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ORIGINAL ARTICLE

Northeast Pacific eelgrass fish communities characterized by environmental DNA represent local diversity and show habitat specificity

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Abstract

Fish biodiversity is an important indicator of ecosystem health and a priority for the National Park Service in Drakes Estero, a shallow estuary within Point Reyes National Seashore, Marin County, California. However, fish diversity has yet to be described following the removal of oyster aquaculture infrastructure within Drakes Estero from 2016 to 2017. We used environmental DNA (eDNA) to characterize fish biodiversity within Drakes Estero. We amplified fish eDNA with MiFish primers and classified sequences with a 12S rRNA reference database. We identified 110 unique operational taxonomic units (OTUs, at 97% similarity) within the estuary from 40 samples across 4 sites. From these 110 OTUs, we identified 9 species and 13 taxonomic groups at the genus, family, order, or class level within the estuary. Species-level assignments are limited by a lack of representative sequences targeted by the MiFish primers for 42% of eelgrass fishes in our region that we identified from a literature review in the Northeast Pacific (NEP) from Elkhorn Slough to Humboldt Bay. Despite this limitation, we identified some common Drakes Estero fishes with our eDNA surveys, including the three-spined stickleback (*Gasterosteus aculeatus*), Pacific staghorn sculpin (*Leptocottus armatus*), surfperches (Embiotocidae), gobies (Gobiidae), and a hound shark (Triakidae). We also compared fish biodiversity within the estuary with that from nearby Limantour Beach, a coastal site. Limantour beach differed in community composition from Drakes Estero and was characterized by high relative abundances of anchovy (*Engraulis* sp.) and herring (*Clupea* sp.). Thus, we can distinguish estuarine and non-estuarine sites (<10 km away) with eDNA surveys. Further, eDNA surveys accounted for greater fish diversity than seine surveys conducted at one site within the estuary. Environmental DNA surveys will likely be a useful tool to monitor fish biodiversity across eelgrass estuaries in the Northeast Pacific, especially as reference databases become better populated with regional species.

KEYWORDS
Drakes Estero, eDNA, eelgrass, fish, seine

1 | INTRODUCTION

Eelgrass meadows are economically important carbon-storing habitats (Fourqurean et al., 2012) that serve as nursery grounds for fishes (Andrews & Liedtke, 2020; Hayduk et al., 2019; Kennedy et al., 2018; Orth et al., 2020; Robinson et al., 2011). Though the value of eelgrass in supporting fish diversity is commonly cited as justification for conservation and restoration efforts, enumerating fish biodiversity can be challenging (Hayduk et al., 2019; Iacarella et al., 2018). Traditional capture-based efforts to document fish diversity require taxonomic experts to identify morphologically similar species and human hours needed to perform surveys. Furthermore, some species are overlooked by traditional methods. For example, some species are cryptic or difficult to discriminate (Hayduk et al., 2019; Iacarella et al., 2018) or can escape or evade nets like large migratory or transient fish species (Closek et al., 2019). Beach seines, a commonly used method of seining (Baker et al., 2016; Iacarella et al., 2018), are further limited to shallow water nearshore or sampling at low tide, where fish species composition may vary throughout the tidal cycle (Kwak et al., 2018; Lee et al., 2014; Sogard et al., 1989). However, advantages to seining and other traditional capture-based approaches include the possibility to estimate size classes, abundances, and biomass of fishes (O'Leary et al., 2021; Wasserman et al., 2020). Seines may also catch benthic invertebrates depending on the mesh size of the net (Bloomfield & Gillanders, 2005; Guest et al., 2003; Martin et al., 2021).

New molecular methods allow for the characterization of fishes present in an environment by the cellular DNA they shed into the water column (environmental DNA, eDNA (Miya et al., 2015; Venter et al., 2004)). While the rates of transport and fate of cellular DNA are hard to quantify in natural systems, discrimination among sites, and habitat types is possible with eDNA surveys (Fernández et al., 2021;He et al., 2022; Oka et al., 2021). Environmental DNA also offers the potential to detect large transient species and small or evasive species that are difficult to detect with traditional visual or net-based surveys (Fernández et al., 2021). Furthermore, sampling of eDNA can be done without disturbing the habitat or habitat-forming species like eelgrass or coral (He et al., 2022; Oka et al., 2021). Standard bioinformatic pipelines permit comparisons across sites, seasons, and years, which is useful for long term monitoring programs (Closek et al., 2019; Djurhuus et al., 2020; He et al., 2022; Oka et al., 2021).

Here, we characterize fish biodiversity in Drakes Estero, a shallow estuary within Point Reyes National Seashore, Marin County, California for the first time using eDNA. Eelgrass, *Zostera marina*, occurs throughout the estuary and characterization of fish biodiversity in this habitat is a priority for the National Park Service (NPS). Use of non-invasive methods like sampling eDNA to characterize fish diversity will prevent disturbance to these productive eelgrass habitats. Limited information exists on fish biodiversity within Drakes Estero from surveys performed in 2002–2004 using a variety of traditional capture-based approaches (Wechsler, 2005). These surveys occurred prior to efforts from 2016 to 2017 to remove oyster aquaculture infrastructure from the estuary and restore the eelgrass habitat (Becker et al. unpublished data). Approximately 6% of the remaining eelgrass habitat in California occurs in Drakes Estero (Hamilton et al. submitted for review, NOAA Fisheries West Coast Region, 2014). Thus, use of a non-invasive reproducible method to monitor fish biodiversity through time in this estuary would be of both local benefit and serve as a proof of concept for using this approach in other eelgrass habitats in this region of California. We compare fish diversity within Drake's Estero to a coastal site just outside of the estuary to determine if estuarine and non-estuarine sites can be distinguished with eDNA surveys. We evaluate whether fish eDNA collected within Drakes Estero reflects the regional pool of estuarine species with reference sequences by performing a literature review for eelgrass estuaries from Humboldt Bay to Elkhorn Slough, CA.

2 | METHODS

2.1 | Site selection

We sampled four eelgrass meadows within Drakes Estero and one non-eelgrass site, along Limantour Beach (38°1′28.524″N -122°52′54.5268″W to 38°01′30.0″N 122°53′04.4″W) just outside the estuary during June 2021. All sites are located in Point Reyes National Seashore (38° N 122° W) in Marin County of Northern California (Figure 1). Three of our eelgrass sites within Drakes Estero were located toward the center of the estuary at the mouth of Creamery Bay (38°3′32.6736″N -122°56′48.5412″W to 38°03′25.0″N 122°56′51.6″W), Schooner Bay (SB west, 38°3′43.7616″N -122°56′21.876″W to 38°03′39.3″N 122°56′18.8″W), and Home Bay (38°3′35.4276″N -122°55′40.1808″W to 38°03′36.3″N 122°55′33.8″W). One eelgrass site was located at the base of Schooner Bay near the access road (SB east, 38°4′53.4864″N -122°55′58.1556″W to 38°04′55.8″N 122°55′58.4″W).

2.2 | Seining

We selected SB east for beach seining due to accessibility of the site and low-water depth at low tide (<1 m). Seining at other sites in the estuary was not feasible due to water depth and distance from shore. Trawling was not possible due to impacts on the habitat. We seined on a falling tide (water depth 0.6–0.9 m) the same day, June 26, 2021, as eDNA sampling at SB east. We began seining immediately upon completion of eDNA sampling to avoid disturbing the water column prior to sampling eDNA. We sampled nekton, including both fishes and decapod crustaceans with a custom beach seine (1 m tall, 3 mm mesh) at low tide when the water above eelgrass habitat was between 0.2 and 0.8 m deep. We sampled a circular area of 11 m^2 with wings of 6 m length, which were then pressed together to chase nekton into the cod end. In total, we performed six seines at least 3 m apart from each other in areas undisturbed by walking

FIGURE 1 Map of the sampling sites in Point Reyes National Seashore, Marin County California. Transects are in white and show the geographic start and end points of sampling within each site. Site labels are color coded to match other figures.

and upstream from previous seines. We counted and identified individuals to the lowest possible taxonomic level (typically species). Of these, we measured the standard length of the first 10 individuals of each fish species to the nearest mm. We plotted histograms of fish standard lengths for each species caught by seine using R v1.2.5042 (R Core Team, 2020) software package ggplot v3.3.2 (Wickham, 2016). It is unlikely that seine surveys at one site within the estuary would capture the same diversity of fishes as surveys conducted across many sites in the estuary. Thus, we also conducted a literature review as described below to describe the potential species pool from non-eDNA surveys in Drakes Estero.

2.3 | Literature review for local and regional species

To identify the local and regional species pool that could be present in our eDNA surveys, we performed a Web of Science topic search for any records of fishes surveyed in Drakes Estero and estuaries from Elkhorn Slough to Arcata Bay. We added species identified with our seine surveys described above to this list of species. Our Web of Science (WOS) search was performed as follows: a separate topic search was performed for each of the following estuaries: Elkhorn Slough, San Francisco, San Pablo, Suisun, Drakes, Tomales, Bodega, Bolinas, Eel River, Russian River, Humboldt, and Arcata. In each search, in addition to the estuary name, the following keywords were included: fish*, teleost*, elasmobranch*, shark*, ray*, bony, cartilaginous, seine*, trawl*, gill*, fyke*, and net*. The asterisk is a wildcard character that represents any character, including no character. Keywords were separated with the "OR" function and paired with the estuary name with the "AND" function. We used primary literature papers or academic theses for generating species lists for each estuary. For the Russian River and the Eel River, we only included fishes caught in estuaries within 1800 and 4000 meters of the Pacific Ocean, respectively, where eelgrass is known to occur (according to the Pacific Marine & Estuarine Fish Habitat Partnership Estuaries Explorer Application at https://www.pacificfishhabitat.org/data/). We did not find any records using these search terms for Arcata Bay and no records occurred within 4000 meters of the river mouth of the Eel River. We only used publications available through the University of California library or publicly available publications to generate a regional and local species list.

2.4 | eDNA sampling

To sample eDNA, we collected 300 mL samples of surface water with sterile Whirlpak bags (532 mL/18 oz. Cat. B01365). We wore clean nitrile gloves at each site while collecting samples. We collected 10

samples per site on falling tides within a three-hour window of low tide at all sites. We sampled Limantour Beach and SB east on June 26th, 2021, by walking along the beach or eelgrass bed while wearing waders. Water depth was approximately 0.1–0.2 m at Limantour Beach and 0.6–0.9 m at SB east at the time and location of sampling. We sampled water at SB east just prior to seining at this site on June 26, 2021, to prevent disturbing the water column prior to eDNA sampling. We washed waders thoroughly with fresh water between sites. We sampled Creamery Bay, SB west, and Home Bay on June 21, 2021, from a NPS 5 m motorized flat-bottom boat; water depth was approximately 1.5–2 m at these sites at the time of sampling. The distance between samples varied among sites, with the greatest distance covered at Limantour Beach and Creamery Bay (approximately 240 m), followed by Home Bay and SB west (approximately 160 m), and SB east (70 m). Differences in distances covered while sampling within a site occurred due to variations in speed and difficulty while sampling by boat and while wading through the eelgrass bed on foot. We kept all water samples in coolers on ice to reduce eDNA degradation (Nagarajan et al., 2022) until filtering, which occurred within four to six hours of collection for all samples. We used Nalgene Analytical Filter Units with 0.22 μM cellulose nitrate filters (Cat. 130–4020) to collect cellular DNA from water samples under vacuum from a hand pump. We additionally filtered 100 mL of deionized water (DI) on each sampling day to serve as negative controls. After filtration, we aseptically transferred filters to sterile DNA/RNA shield collection tubes (Zymo Research, Cat. R1102) to preserve DNA. We extracted DNA from samples within 20–25 days of collection.

2.5 | eDNA extraction

We extracted cellular DNA from cellulose nitrate filters preserved in DNA/RNA Shield (Cat. R1102) on July 12–16, 2021. We aseptically cut filters into 2–3 mm slices and placed sliced filters back in their original DNA/RNA shield collection tubes to be vortexed (15 s) prior to extraction of DNA from the DNA/RNA shield solution. We used the ZymoBIOMICS DNA Microprep Kit (Cat. D4305) to extract DNA with some changes to the manufacturer's protocol. First, we transferred 490 μL DNA/RNA shield containing sample DNA into a sterile 1.5 mL tube and incubated the sample with 10 μL of Proteinase K (Cat. D3001-2-20; 20 mg/mL) for 30 min at 55°C to break down proteins. No bead beating was performed due to a supply chain shortage of Zymobiomics tubes with beads. All other steps followed the manufacturer's instructions except a final elution volume of 40 μL rather than 20 μL of DNase/RNase free water. We quantified total DNA concentration with Qubit dsDNA HS Assay Kit (Cat. Q32854) and diluted DNA to 4 ng/μL prior to library preparation.

2.6 | Library preparation and sequencing

We used universal fish primer set MiFish-U and MiFish-E to amplify teleost and elasmobranch DNA, respectively (Miya et al., 2015). These primers amplify a hypervariable region (*ca* 170 bp) flanked by highly conserved regions (*ca* 20–30 bp) of the 12S rRNA mitochondrial genome (mitogenome) that allows for differentiation among species of bony and cartilaginous fishes (Miya et al., 2015). Following amplification of this gene region we proceeded with a second amplification step to add custom barcode primers (Miya et al., 2015) to multiplex our samples into one sequencing run on an Illumina MiSeq with a 500-cycle kit for paired-end sequencing. We sterilized benches and pipettes with 10% bleach, used filtered tips, and wore nitrile gloves sterilized with 10% bleach prior to all library prep steps. The first polymerase chain reaction (PCR) mixture contained forward and reverse MiFish-U and MiFish-E primers (0.08 μM–0.29 μM final, varied to optimize amplification success), 3 μL of template DNA, Bovine Serum Albumin (BSA, 0.2 μg/μL final concentration, Cat. B9000S), Phusion Hot Start Flex 2X Master Mix (6 μL, Cat. # M0536S), and molecular grade water (Ambion Cat. AM9938) to reach a final volume of 12 μL. We ran triplicate reactions (technical replicates) for each sample under the following conditions: initial 3 min denaturation at 95°C followed by 35 cycles with denaturation at 98°C for 20 s, annealing at 65°C for 15 s, and extension at 72°C for 15 s and a final elongation at 72°C or 5 min (Miya et al., 2015). We ran PCRs in batches of eight samples with one no-template control for each batch. We visualized PCR products by gel electrophoresis (4% agarose) stained with GelStar (Cat. 50,535, 1 μL Gel Star to 1 mL DI H_2O). No product was observed in any of our no-template controls. If low product was observed for a sample, we repeated the PCR reaction and varied the final primer concentrations. Upon amplification success, we pooled triplicate PCRs for each sample and diluted the PCR product 10 times with molecular grade water to use as template in the second step PCR.

The second PCR appended unique barcode indices (forward and reverse) to each sample for multiplexing samples onto one sequencing run (Miya et al., 2015). We set up this reaction in batches of 12 samples with one no-template control per batch. Reaction mixtures contained forward and reverse barcode primers (0.29 μM), 4 μL of template DNA, Phusion Hot Start Flex 2X Master Mix (24 μL, Cat. # M0536S), and molecular grade water (Ambion Cat. AM9938) to reach a final volume of 48 μL. We ran PCRs with initial 3-min denaturation at 95°C followed by 12 cycles with denaturation at 98°C for 20 s, annealing and extension at 72°C for 15 s and a final elongation at 72°C for 5 min (Miya et al., 2015). We visualized PCR products by gel electrophoresis (4% agarose) stained with GelStar (Cat. 50,535, 1 μL Gel Star to 1 mL DI H_2O). No product was observed in any of our no-template controls. Following amplification of all samples, we tested all unique barcode primers (5 unique forward primers and 12 unique reverse primers) for contamination by performing additional no-template control PCR reactions. We visualized products with gel electrophoresis but did not observe any bands.

To address potential contamination from any point in our processing pipeline, we included two negative control samples throughout our processing steps from sample collection to DNA extraction,

library preparation, and sequencing. No product was observed with gel electrophoresis for these two negative controls in the first or second PCRs. However, we included these two negative controls on our sequence run to check for any low-abundance PCR product that may not be observed by gel electrophoresis or for any contamination that may occur during Illumina sequencing.

We cleaned and normalized barcoded PCR products with SequalPrep Normalization Kit (Cat. A10510-01) prior to sequencing at the UC Davis Genome Center. We pooled the normalized product and quantified DNA concentration with Qubit dsDNA HS Assay Kit (Cat. Q32854). Personnel at the UC Davis Genome center verified sample purity and length of DNA fragments with a bioanalyzer trace. UC Davis Genome center personnel performed sequencing with a MiSeq 500 cycle (250 paired-end) sequencing kit with 15% PhiX added to balance nucleotide content.

2.7 | Data preparation

We performed initial quality filtering of sequence reads as follows. We used TrimGalore! software (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) to trim the ends of sequence reads with low-quality scores (Phred score cutoff 20), remove sequencing adapters, and remove reads with fewer than 20 base pairs (bp). TrimGalore! software wraps two other software programs, FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) for quality trimming and cutadapt (Martin, 2011) for removing adapters. We imported trimmed reads into QIIME2 version 2020.2 bioinformatic software (Bolyen et al., 2019) using a manifest file and used DADA2 on unmerged paired reads to remove erroneous sequences (Callahan et al., 2016). We trimmed forward and reverse reads to 200 bp (median quality score was 38 for reverse reads and 37 for forward reads at this position) with DADA2. The minimum overlap for merging reads with DADA2 was 12 bp. From 11,096,674 reads, 2,828,053 reads remained after removing erroneous sequences with DADA2.

To characterize fish species, we used clustering and classification of sequences as follows. We performed de novo clustering at 97% similarity of sequences using vSEARCH (Rognes et al., 2016) with QIIME2 function (qiime vsearch cluster-features-de-novo). While single nucleotide differences can capture inter- and intra-specific variation in the 12S rRNA marker gene, a well-populated and curated reference database is needed to adequately describe this variation within and across species (Oka et al., 2021; Sigsgaard et al., 2016; Tsuji et al., 2020). Unfortunately, many fishes from eelgrass estuaries in our region lack reference sequences (as reported in our results section: Literature review for local and regional species). Thus, we chose to use clusters of 97% similarity (operational taxonomic units, OTUs) to describe fish diversity in our samples, which are useful for identifying fish species from sequence data (Closek et al., 2019; Deiner et al., 2017; Miya et al., 2015). We classified OTUs with QIIME2 function 'qiime feature-classifier classify-consensus-blast' with the default parameterization for query coverage (--*p*-query cov 0.8), the maximum number of hits to keep for each query (--*p*-maxaccepts 10), and percent consensus among the hits (--*p*-min-consensus 0.51) to be accepted as the consensus taxonomy. In other words, at least 51% assignments must match the top hit for the assignment to be accepted as the consensus. We altered the e-value to a more stringent value that matches recommendations (--*p*-evalue 0.00001) by the developers of the primers used in our study (Miya et al., 2015). We used percent similarity values for our blast algorithm in a stepwise manner, starting with 99% similarity down to 80% similarity to the reference sequences (--*p*-perc-identity 0.99, 0.97, 0.95, 0.90, 0.80). We used this algorithm with the March 2022 release of the reference datasets (12S, 16S, and 18S rRNA sequences) from the Mitohelper public repository (10.5281/zenodo.6336244). The 12S rRNA sequences in this repository include those from MitoFish (Lim & Thompson, 2021) monthly releases (http://mitofish.aori.u-tokyo.ac.jp). 16S and 18S rRNA sequences in this repository originate from the SILVA database v138 release (Quast et al., 2012) available at https://docs.qiime2.org/2020.11/data-resources/ and we used these to identify non-fish sequences that may be present in our sequence data. We assigned taxonomic labels to OTUs with the highest percent similarity available. For example, OTUs which remained unassigned at 99% similarity to reference sequences were assigned labels from 97% similarity results and OTUs which remained unassigned at 97% similarity to reference sequences were assigned labels from 95% similarity results. While it is unclear at this time what a reliable percent similarity level is for each taxonomic level (class, order, family, genus) for sequences amplified with the MiFish primers, 97% similarity to reference sequences can accurately identify fish species (Miya et al., 2015). Thus, we applied the following rules for our taxonomic labels to conservatively assign species names to our OTUs: ≥97% similarity to reference sequences are assigned species labels, ≥95% to reference sequences are assigned genus labels, ≥90% to reference sequences are assigned family labels, and ≥80% to reference sequences are assigned class and order labels. If multiple OTUs were assigned to a genus, family, order, or class but not to the species level, we used "spp." to indicate that multiple OTUs were present. By using the pool of regional species obtained from our literature survey, we assigned species labels if there was only one species found within a genus or family. Species assigned based upon this regional knowledge are indicated with an asterisk after the species name. We removed any sequences which remained unassigned at 80% similarity with the QIIME2 function (qiime taxa filter-table –*p*-exclude Unassigned). This excluded 7 OTUs from 140 total OTUs present in this dataset. We manually blasted these 7 OTUs with the interactive BLASTN v. 2.13.0+ (Altschul, 1997) software for nucleotide sequences on the National Center for Biotechnology Information (NCBI) website. These 7 OTUs had top hits at 97% similarity or higher to Western gull, *Larus occidentalis* (3 records), and dog, *Canis lupis familiaris* (4 records). This is consistent with an earlier study that discarded non-fish sequences which remained unassigned at 80% similarity classification against the MitoFish reference database (Oka et al., 2021). For comparison purposes, we characterized the number of sequence variants that could be assigned taxonomic labels using the same blast parameters described above at 97% similarity to the reference database.

Given that the MiFish primers do not adequately resolve species in some cases (for example, *Thunnus* spp., tunas, (Miya et al., 2015)) and numerous OTUs in our dataset could not be assigned taxonomic labels with ≥97% similarity, we checked all final taxonomic assignments for biological relevance with known species in the Northeast Pacific (NEP). We found 4 cases that did not follow biological distributions of fishes. OTUs were misidentified at 97% similarity to *Clupea harengus*, which is not known to occur in estuaries of the NEP. *Clupea harengus* is a congener of a known local species; therefore, we changed the taxonomic label to '*Clupea* sp.' We identified five OTUs as *Cottus sp.* (no species name provided) assigned at 97% similarity. *Cottus* is not known to occur in estuaries in the NEP unless there is significant riverine input, which Drakes Estero lacks. However, several members of the Cottidae family do occur in eelgrass estuaries. For this reason, we changed the taxonomic label from '*Cottus* sp.' to 'Cottidae spp.'. We identified one OTU as genus *Etropus* assigned at 95% similarity. No *Etropus* fishes occur in the NEP. Thus, we assigned this one OTU at the family level (Paralichthyidae). Lastly, we identified one OTU at 99% similarity to *Pholis gunnellus*, rock gunnel, which is not known to occur in the NEP, though congeners do occur in the NEP. We assigned this one OTU to the genus level (*Pholis* sp). All other taxonomic labels remained the same. All original taxonomic labels from our blast algorithm and updated labels to account for misidentifications are included in Table S1.

We used a phylogenetic tree to visualize clade patterns between our OTUs and reference sequences, as a recommended method to further validate taxonomic assignments (Miya et al., 2020). We included up to five reference sequences per species from our list of regional species (from our Web of Science literature search described above) present in the mitohelper 12S rRNA reference database for generating a phylogenetic tree. We performed MAFFT alignments (Katoh & Standley, 2013) with function 'qiime alignment mafft', removed noisy positions with function 'qiime alignment mask', and used fasttree (Price et al., 2010) to build a phylogenetic tree with function 'qiime phylogeny fasttree'. We midpoint rooted the tree with the function 'qiime phylogeny midpoint-root.' We visualized the phylogenetic tree with R v4.1.1 (R Core Team, 2020). We imported the tree with the read.newick command from the phytools package v1.0.3 (Revell, 2012) and visualized the tree with the ggtree command from the ggtree package v3.2.1 (Yu et al., 2017).

We rarefied OTU tables to 29,000 sequences per sample prior to calculating alpha and beta diversity to avoid spurious conclusions regarding OTU diversity or composition between sites occurring due to differences in sequencing depth between sites (Knight et al., 2018; Weiss et al., 2017). While rarefaction can lead to lower sensitivity, it overcomes numerous other limitations and problematic outcomes of other methods of normalization as discussed in Weiss et al. (2017). Further, our samples differed by 47-fold (2318 to 109,410) in sequences per sample and normalization was necessary. We rarefied tables with the function 'qiime diversity core-metrics' and passed the parameter of '--*p*-sampling-depth 29000' which rarefies without replacement (Bolyen et al., 2019). We retained 84% of samples and 44% of our reads at a rarefaction depth of 29,000 sequences per sample. Samples with fewer than 29,000 sequences per sample occurred in SB east (2 samples), SB west (3 samples), Limantour Beach (2 samples) and Home Bay (1 sample). Zero samples from Creamery Bay had fewer than 29,000 sequences.

2.8 | Fish community alpha and beta diversity analysis from eDNA

We used the R v1.2.5042 (R Core Team, 2020) package phyloseq v1.3.4 (McMurdie & Holmes, 2013) 'estimate_richness' function to calculate alpha diversity metrics, richness and Shannon Diversity on rarefied OTU tables. We visualized differences in alpha diversity with the R package ggplot v3.3.2 (Wickham, 2016). We used base R to test for differences in alpha diversity between sites with a Kruskal–Wallis rank sum test and the package FSA v0.9.3 to perform a Dunn test for post hoc comparisons among sites (Ogle et al., 2022). We calculated Bray Curtis dissimilarity of fish communities from eDNA samples with the R package phyloseq v1.3.4 (McMurdie & Holmes, 2013), and performed principal coordinate ordination (PCoA) on resulting distance matrices to visualize differences between samples and across sites. We performed multivariate analysis of variance (PERMANOVA, Anderson, 2001; McArdle & Anderson, 2001) with the 'adonis2' function in the R package phyloseq v1.3.4 (McMurdie & Holmes, 2013) to test for differences in fish community composition across sites. We used the function 'betadisper' in the R package vegan v2.5.6 (Oksanen et al., 2007) to test for differences in fish community dispersion, a measure of how variable fish communities are among samples and between sites. Dispersion is calculated by per sample distance from the centroid for any grouping variable in ordination space.

2.9 | Species and OTU accumulation curves from seine and eDNA surveys

We used the 'specaccum' function in the R package vegan v2.5.6 (Oksanen et al., 2007) to calculate species and OTU richness curves for seines ($n = 6$ per site) and eDNA samples ($n = 10$ per site), respectively. Samples were randomly drawn from each site and richness was determined from one hundred permutations for eDNA samples and by the maximum (exact) number of permutations permitted for the seine samples. We plotted richness curves with R package ggplot v3.3.2 (Wickham, 2016).

2.10 | Querying the Mitohelper reference database for representative sequences of local and regional species identified by our literature review

To determine which species lacked reference sequences in the reference database, we used the publicly available mitohelper python

tool (Lim & Thompson, 2021) to query the reference database. We used the 'getrecord' python script to query the March 2022 release of the reference 12S rRNA mitogenome dataset from the Mitohelper public repository (10.5281/zenodo.6336244) for local and regional fish species. Our query included species identified from our Web of Science literature search, from beach seines conducted as part of this study, and from earlier beach seining efforts in Drakes Estero (unpublished data, Collin Gross). We removed reference records that aligned to regions other than the 12S mitogenome targeted by the MiFish primers by using the 'getalignment' mitohelper python script to align our sequences to the reference 12S rRNA sequence of *Danio rerio* supplied by https://github.com/aomlomics/mitohelper and using the filter function in R (R Core Team, 2020) dplyr package v.1.0.7 (https://github.com/tidyverse/dplyr). We removed sequences with a 'start' location at 300 bp or greater or an 'end' location at 330 bp or less on the *D. rerio* reference sequence. Our sequences occurred at locations 229–447 ± 0.9–1.8 (mean ± standard deviation) on the *D. rerio* reference sequence. We then used the filter function to remove records with sequence lengths of 100 bp or less, even if they occurred within the region targeted by the MiFish primers. By doing so, we generated a list of local and regional species for which there are reference sequences (minimum length 101 bp) for the region targeted by the MiFish primers in the Mitohelper reference database.

3 | RESULTS

3.1 | Sequencing and classification of OTUs

We successfully sequenced fish DNA from all sites and maintained high sequencing depth per sample after denoising and rarefaction. After quality filtering and removing sequences of non-biological origin, we obtained 2,828,053 sequences and 1519 unique sequence variants. Of these, 1211 sequence variants occurred within the estuary, ranging from 289 to 398 sequence variants per site (Figure S1a,b). Our two negative control samples that went through processing steps from sample filtration, DNA extraction, and library prep had zero sequences after quality filtering and denoising. The total number of sequences per sample varied among sites (Figure 2) and thus, we rarefied samples to an even sequencing depth per sample prior to alpha and beta diversity analyses. Rarefaction of 29,000 sequences per sample allowed us to retain 84% of our samples for downstream analyses. During initial exploratory analysis, only 48% of sequences within the estuary could be assigned species labels at ≥97% similarity. The number of sequence variants ranged from 2 to 373 per species. Given that we could not assign species labels to 52% of sequences from estuarine samples, we proceeded with OTU clustering for taxonomic assignment and diversity estimates.

We found support for our bioinformatic pipeline and the use of OTU clusters in characterizing fish at varied taxonomic levels. Following taxonomic assignment of OTU clusters using the Mitohelper reference database that contained teleost and elasmobranch fishes (12S rRNA gene), bacteria (16S rRNA gene), and other eukaryotes (18S rRNA gene), only 7 OTUs remained totally unassigned from a total of 140 OTUs. We removed these unassigned sequences from downstream analyses. Of the remaining 133 OTUs, we identified 127 OTUs of class Actinopteri (ray-finned fishes) and 6 OTUs of class Chondrichthyes (cartilaginous fishes). One hundred ten of these OTUs occurred in Drakes Estero (23 OTUs were specific to Limantour beach samples). We identified 13 unique fish species across all samples, with remaining OTUs classified at the genus, family, order, or class level. In contrast, we only identified eight unique species across all samples prior to clustering sequences into OTUs. The clustering patterns of our OTUs within an approximately

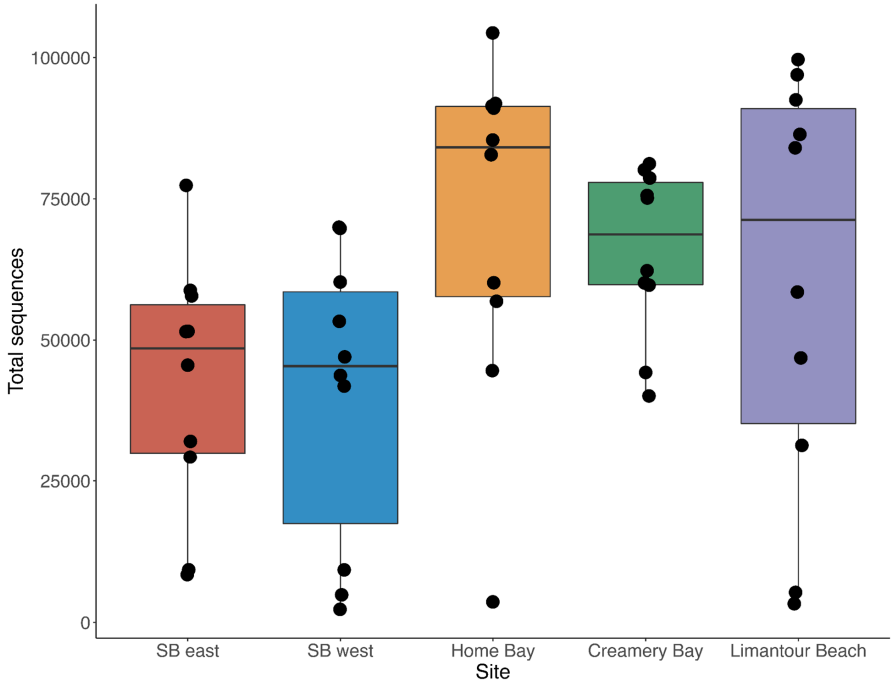

FIGURE 2 Box and whisker plots showing the median sequencing depth per site after denoising samples with DADA2 and removing sequences that could not be taxonomically assigned with our bioinformatic pipeline. Each data point represents a sample.

maximum-likelihood phylogenetic tree containing region-specific reference sequences supported most of our OTU taxonomic assignments (Figure S2). However, some OTUs identified at lower percent similarity showed some phylogenetic incongruencies (e.g., *Embiotoca* sp. at 95% similarity appears more closely related to confamilial species *Brachyistius frenatus* rather than congeners *Embiotoca jacksoni* or *Embiotoca lateralis* (Figure S2)). Though, some of the branch tips within the Embiotocidae family have low support (<0.50) and thus, branching patterns at these tips are not reliable. We also found *Cottus* and *Leptocottus* clustering separately from *Oligocottus*, *Clinocottus*, and *Artedius* (Figure S2), a phylogenetic pattern among Cottidae fishes that is consistent with recent findings by Rabosky et al. (2018).

3.2 | Literature review for local and regional species

Using data from our seining effort ($n=6$) at the same time of eDNA collection, seining efforts in earlier years (unpublished data, Collin Gross), and our Web of Science search, we identified 31 total fish species documented in Drakes Estero (including an unidentified rockfish, *Sebastes* sp.). By widening our search to include estuarine fish species (identified by various non-eDNA methods) from areas where eelgrass occurs regionally from Elkhorn Slough to Humboldt Bay, we found a total of 106 potential species. Of the 31 species found in Drakes Estero (Table 1) using non-eDNA approaches, 9 lacked representative sequences in the reference database. This included *Atherinops affinis* (topsmelt), *Atherinopsis californiensis* (jacksmelt), *Cebidichthys violaceus* (monkeyface prickleback), *Hyperprosopon argenteum* (walleye surfperch), *Hypsopsetta guttulata* (diamond turbot), *Micrometrus minimus* (dwarf perch), *Paralichthys californicus* (California halibut), *Porichthys notatus* (plainfin midshipman), and *Triakis semifasciata* (leopard shark). Of the 106 regional species, 44 species lacked reference sequences in the database. Species identified regionally and locally, method of sampling, time of year, and whether they have reference sequences that overlap the region targeted by the MiFish primers (of at least 101 bp in length) are identified in File S1. The full citation list of species found from our Web of Science search can be found in File S2.

Table 1 Fishes of Drakes Estero (a) Fishes detected in non-eDNA surveys only. (b) Fishes detected in eDNA surveys only. (c) Fishes detected in both non-eDNA and eDNA surveys. The lowest taxonomy assigned column shows the lowest assignments from our bioinformatic pipeline (i.e., species when available, otherwise the genus, family, or order is provided).

3.3 | Alpha diversity

OTU richness and Shannon diversity varied across sites (Figure 3a,b, Kruskal–Wallis rank sum test df = 4, $p=0.002$, and $p=0.008$, respectively). Richness was lower at SB east and Limantour Beach compared with Home Bay (Table S2 Dunn post hoc comparisons). Shannon Diversity estimates, which consider richness and evenness of the fish community followed a similar pattern with lower diversity at SB east compared to Home Bay (Table S3 Dunn post hoc comparisons).

3.4 | Beta diversity

We detected differences in fish community composition, but not dispersion (variability of communities), across sites (Figures 4 and 5, PERMANOVA $R^2=0.48$, $p=0.001$; permdispersion $p=0.079$). Fish community composition was notably different at our open coast site at Limantour Beach compared to those within Drakes Estero. Limantour Beach is characterized by the high-relative abundances of barred surfperch (*Amphistichus argenteus*), Californian anchovy (*Engraulis mordax*) and herring (*Clupea* sp.). Further, SB east at the head of Schooner Bay differed from all sites that occur at the mouths of each bay (Creamery Bay, Schooner Bay, and Home Bay) within the estuary. SB east has lower OTU diversity (Figure 3) and is characterized by high-relative abundances of silversides (Atherinopsidae). SB west, Creamery Bay, and Home Bay overlapped in community composition, characterized by high-relative abundances of shiner perch (*Cymatogaster aggregata*) and gobies (Gobiidae). We found some species, like the three-spined stickleback (*Gasterosteus aculeatus*) and Pacific staghorn sculpin (*Leptocottus armatus*), at all four sites within the estuary at similar relative abundances.

3.5 | eDNA and seine comparisons

eDNA surveys at SB east provided greater species richness than seine surveys and detected species or family groups identified by seine surveys. This included the three-spined stickleback (*Gasterosteus aculeatus*), the Pacific staghorn sculpin (*Leptocottus armatus*), and family group *Syngnathidae* (pipefishes, like the bay pipefish detected in seines, Figure 4, SB east, Figure S3, and Figure 6). Two fish species detected in seines (Figure 6), plainfin midshipman (*Porichthys notatus*, family Batrachoididae) and arrow goby (*Clevelandia ios*, family Oxudercidae) were not detected in eDNA surveys (Figure 4, and Figure S3). Of these two species, plainfin midshipman lacked reference sequences in the reference database. The OTU diversity detected per sample was higher with eDNA surveys at SB east (Figure 3, min 6 to max 15 OTUs per sample) compared to seine surveys at SB east (Figure 6, min 2 to max 5 species per seine). Total richness (across all samples) for SB east was much higher for eDNA surveys (36 to 40 OTUs with rarefied and non-rarefied data, $n=8$–10) compared to seines (5 species, $n=6$ seines, Figure 7a). However, only four OTUs could be taxonomically assigned to species in SB east eDNA samples (*Embiotoca lateralis*, *Gasterosteus aculeatus*, *Leptocottus armatus*, *and Paralichthys californicus*), with the remaining OTUs classified at the family level across 4 families (Atherinopsidae, Cottidae, Gobiidae, and Syngnathidae).

TABLE 1 Fishes of Drakes Estero identified by (A) non-eDNA surveys only, (B) eDNA surveys only, or (C) both eDNA and non-eDNA surveys.

(A)					
Common name	**Genus species**	**Reference record**	**Lowest taxonomy assigned**	**Detected in our seine surveys (year detected)**	**Identified in our literature review**
Tubesnout	*Aulorhynchus flavidus*	Yes	NA	No	Yes
Monkey-faced prickleback	*Cebidichthys violaceus*	No	NA	No	Yes
Striped kelpfish	*Gibbonsia metzi*	Yes	NA	No	Yes
Surf smelt	*Hypomesus pretiosus*	Yes	NA	No	Yes
Pacific tomcod	*Microgadus proximus*	Yes	NA	No	Yes
Plainfin midshipman	*Porichthys notatus*	No	NA	Yes (2021)	Yes
(B)					
Common name	Genus species	Reference record	Lowest taxonomy assigned	Detected in our seine surveys (year detected)	Identified in our literature review
Californian anchovy	*Engraulis mordax*	Yes	*Engraulis mordax*[c]	No	No
	Myliobatiformes	Yes	Myliobatiformes	No	No
Bat eagle ray	*Myliobatus californicus*	No	*Myliobatus californicus*[b,c]	No	No
(C)					
Common name	Genus species	Reference record	Lowest taxonomy assigned	Detected in our seine surveys (year detected)	Identified in our literature review
Topsmelt	*Atherinops affinis*	No	Atherinopsidae family	Yes (2019)	Yes
Jacksmelt	*Atherinopsis californiensis*	No	Atherinopsidae family	No	Yes
Kelp surfperch	*Brachyistius frenatus*	Yes	Embiotocidae family	No	Yes
Speckled sanddab	*Citharichthys stigmaeus*	Yes	*Citharichthys* genus	No	Yes
Arrow goby	*Clevelandia ios*	Yes	Gobiidae family	Yes (2019, 2021)	No
Wooly sculpin	*Clinocottus analis*	Yes	Cottidae family	No	Yes
Pacific herring	*Clupea pallasii*	Yes	*Clupea* genus[a]	No	Yes
Shiner surfperch	*Cymatogaster aggregata*	Yes	*Cymatogaster aggregata*	Yes (2019)	Yes
Black surfperch	*Embiotoca jacksoni*	Yes	*Embiotoca jacksoni*	No	Yes
Three-spine stickleback	*Gasterosteus aculeatus*	Yes	*Gasterosteus aculeatus*	Yes (2019, 2021)	Yes
Brown Irish Lord	*Hemilepidotus spinosus*	Yes	Cottidae family	No	Yes
Walleye surfperch	*Hyperprosopon argenteum*	No	Embiotocidae family	No	Yes
Diamond turbot	*Hypsopsetta guttulata*	No	Pleuronectidae family	No	Yes
Butter sole	*Isopsetta isolepis*	Yes	Pleuronectidae family	No	Yes
Bay goby	*Lepidogobius lepidus*	Yes	Gobiidae family	No	Yes
Staghorn sculpin	*Leptocottus armatus*	Yes	*Leptocottus armatus*	Yes (2019, 2021)	Yes
Dwarf surfperch	*Micrometrus minimus*	No	Embiotocidae family	No	Yes
California halibut	*Paralichthys californicus*	No	*Paralichthys californicus*[c]	Yes (2019)	No
Saddleback gunnel	*Pholis ornata*	Yes	*Pholis* genus	No	Yes
Starry flounder	*Platichthys stellatus*	Yes	Pleuronectidae family	No	Yes

TABLE 1 (Continued)

(A)

Common name	Genus species	Reference record	Lowest taxonomy assigned	Detected in our seine surveys (year detected)	Identified in our literature review
Pile surfperch	*Rhacochilus vacca* (now *Phanerodon vacca*)	Yes	*Phanerodon vacca*	No	Yes
Cabezon	*Scorpaenichthys marmoratus*	Yes	Cottidae family	No	Yes
Rockfish	*Sebasates sp.*	Yes	*Sebasates sp.*	No	Yes
Bay pipefish	*Syngnathus leptorhynchus*	Yes	Syngnathidae family	Yes (2019, 2021)	Yes
Leopard shark	*Triakis semifasciata*	No	Triakidae family	No	Yes

[a]*Clupea pallasii* was only detected in eDNA samples from Limantour Beach. *Clupea* sp. was detected in Drakes Estero.

[b]Bat eagle rays, *Myliobatis californica*, are commonly observed by NPS staff in Drakes Estero. However, there are not any records of this fish from our literature review.

[c]Likely detected with eDNA. We annotated these species-level assignments using knowledge of the regional species pool in cases where only one species within a given family or genus is known to occur in the region.

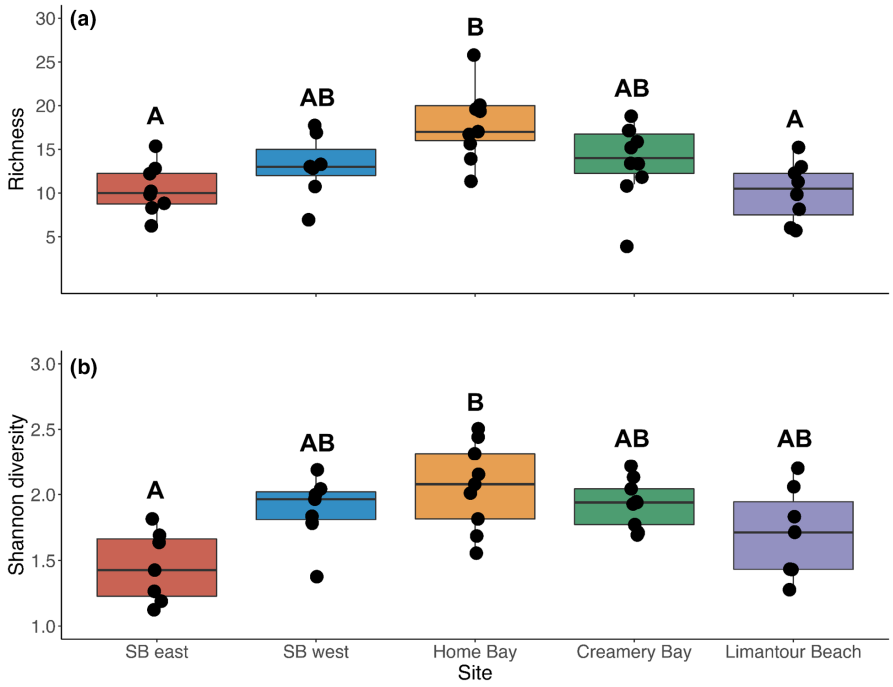

FIGURE 3 OTU diversity estimates (a, richness and b, Shannon Diversity) from eDNA surveys from rarefied tables varied among sites (Kruskal–Wallis rank sum test df = 4, $p = 0.002$, and $p = 0.008$, respectively, see Tables S2 and S3 for post hoc comparisons). Each data point represents a sample.

Paralichthys californicus did not occur in rarefied eDNA samples from SB east (Figure 4 and Figure S3). Seines allowed for characterizations of fish size class distributions that are not possible with eDNA surveys (Figure S4). Across all four sites within Drakes Estero, eDNA detected Myliobatidae (likely bat eagle ray), Myliobatiformes, and *Engraulis* sp. (anchovy) that are not yet reported from non-eDNA surveys (i.e., seine data from this study, earlier unpublished seine data, and results from our literature review, Table 1b).

3.6 | Species accumulation curves

Although fish community composition is more similar within a site than between sites (Figure 5; Figure S3), species and OTU richness increases with sampling effort for both seines and eDNA surveys, respectively (Figure 7a). After six seines, the slope of the species accumulation curve (Figures 7a and 8a) asymptotes with a maximum species richness of five. Thus, increasing the sampling effort at the SB east site, will not lead to increased species detection. However, if we expanded the spatial extent of seining to other sites in the estuary, additional species would likely be identified with this method. The OTU accumulation curves for eDNA surveys (Figure 7a) did not asymptote. Thus, increasing the eDNA sample size per site beyond 10 samples would likely increase the number of species (OTUs) detected at each sampling site. Further, when considering all sites ($n = 4$) within the estuary together (Figure 7b, $n = 40$), the slope of the OTU accumulation curve did not asymptote. This further supports that additional fish species could be detected within the

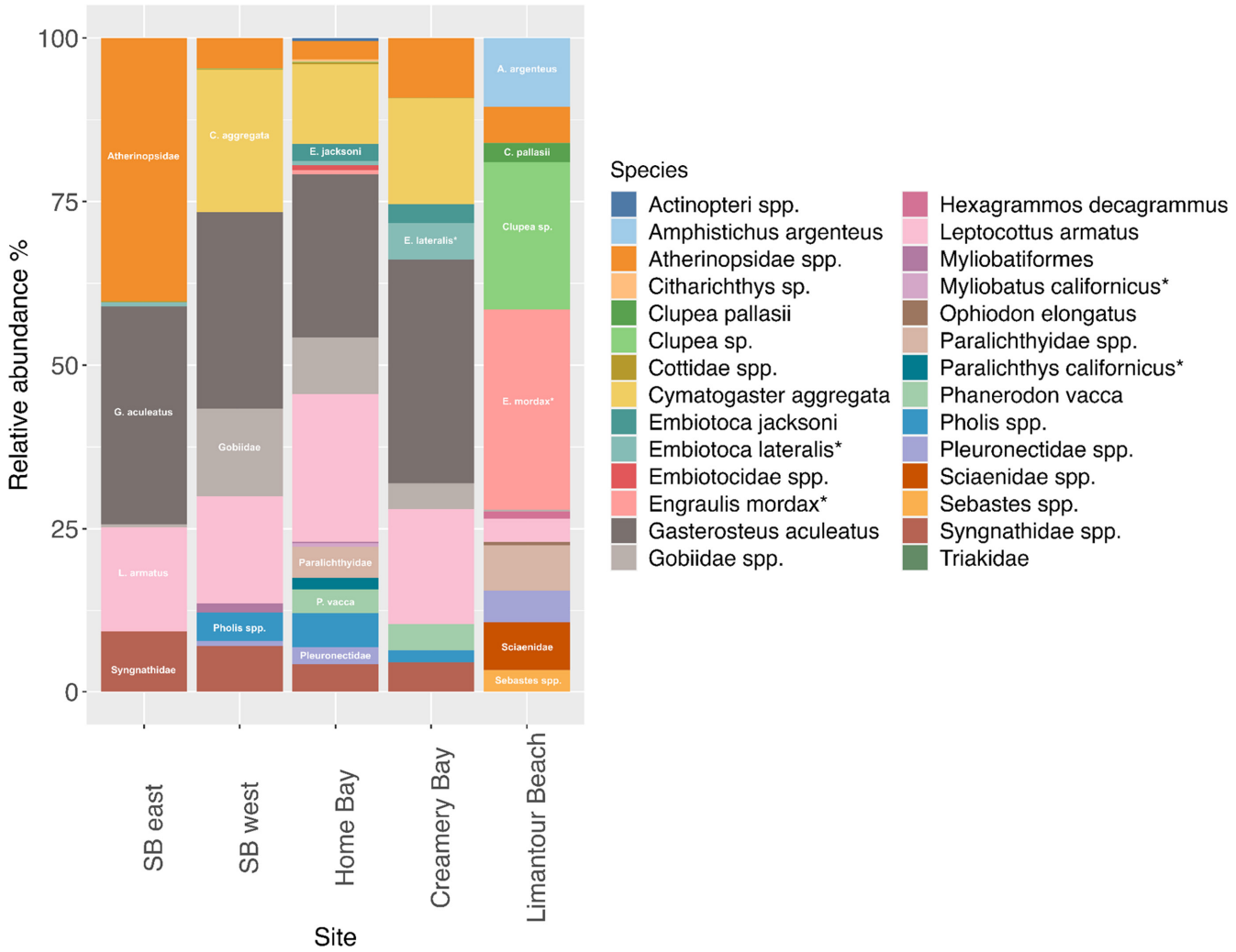

FIGURE 4 Mean relative abundances of fish taxa ($n = 7$–10 samples per site) from eDNA surveys after rarefying tables to an even sequencing depth for all samples. All sites occur within Drakes Estero, except for Limantour Beach, an open ocean coastal site.

estuary with greater sampling effort. However, our current ability to characterize which species these OTUs represent is limited. We show accumulation curves of unique taxonomic groups assigned to OTUs within each site in the estuary (Figure 8a, $n = 10$ per site) and across all sites in the estuary (n = 40, Figure 8b). While the maximum number of OTUs that occur within Drakes Estero is 110 ($n = 40$, non-rarefied data, Figure 7b), only 22 unique taxonomic groups could be identified ($n = 40$, Figure 8b).

4 | DISCUSSION

We characterized fish biodiversity within eelgrass meadows of Drakes Estero using the recently developed MiFish primers that target elasmobranch and teleost fishes (Miya et al., 2015) and we generally found support for the use of this tool to survey fishes in our region. Within Drakes Estero we identified 9 species and observed similar common species from our eDNA surveys as those from non-eDNA surveys. Our literature search identified 106 fishes in our regional species pool. Of these, 42% lacked representative sequences for the MiFish target of the 12S rRNA gene in the reference database limiting our ability to make species-level assignments. However, after clustering sequences into operational taxonomic units (OTUs) of 97% similarity, we identified 110 OTUs within Drakes Estero. This number is similar to the regional species pool, suggesting that we captured a high amount of the regional diversity with our surveys. Further, we observed differences in alpha diversity among sites and differences in fish community composition between our estuarine and non-estuarine sites that are representative of these habitats. We discuss these findings in the context of the growing literature of fish eDNA studies and as they relate to our local and regional knowledge of eelgrass fishes.

We identified similar common species in both eDNA and non-eDNA surveys, but misassignments to fishes not found in our region mean that careful review and curation of eDNA results are necessary. Across all samples, we identified 13 species and 15 additional unique taxonomic groups with lower taxonomic resolution (genus to class). These 13 species are known to occur in eelgrass estuaries in

the Northeast Pacific. However, several OTUs were misidentified at 97% similarity to either a species (*Clupea harengus*) or genus (*Cottus sp.*, no species name provided) not known to occur in eelgrass beds in the Northeast Pacific. *Clupea harengus* is a congener of a local species, *Clupea pallasii*, which is present in the reference database and identified in our eDNA samples, so it is unclear why OTUs were not assigned to the local species. Similarly, *Cottus*, is a genus that is not expected to occur in a primarily oceanic estuary like Drakes Estero but was identified in our samples with 97% similarity. The nearest relative of this genus within the family Cottidae found in our samples is the Pacific staghorn sculpin (*Leptocottus armatus*; Rabosky et al., 2018). Miya et al. (2015) noted that the MiFish primers do not discriminate between some closely related congeners, and this may be the reason for the observed misidentifications in our study.

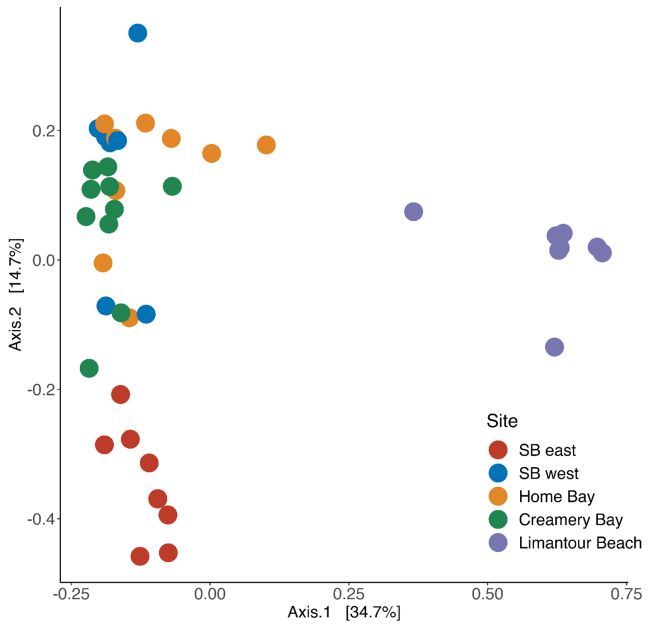

FIGURE 5 Principal coordinate analysis of fish communities from eDNA surveys across sites ($n = 7$–10 per site). Bray Curtis dissimilarity was calculated on rarefied OTU tables clustered at 97% similarity prior to ordination. All sites occur within Drakes Estero, except for Limantour Beach, an open ocean coastal site. Each data point represents a sample.

Creating a region-specific reference database can help prevent misidentifications to non-resident taxa (Gold et al., 2021). However, this comes with the cost of reduced sensitivity (Gold et al., 2021), for example, the inability to detect new range expansions, invasions, or species yet to be identified due to their transient use of the habitat. Despite these challenges, we identified some of the most common species in Drakes Estero from non-eDNA surveys, like the three-spined stickleback, Pacific staghorn sculpin, and the shiner surfperch (Wechsler, 2005) as common in our eDNA surveys. This supports the potential of eDNA surveys to provide similar pictures of fish community composition for eelgrass habitats as those from non-eDNA survey approaches.

A well-populated reference database is necessary for characterizing biodiversity from eDNA surveys. Thus, to identify which eelgrass fishes from our regional species pool lack representative sequences we queried the reference database using the mitohelper tool (Lim & Thompson, 2021). Of the 106 regional species, 44 lacked reference sequences and 9 of these are known to occur in Drakes Estero from non-eDNA surveys. Thus, 58% of fishes known to occur in eelgrass estuaries from Elkhorn Slough to Humboldt Bay and 70% of fishes known to occur in Drakes Estero have representative sequences in the reference database. This is considerably lower than for the California Current Ecosystem (CCE), where 93% of common fishes have representative sequences following recent efforts to populate the reference database for resident taxa (Gold et al., 2021). This suggests that eDNA biomonitoring programs of eelgrass ecosystems in California would benefit from collecting voucher specimens and generating reference sequences for the newly developed MiFish primers. Indeed, overlap in habitat use by fishes in nearshore estuaries and the CCE and recent efforts to generate reference sequences for CCE fishes by Gold et al. (2021) may be the reason why our regional species pool has higher reference sequence representation (58%) than the global average (24%) for the MiFish target (Miya et al., 2020). For fishes that lack representative sequences, using knowledge of the local or regional species pool may allow for curation of OTUs within eDNA datasets. For example, if sequences are identified with high similarity to a genus with only one known local species, manual annotation may be possible. As representative sequences are added to the reference database, the usefulness of

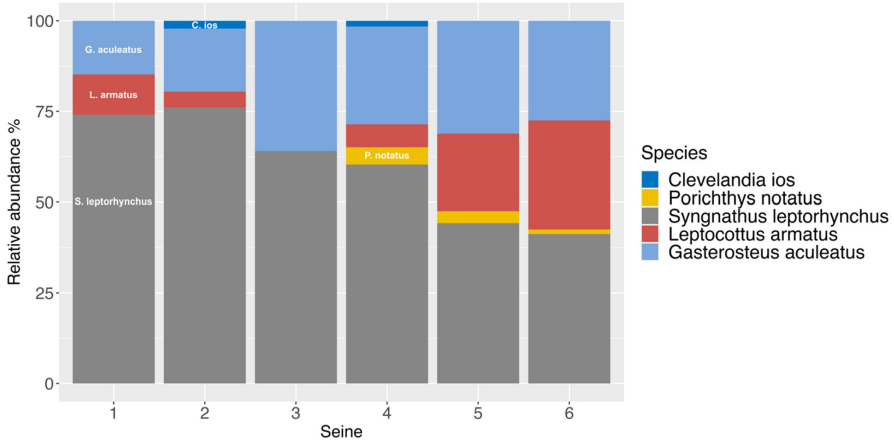

FIGURE 6 Relative abundances of fishes caught in six seine surveys at SB east.

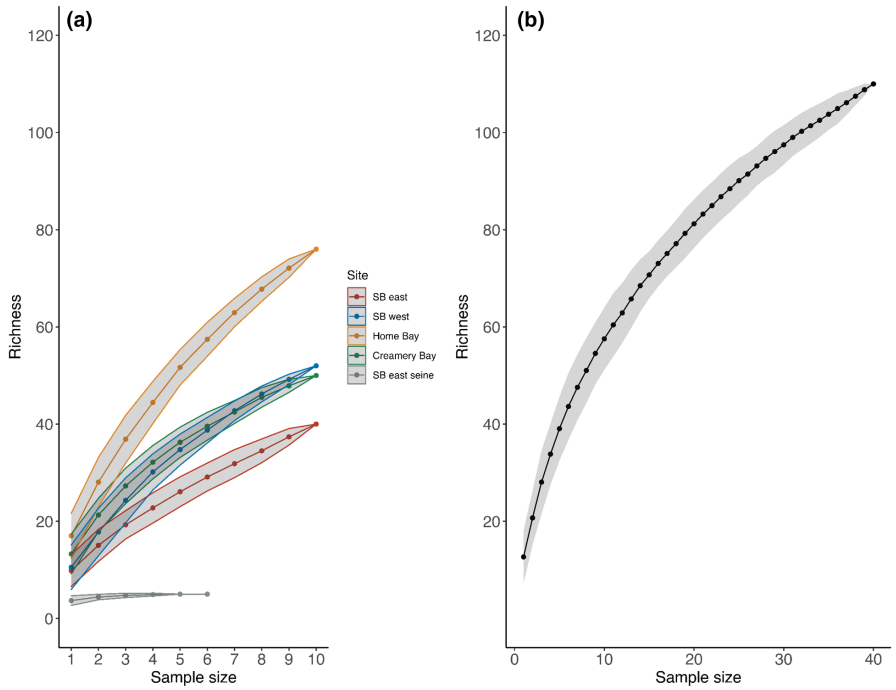

FIGURE 7 OTU (eDNA surveys) and species (seine surveys) richness increases as sample size increases within the estuary. (a) Mean richness (± standard deviation) in eDNA surveys per site ($n = 10$, SB east, SB west, Home Bay, and Creamery Bay, non-rarefied data), and in seine surveys ($n = 6$, SB east seine). (b) Mean richness (± standard deviation) across all eDNA surveys within the estuary ($n = 40$, non-rarefied data).

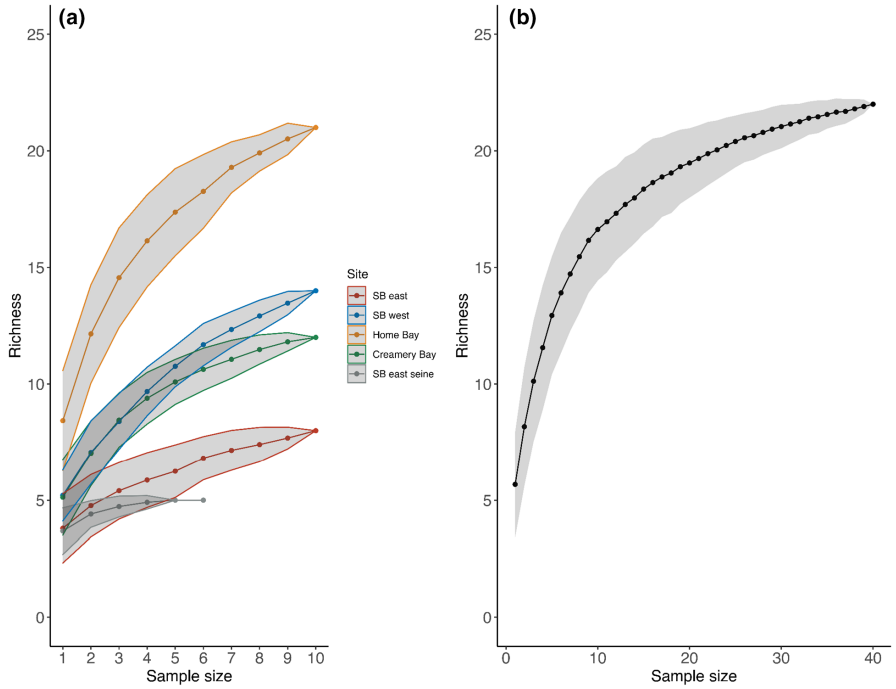

FIGURE 8 Taxonomic (eDNA surveys) and species (seine surveys) richness increases with sample size. Taxonomic richness of eDNA surveys represent unique taxonomic groups at the highest resolution available (i.e., species when available, otherwise, genus, family etc.). (a) Mean richness (± standard deviation) in eDNA surveys per site ($n = 10$, SB east, SB west, Home Bay, and Creamery Bay, non-rarefied data), and in seine surveys ($n = 6$, SB east seine). (b) Mean richness (± standard deviation) across all eDNA surveys within the estuary ($n = 40$, non-rarefied data).

this tool will improve (Fernández et al., 2021; Gold et al., 2021; Oka et al., 2021). However, even with additional reference sequences for the MiFish target of the 12S rRNA gene, some taxonomic groups may not be resolved to the species level (Gold et al., 2021; Miya et al., 2015; Miya et al., 2020). For example, the MiFish target may not capture enough variation to distinguish rapidly evolving species, like those observed in the adaptive radiation of rockfishes, genus *Sebastes* (Gold et al., 2021; Miya et al., 2020). Indeed, we observed incongruencies among some of the rapidly evolving Embiotocidae fishes in our phylogenetic tree, suggesting that some groups of Embiotocid fishes may be difficult to resolve with the MiFish locus. Alternatively, these phylogenetic incongruencies could be due to incorrectly annotated reference sequences or the need for taxonomic revision within this group, as others have recently suggested (Longo & Bernardi, 2015). In cases where congeners cannot be resolved with the MiFish locus, developing species-specific primers (Brandl et al., 2014; Miya et al., 2020) for a more targeted monitoring approach may be useful.

Recent computational developments may allow for the characterization of both intra- and inter-specific diversity in eDNA studies. For example, denoising removes sequences of non-biological origin and allows researchers to resolve single nucleotide differences or

sequence variants within sequence data (Callahan et al., 2016; Miya et al., 2020). Single nucleotide variation can capture species diversity and within species (intraspecific) genetic diversity (Sigsgaard et al., 2016; Tsuji et al., 2020). However, OTU clustering can still be useful in cases like ours where reference databases are underpopulated for a given survey area (Closek et al., 2019; Deiner et al., 2017; Oka et al., 2021). For example, in initial exploratory analyses, we identified six fish species within the estuary across 582 sequences, with the number of sequence variants per species ranging from 2 to 373. Thus, given sufficient sequencing depth, both inter- and intraspecific diversity can be uncovered from sequence data generated with the MiFish primers. This is consistent with recent findings that fish intraspecific genetic diversity from single nucleotide variation can be uncovered from eDNA studies with the use of denoiser computational tools (Sigsgaard et al., 2016; Tsuji et al., 2020). However, a well populated reference database is needed to disentangle intra- and inter-specific diversity within eDNA sequence data and to provide estimates of species richness. Further, many reads within the MitoFish reference database align to regions of the 12S rRNA gene that are outside of the area targeted by the Mifish primers (Gold et al., 2021 and observed in our study). Indeed, we found 42% of fishes from our regional species pool lacked reference sequences for the MiFish target and this is similar to the percentage of estuarine sequences (52%) which could not be assigned species labels. This means we are unable to disentangle species intra- versus inter-specific diversity for the remaining 48% of sequence data from Drakes Estero. Thus, until the reference database is better populated with region-specific species for the MiFish target, OTU clustering is a useful approach to estimate of species richness and composition, especially when species-level taxonomic assignments cannot be resolved (Deiner et al., 2017).

Despite uncertainty regarding some taxonomic assignments, there are notable advantages of eDNA surveys. We identified greater fish diversity with eDNA surveys compared to seine surveys at our SB east site within the estuary, even after considering differences in sampling effort. This indicates that eDNA surveys offer an advantage to traditional methods in enumerating fish diversity by catching additional species and potentially fishes of larger size classes that are missed by seining efforts. For example, fishes caught in our seines were 20 cm or smaller in standard length. Thus, our seines may miss larger size classes of fishes or certain species like elasmobranch fishes that would likely evade seines. We also found fish species from our seine surveys present in our eDNA surveys, albeit at lower taxonomic resolution for some species, except for plainfin midshipman (family Batrachoididae) and arrow goby (family Oxudercidae). However, we only caught two arrow gobies across six seines at SB east, which is far fewer individuals than caught for other species like the three-spined stickleback, Pacific staghorn sculpin, and bay pipefish. Thus, low abundance of arrow gobies may have contributed to the lack of detection of this species in our eDNA surveys. Additionally, plainfin midshipman lacks reference sequences in the database. Therefore, identification of this species with eDNA is not possible without adding reference sequences to the database. However, for remaining species, eDNA surveys captured phylogenetically similar or the same fish species (like the three-spined stickleback, *Gasterosteus aculeatus*, and the Pacific staghorn sculpin, *Leptocottus armatus*) to those caught in our seines, as well as new species missed by seining efforts. When we included earlier fish surveys in Drakes Estero from our literature review and combined these with our seine surveys, we found a total of 30 unique species documented within Drakes Estero in the last 20 years. We identified 7 of these 30 species in our eDNA surveys from June 2021, with 17 of the remaining species represented at lower levels of resolution (genus or family) in our eDNA surveys. Further, some of the most abundant fishes from an earlier study in Drakes Estero (Wechsler, 2005) including topmelt, three-spined stickleback, Pacific staghorn sculpin, bay pipefish, and shiner perch are common in our eDNA surveys. While bay pipefish and topsmelt are not represented at the species level, we did identify their family groups (Syngnathidae and Atherinopsidae) in our eDNA surveys. In total, we identified 22 unique taxonomic groups (from species to class level assignments) from 110 OTUs within Drakes Estero from our eDNA surveys. This further supports that eDNA has the potential to characterize higher fish biodiversity than traditional methods, especially as reference databases become better populated with reference sequences.

Interestingly, we observed differences in beta diversity (community composition) across sites within the estuary and between the estuary and our non-estuary site. We found different fish communities between sites near the center of the estuary (at the opening of Creamery, Schooner, and Home Bay) compared to the site at the very narrow head of Schooner Bay. This may reflect differences in abiotic conditions (e.g., water temperature, water mixing, or retention) and bathymetry of these sites. However, given that our spatial coverage of samples at SB east was lower than at sites in the center of the estuary, lower diversity at SB east may also be due to this reduced spatial coverage of sampling. Fish species composition within the estuary is notably different from that just outside the estuary at our open coastal site, Limantour Beach, less than 10 km away. This indicates that eDNA surveys are a viable method to characterize species found within the estuary and to discriminate estuarine sites from non-estuarine sites. For example, species like anchovy and herring that are common to (and detected at high-relative abundances by eDNA surveys at) Limantour Beach, were not common in the estuary eDNA surveys (detected in only 1 and 2 samples at 6% and ≤1% relative abundance, respectively). This is consistent with non-eDNA surveys showing that anchovy and herring are rare community members within the estuary despite year-round surveys (Wechsler, 2005). This suggests that fish communities described with eDNA surveys within Drake Estero are reflective of eelgrass fish diversity and not simply cellular DNA washed into the estuary from outside of the estuary at high tide. Water residence time within the estuary, although unknown, may be longer due to the narrow opening of the estuary and distance from this opening to each bay within Drakes Estero. Ultraviolet radiation and warmer temperatures are associated with higher rates of eDNA degradation (Harrison et al., 2019;

Nagarajan et al., 2022). Given that our sites are in the cooler temperate region of California, eDNA may degrade more slowly here compared to eelgrass estuaries in warmer regions. However, collection of only cellular or organelle-housed DNA (not free DNA that would pass through the 0.22 uM filters) in our surveys, may have limited the amount of non-estuarine species caught in our estuarine eDNA surveys (see Nagarajan et al., 2022 for discussion of estuarine eDNA transport and degradation rates). Our findings are similar to other eDNA fish surveys that show differences between sites and habitat types in marine and estuarine systems (He et al., 2022; Oka et al., 2021).

Species accumulation curves provide a sense of the adequacy of our sampling effort in detecting differences among sites and in capturing the full fish diversity of the estuary. The highest rate of increase in species richness (OTU richness) with sampling effort occurred with the first 10–20 samples within Drakes Estero. However, even at 40 samples the slope of the OTU richness accumulation curve did not level off, suggesting that further sampling would recover additional low abundance or rare OTUs. Nonetheless, we did detect some species known from these estuaries at low-relative abundance, especially sharks and rays. We identified six OTUs of Chondrichthyes (cartilaginous fish, including one Triakid shark, two Myliobatiformes, and three Myliobatidae, likely *Myliobatus californicus*, the only Myliobatidae species found in the Northeast Pacific), but these occurred at lower relative abundances and in fewer samples compared to teleost fish. Additional sampling effort may allow for a better understanding of their occurrence and distribution throughout space and time.

In summary, eDNA surveys with the 12S MiFish primers prove a useful tool for characterizing fish diversity in eelgrass beds in the Northeast Pacific. In describing diversity and species relative abundances it is likely superior to non-eDNA techniques, but the inability to assess size structure or absolute abundances of fishes will mean that traditional non-eDNA survey methods will be needed for some applications. Nevertheless, eDNA surveys currently capture a large fraction of the fish diversity in eelgrass beds and targeted efforts to add common species to the reference database will increase our ability to characterize fish diversity not only in Drakes Estero but also more broadly in the Northeast Pacific.

AUTHOR CONTRIBUTIONS

DSB and JJS designed the experiment. DSB, ED, and CG collected samples and seined for fish. DSB performed DNA extractions and ED prepared samples for sequencing with guidance from DSB. DSB performed all bioinformatic and statistical analyses. DSB and ED made figures for the manuscript. ED performed the literature review with input from DSB, CG, and JJS. DSB wrote the manuscript with input from ED, CG, and JJS.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

We deposited raw fastq sequencing files at the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under bioproject number PRJNA877692. Code for this project is archived at https://doi.org/10.5281/zenodo.7901641.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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