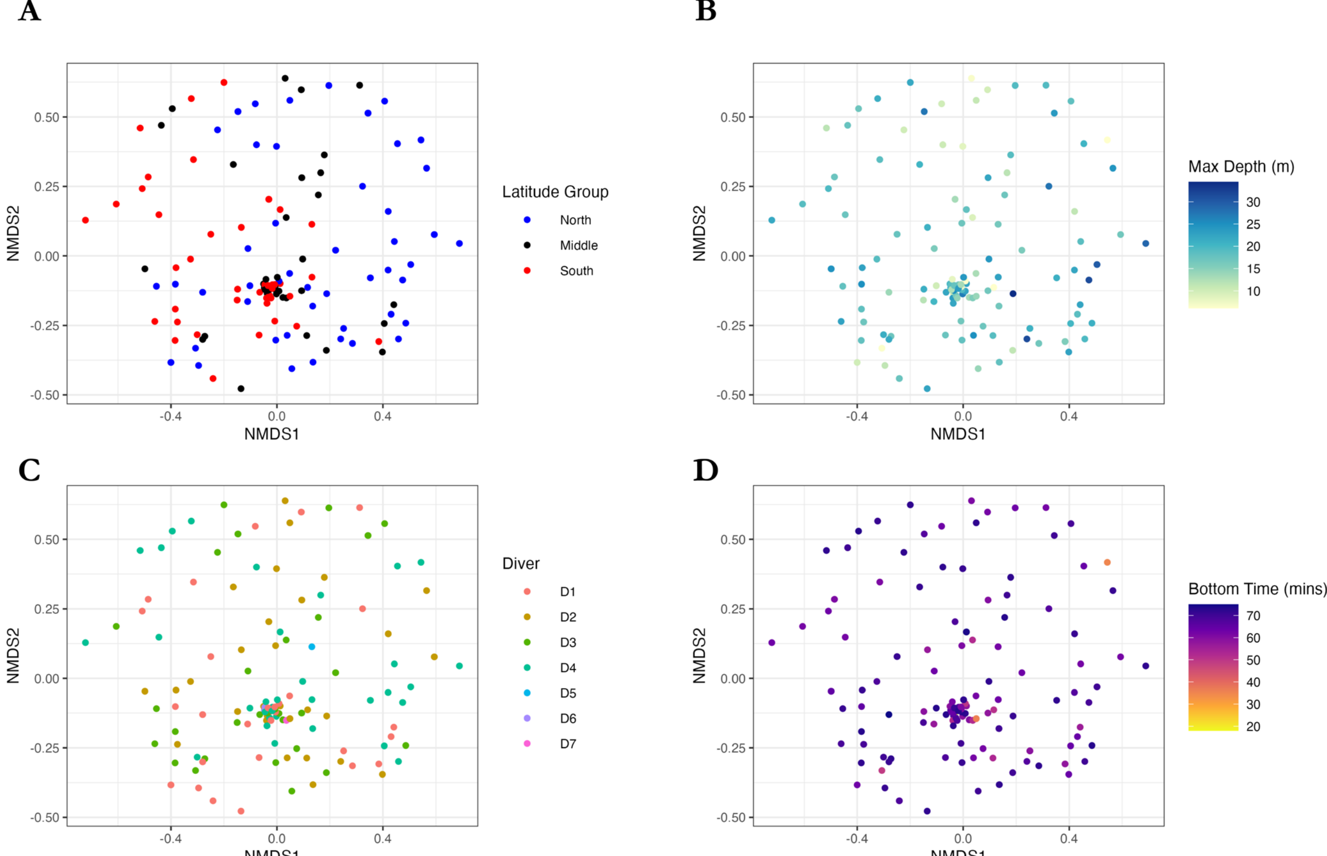


FIGURE 5 | NMDS beta diversity plots for PEDS following Hellinger transformation of data using Euclidean distance for latitude group (A), maximum depth (B), diver (C), and bottom time (D).

primers, with subsequent species detections that partially overlapped with visually detected species, and that expanded the total richness of the species observed in our study. The PEDS protocol required minimal time and no specialized scientific knowledge: divers needed only basic materials and knowledge of sample handling. Although the current PEDS design implemented here had limitations in sampling coverage and efficiency compared to RVS, these findings highlight the potential of diver-mounted passive eDNA collection to democratize biodiversity monitoring by offering an accessible and scalable method for engaging non-specialists in marine research.

4.1 | PEDS Complementing RVS

Overall, RVS and PEDS had 44 species of overlap with over half of these species sharing identical presence/absence data in both RVS and PEDS in 13 of the 33 sites. Interestingly, PEDS also detected 29 species that were not observed in RVS, including *Engraulis mordax* (Northern Anchovy), *Hemanthias signifer* (Damsel Bass), and *Umbrina roncador* (Yellowfin Croaker). *E. mordax* was not speciated by RVS: 25 entries in RVS are identified only as "Atheriniformes sp./Clupeiformes sp.," or as "Unidentified Silvery Fish (Silversides/Anchovies/Herrings)." Forage fishes such as anchovies can be hard to visually distinguish at the species level on visual dive surveys given the morphological similarity and small body size, indicating one possible advantage of PEDS over RVS. As for *H. signifer* and *U. roncador*, the former occupies habitats too deep for divers (Eschmeyer et al. 1983), while the latter spends much of its time in the surf zone, too shallow to be observed by the RVS divers (Pondella et al. 2008). Thus, our results also suggest that PEDS integrate over a larger ecological footprint compared to RVS transects, in line with previous observations of eDNA (Canals et al. 2021; Andruszkiewicz et al. 2017). These results suggest that PEDS captured similar underlying fish communities while also providing important complementary observations to RVS.

While the marine mammal D-loop sequencing only yielded three species, the *Arctocephalus townsendi* (Gudalupe fur seal) was unexpected. This species was believed to be driven to near extinction by 19th century hunting but was seen again in Baja California in the 1950s. Since then, the Guadalupe fur seal has recovered slowly, and it is included in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) 2017 Appendix (García-Aguilar et al. 2018). Three years prior to our study, in 2019, researchers found evidence of a new colony forming in the Gulf of California (Elorriaga-Verplancken et al. 2021). Although other studies have had mixed results for the ability of PEDS to detect rare species (Bessey et al. 2022; Nichols and Marko 2025; Jeunen et al. 2022), our PEDS' detection of the Guadalupe fur seal shortly after its discovery through traditional survey methods supports the tool's ability to identify threatened species and support their ongoing monitoring.

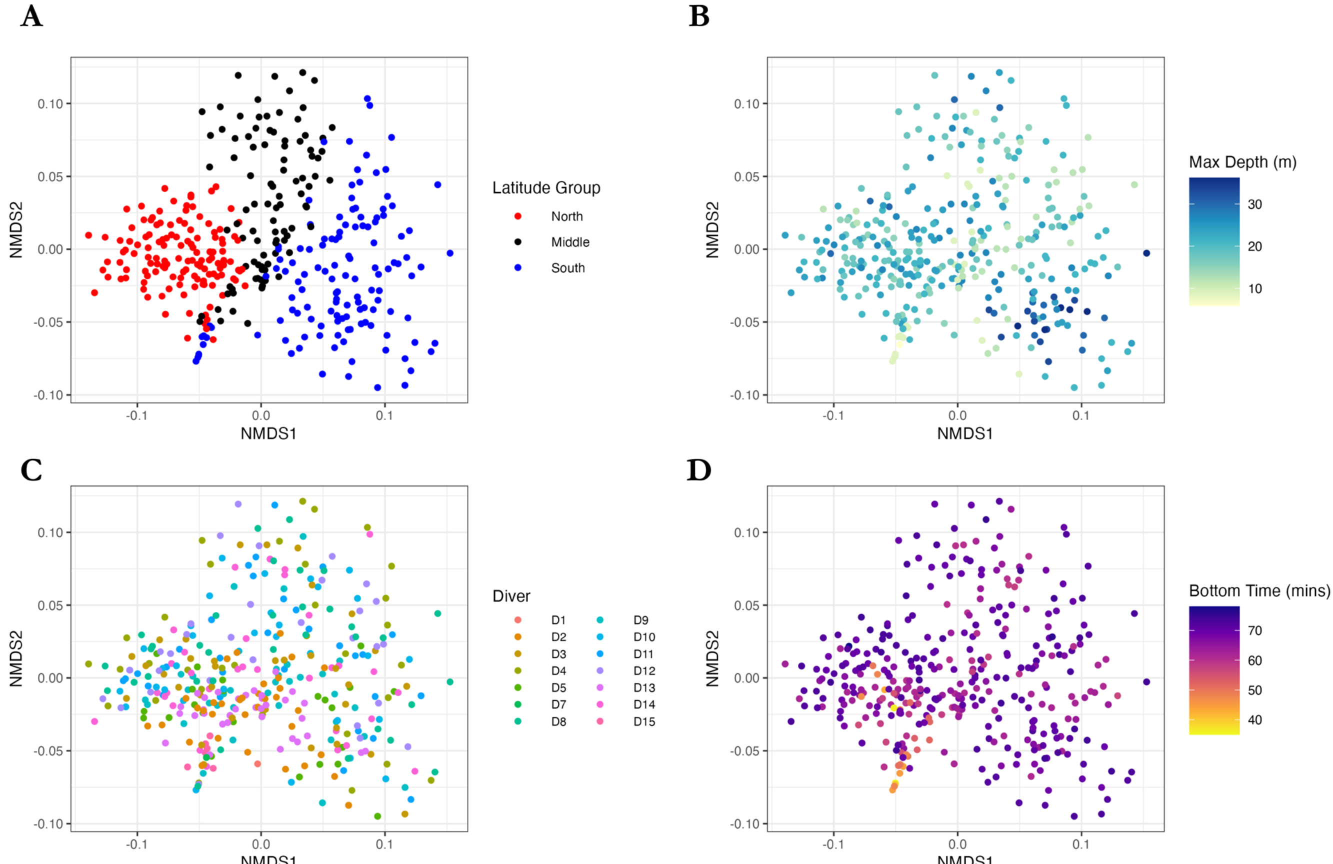


FIGURE 6 | NMDS beta diversity plots for RVS using Gower distance for latitude group (A), maximum depth (B), diver (C), and bottom time (D).

TABLE 2 | *F*-statistics and *p* values for PERMANOVAs of five metadata variables for PEDS and RVS, and their respective NMDS values.

	PEDS		RVS	
	F	***p***	***F***	***p***
Latitude group	2.5521	0.001*	89.4389	0.001*
Depth	2.2166	0.005*	18.9197	0.001*
Diver	0.9437	0.640	4.4663	0.001*
Bottom time	1.2532	0.184	12.2521	0.001*

*Statistical significance with threshold $p < 0.05$.

For beta diversity analyses, diver and bottom time did not emerge as significant variables for the PEDS but did for RVS. The significance of diver for RVS beta diversity suggests observer bias among the citizen scientist divers. Other research has previously documented observer bias in visual survey detection probabilities of marine fishes, likely due to a combination of differences in taxonomic identification skill and diving behaviors (e.g., individuals may be more or less likely to go to greater depths on a given dive) (Williams et al. 2006; Bernard et al. 2013). The lack of diver effect in PEDS could reflect a genuine reduction in observer-driven bias, a potential strength of using passive eDNA tools. As for the positive relationship between RVS diversity and dive time, longer dives likely increased the possibility of encountering more site-specific species. It is notable that PEDS did not identify dive bottom time as a statistically significant factor for beta diversity metrics, despite the wide variation in dive durations, which ranged from 18 to 75 min. This is in line with Bessey et al. (2022), who found that longer PEDS submersion times do not increase eDNA collection, and that in as few as 5 min of submersion, PEDS can obtain comparable fish species richness as active eDNA filtration methods (Bessey et al. 2022). Nichols and Marko (2025) similarly found that PEDS exposure time did not significantly impact eDNA capture rates. Future studies could investigate whether our PEDS saturate as quickly, or whether it is beneficial for divers to carry PEDS throughout the courses of their dives.

4.2 | Limitations of PEDS

RVS data provided a more comprehensive estimate of site-level species richness with fewer surveys (Figure 4). The saturation trend of the species accumulation curve for RVS highlights that the existing survey effort captured nearly all of the observable species within a given site. Conversely, the species accumulation curve for PEDS had much shallower asymptotic behavior, suggesting that many more PEDS samples would be required to approach saturated species richness relative to RVS, which would then increase laboratory and sequencing costs. Furthermore, as RVS maintains lower turnover and higher retention than PEDS, the species detected in RVS are more consistently found across surveys and sites. The steeper zeta diversity decline of

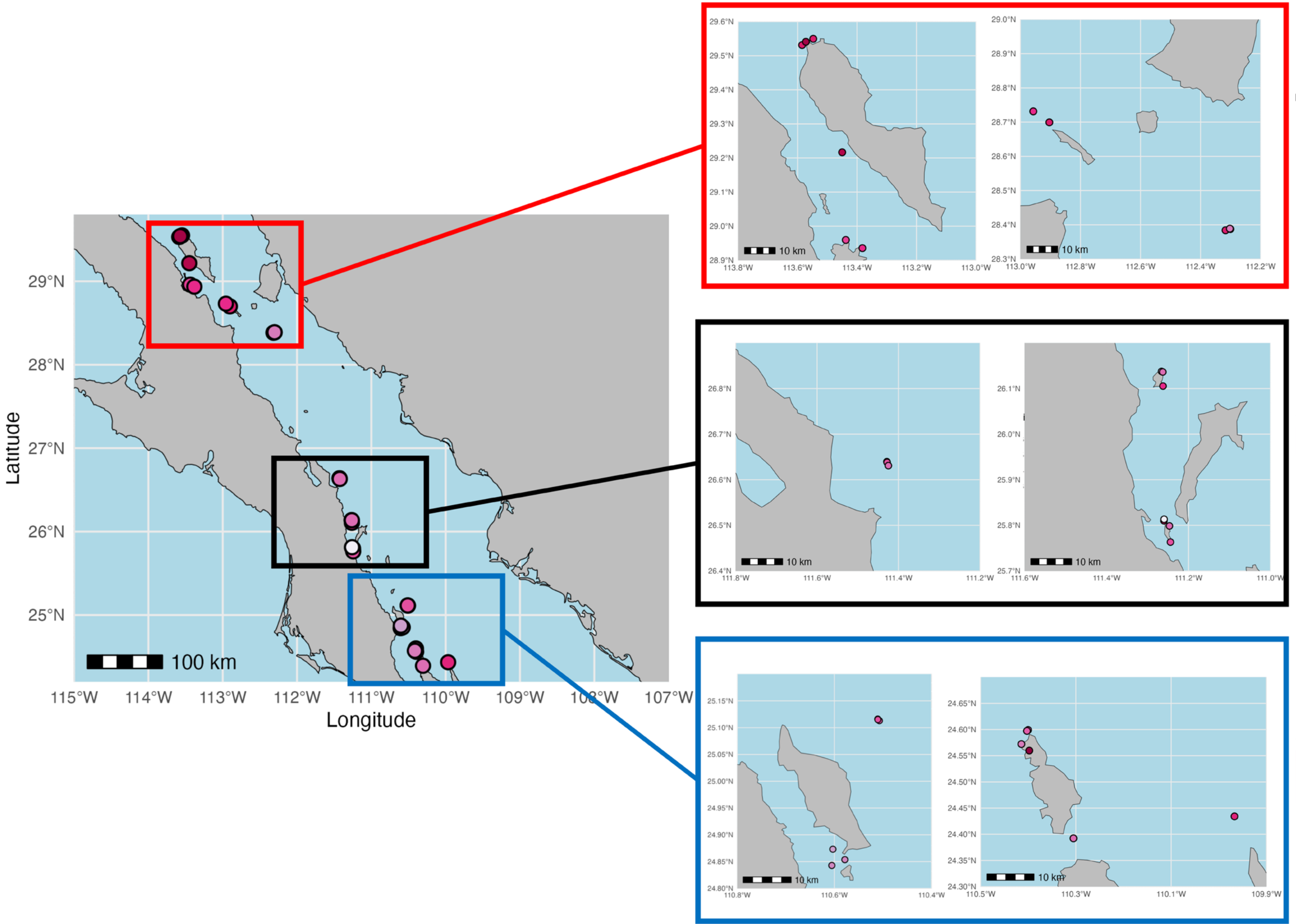


FIGURE 7 | Map of sampling sites in which each point is colored by similarity, or the number of species in each site for which PEDS and RVS had matching presence/absence data. Each similarity score represents the number of common species present in both or absent in both methods for a given site. The left panel shows all sites, while the paired panels on the right present zoomed views of sites within each latitude group: North (red; 11 sites), Middle (black; 10 sites), and South (blue; 12 sites).

PEDS indicates that fewer species were shared across replicates, reflecting either poor sampling of underlying homogeneous eDNA signatures or highly patchy eDNA distribution (Bessey et al. 2020).

RVS observations of fish communities appear more responsive to both fine-scale habitat variation and broader geographic gradients. Specifically, RVS was significantly influenced by both geographic location (latitude group) and diver behavior (maximum depth), with much higher pseudo-F statistics in PERMANOVA tests than those for PEDS (e.g., 89.4 vs. 2.6 for latitude group, 18.9 vs. 2.2 for depth). This greater sensitivity likely reflects the fact that many reef fish species are habitat specialists, confined to particular depths or site-specific features that divers encounter directly (Stefanoudis et al. 2019). In contrast, PEDS integrates eDNA across a diver's full path, likely smoothing over such local heterogeneity. Additionally, the higher sensitivity of RVS to latitude groups is largely a function of decreased variance across replicate diver surveys, reflecting increased power to capture biogeographic differences in species composition across regions. In contrast, biogeographic patterns were weaker in PEDS data due to higher variance across recovered communities. Similarly, the lack of significant effects of diver and dive time in the PEDS data may reflect the method's reduced sensitivity to fine-scale variation, limiting its ability to detect diver-specific and temporal patterns even if they are present. In other words, while PEDS offers a complementary perspective on biodiversity, RVS may provide finer resolution of spatial community structure in reef fish assemblages under the sampling effort applied here.

Unlike previous eDNA studies that documented equal richness to or higher richness than paired visual surveys (Valdivia-Carrillo et al. 2021; Xanthopoulou et al. 2025; Roblet et al. 2024), our study documented lower richness in eDNA results. However, within the Gulf of California, a 2021 study by Valdivia-Carrillo and colleagues found that active filtration of eDNA detected 119 Actinopterygii operational taxonomic units (OTUs), representing 64 fish species, compared to 97 species from visual censuses (Valdivia-Carrillo et al. 2021). Although we did not benchmark PEDS against traditional active eDNA sampling, we detected 70 marine fish species from PEDS, indicating that our passive sampling resulted in similar numbers of observed species as previous eDNA methods in the region. However, we observed greater variance in species detected in replicate PEDS than observed in replicate active eDNA filters by Valdivia-Carrillo et al. 2021. These results suggest that the passive filters deployed here may not be as effective at capturing eDNA as active approaches.

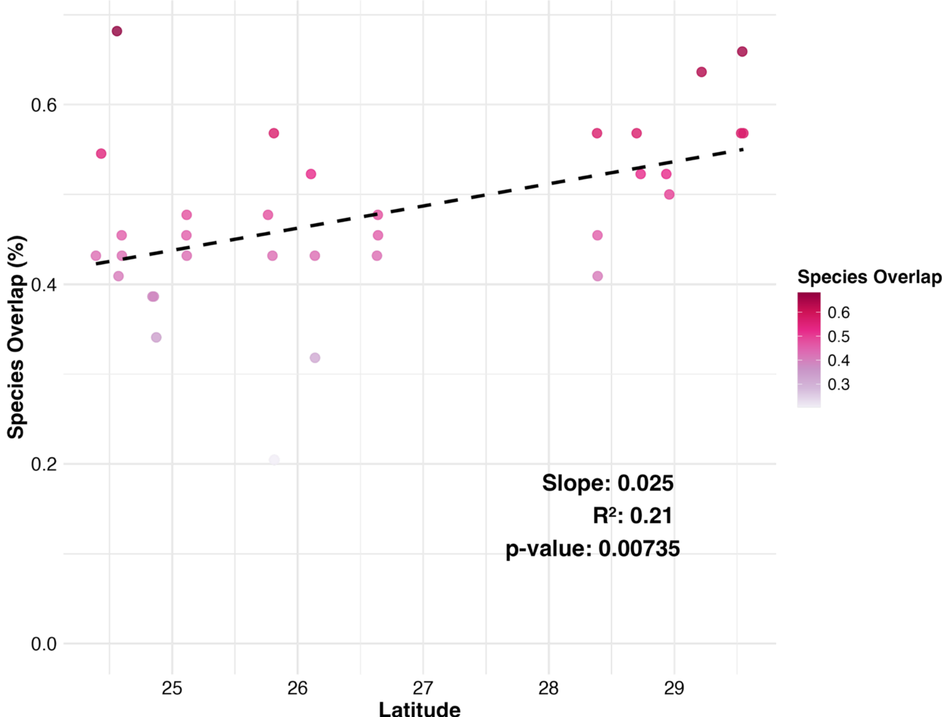


FIGURE 8 | Scatter plot with a linear regression between species overlap numbers and latitude. Slope is 0.025, R (Taberlet et al. 2018a) value is 0.21, and *p* value of the regression is 0.007.

Additionally, Nichols et al. (2025) suggest attaching PEDS away from the bubble streams of divers' exhalations, which may have impacted the retention of eDNA on our PEDS given that they were attached to divers' first stage regulators. PEDS methods may be improved in the future by using alternative absorbent materials, greater surface areas, and optimal placement on divers.

Collectively, these results demonstrate the importance of continued optimization of PEDS performance by investigating the efficacies of different sampler materials and deployment strategies to achieve similar success as observed elsewhere (Min et al. 2023; Xu et al. 2024).

4.3 | Critical Importance of Reference Barcoding

A major insight from our study—combining visual surveys with eDNA analysis—was the importance of comprehensive reference databases for accurate identification of fish species in the Gulf of California. 95 of the 183 species observed by divers were not available in our initial reference database generated from GenBank in October 2023 (Gold et al. 2023; Curd et al. 2024). By constructing reference barcodes for an additional 64 fish species, we were able to improve PEDS taxonomic assignments for 15 species, aligning with previous work demonstrating the critical importance of reference databases for molecular based taxonomic assignments (Stoeckle et al. 2020; Weigand et al. 2019). The publicly available sequence databases generated here will aid future eDNA and DNA-based research on fish biodiversity in the Gulf of California and other regions where those species are found. Prior to the construction of the expanded reference database, 26% of species detected by PEDS ($n = 17$) had never been documented in the Gulf of California. Six of these erroneous assignments were successfully corrected to more biogeographically appropriate species using the expanded reference database (Table S6). However, our targeted reference barcoding efforts did not solve all potentially erroneous taxonomic assignments. For example, one of the species detected by eDNA was *Caranx hippos*, or the Crevalle Jack. However, this species is exclusively found in the Atlantic Ocean (Smith-Vaniz and Carpenter 2007). When we ran our ASVs against GenBank (GenBank 1982) using BLAST (Altschul et al. 1990), we found that in addition to its 100% identity match to *Caranx hippos*, the sequence also had a 98.82% identity match to *Caranx sexfasciatus* reference sequences. Of note, *C. sexfasciatus* was observed in RVS on the same dive as PEDS detected *C. hippos*. It is thus likely that the *C. hippos* detection arose from the paucity of *Caranx* reference barcodes in publicly available sequence databases, which either fail to capture intraspecific variation of *C. sexfasciatus* or represent interspecific variation within the *Caranx* genus that has yet to be sequenced. Together, these results add to a growing body of work demonstrating the importance of comprehensive reference databases capturing both inter- and intra-specific variation to enable trusted and accurate taxonomic assignments from eDNA datasets (Coissac et al. 2012; Keck et al. 2023; Leray and Knowlton 2016).

Another potential limitation of our PEDS was our use of the MiFish-U primers, which may not have sufficient resolution to enable taxonomic identification of certain species. Recent studies have shown that other primers can work better for certain fish species, particularly for more recent adaptive radiations (Fontes et al. 2024; Ferreira et al. 2024). The application of multiple markers or novel longer amplicon sequencing technologies can improve taxonomic assignment from PEDS in the future (Lin et al. 2024).

Our results underscore the importance of developing curated local databases and optimized primer sets, particularly when there are closely related but geographically distinct species and regionally-relevant adaptive radiation (Stoeckle et al. 2020; Weigand et al. 2019; Gold et al. 2021). Efforts to expand fish barcoding in the Gulf of California and evaluate the effectiveness of different metabarcoding loci are needed to improve taxonomic assignment of eDNA metabarcoding data.

4.4 | Ecological Inference From PEDS and RVS in the Gulf of California

One of the key takeaways from our diversity analyses is that latitude is an important variable for both PEDS and RVS. The Gulf of California has several features that could account for the significance of latitude on beta diversity. First, much of the surface circulation of the southern gulf (specifically the peninsula side, where all of the sites in this study were located) is driven by mesoscale eddies in the summer (Lavín et al. 2014), and these eddies last from August until October (Lavín et al. 2013), which encompasses the dates of PEDS and RVS data collection. While eDNA signatures generally degrade on the order of hours to days and are unlikely to be transported over long distances by eddies (Harrison et al. 2019; Sassoubre et al. 2016), the mixing and environmental variation caused by these mesoscale features may influence local ecological communities and, consequently, the composition of eDNA present in the water. Additionally, a previous study compared fish biodiversity between three latitude groups in the Gulf of California and found that Shannon diversity values showed statistically significant differences among latitude groups. Their northern latitude group matches the one in this study ($\geq 28°$N), although their "central zone" encompasses both of our southern and central zones and ranges from 27°N to 24°N (Fernández-Rivera Melo et al. 2018). These features could explain the results of the linear regression linking higher latitudes to greater similarity in detection between the two methods: higher latitudes have lower biodiversity, leading to more shared absences between the two methods, as well as less water mixing and eDNA transport, leading to more shared presences between the two methods. Other studies have also found latitudinal gradients or biogeographic regionalization of species composition in the Gulf of California using both eDNA metabarcoding and underwater visual surveys (Valdivia-Carrillo et al. 2021; Mac Loughlin et al. 2024), further supporting the significance of latitude on diversity values in the present study.

4.5 | Importance of Citizen Science for Monitoring Marine Biodiversity

Our study illuminates a novel approach for citizen scientists to aid in marine biodiversity monitoring: by attaching PEDS to their SCUBA gear, divers can passively collect eDNA as they swim. This work expands upon a rich history of citizen scientists collaborating with researchers to observe, monitor, and track patterns of biodiversity over time (Pocock et al. 2018; Chandler et al. 2017; Amano et al. 2016). In recent years, eDNA has increasingly enhanced the reach and resolution of these efforts: in the past year alone, citizen scientists have successfully used eDNA methods to aid Mediterranean monk seal conservation (Bonicalza et al. 2024), contribute to novel ecological insights for marine fish conservation (Kvalheim et al. 2024), and advance genetic research on southern right whales (through blow samples) (Neveceralova et al. 2025). In our study, PEDS complemented traditional diver-led visual surveys, offering observer-independent biodiversity data that required no taxonomic expertise. While PEDS have limitations, they are uniquely well suited to citizen-science scaling: recreational divers can easily deploy multiple PEDS replicates, whereas increasing effort in active eDNA filtration or visual surveys demands substantially more equipment, training, and effort. As reference databases improve, PEDS could further expand biodiversity detection in fishes, marine mammals, and other groups, reinforcing their value for community-wide monitoring.

Ultimately, PEDS-based eDNA sampling offers a scalable, low-barrier entry point for engaging citizen scientists in marine monitoring—especially for cryptic, rare, or visually inaccessible species. With continued optimization of sampling methods and reference databases, passive eDNA could help diversify and democratize biodiversity assessments while enhancing data quality and taxonomic breadth.

Author Contributions

The following authors made significant contributions to each section of the study: (i) Zachary Gold, Julie Dinasquet, Nastassia V. Patin, Christy Pattengill-Semmens, and Brice Semmens: conception and design of the study, (ii) all authors: acquisition, analysis, and interpretation of the data; and (iii) Theodora T. Mautz: writing and all authors for the editing and reviewing of the manuscript.

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Disclosure

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The raw sequences generated for this project are available on the NCBI Sequence Read Archive under BioProject #PRJNA1234680. All tissue voucher sequences have been deposited in NCBI PV263179-PV263242

(File S1). All code used to process and analyze sequences is available on GitHub at https://github.com/theodoramautz/baja_edna_git.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Fish tissue voucher sequences that were deposited in NCBI and added to the expanded rCRUX reference database, with accession numbers PV263179-PV263242. **Table S2:** (first tab) and **Table S6** (second tab). **Table S2**. Original species detected in PEDS and whether they are documented in the Gulf of California. **Table S6:** Final species detected in PEDS using the expanded reference database and whether they are documented in the Gulf of California. **Table S3:** List of species detected in RVS. **Table S4:** (first tab) and **Table S7** (second tab). **Table S4**. Original species detected by both PEDS and RVS. **Table S7:** Final species detected by both PEDS and RVS using the expanded reference database. **Table S5:** Species detected in field negative controls and whether they are documented in the Gulf of California. **Table S8:** Similarity between PEDS and RVS methods at each site, measured by the numbers of species either detected or not detected by both methods per site (Species Overlap column), and their proportions to the total number of species detected by both methods (% Common Species column).