BIODIVERSITY OF ESTUARINE SPECIES: COMPARING MULTI-DEPTH EDNA SAMPLING TO FOUR TRADITIONAL SAMPLING GEARS

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Abstract

Estuaries are physically and biologically complex habitats which demand higher sampling efforts that either freshwater or marine systems to fully document aquatic species and understand ecological relationships. A lack in sampling effort for estuarine biota is typical which may lead to negligent resource management decisions and a limited view of estuarine systems. Environmental DNA (eDNA) metabarcoding is a developing method used to detect multiple taxonomic groups in a single water sample, reshaping biodiversity surveys in aquatic systems. However, sampling methodologies for eDNA surveying in estuaries and their operational constraints remain ambiguous. Previous comparative studies examined solely surface water eDNA samples with a single traditional sampling gear. These studies did not address the need for more diverse traditional sampling methods and the additional possible bias of taking only surface samples in relatively physiochemically complex water columns, e.g., salt wedges. With an aim to provide reliable estimates of biodiversity and best practices of eDNA surveying methodologies in estuaries, this study compared four manual sampling gears, i.e., underwater baited camera, cast net, minnow trap, and angling, to multi-depth eDNA sampling using two targeted genetic regions (COI, 12S) across five stream mouths of the Pearl Harbor estuary on the island of O‘ahu in the Hawaiian Archipelago. In doing so, this study compared alpha and beta biodiversity estimates between methods, identified unique and overlapping species of each sampling component, measured the agreement of species detected between methods, and assessed the need to take eDNA samples at multiple depths. This study found that sampling multiple locations in an estuarine water column offers a more efficient means of capturing rare species than extended sampling at surface waters. Both traditional and eDNA methods revealed similar spatial and temporal patterns of species composition between surveys in Pearl Harbor but had conflicting views of total alpha diversity for stream sites. Inverse relationships for streams between methods were due to differences in each method’s ability to capture species richness, rare species, and dominant species, affecting computations of diversity. The exclusive use of one method can give incomplete views of species composition and estimates of diversity, which can alter management perspectives of estuarine ecosystems.

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1.0 Introduction

Estuaries demand greater sampling efforts to describe generalities concerning pattern and process as well as to provide the taxonomic coverage required in these biodiversity hotspots (Butler et al., 2001; Rotherham et al., 2007; A. D. Jassbya, B. E. Col Jassby; Livingston 1987). The spatial and temporal variability of freshwater input, tide, and sediment deposition result in tremendous habitat heterogeneity on a range of scales, making the development of standardized survey designs challenging (Livingston, 1987). Water chemistry which influences vertical and horizontal gradients found within estuaries can change over the course of a single day (R.S.K. Barnes, 1974; Perkins 1974; Livingston, 1987). Despite recommendations to use multiple stationary and mobile sampling methods (henceforth termed "gears") to capture species that differ in behaviors and depth, most monitoring efforts rely on a single or paired traditional gear type (Rozas & Minello, 1997; Butler et al., 2001.; Allen et al., 2006; Ficetola et al., 2007; Rotherham et al., 2007;). Currently, experts in traditional taxonomic identification are becoming antiquated in the field of systematics (Sluys, 2013; Padial & De La Riva, 2010). For all above reasons, inventorying freshwater, brackish, and marine species is more difficult to complete for natural resource managers and long-term monitoring programs that track invasive species. If continued to go unchecked, the lack of sampling design and sampling effort that reflects the heterogeneity of estuaries may lead to negligent resource management (Estuarine Pollution Control and Assessment, 1974; Livingston 1987; Rotherham et al., 2007a).

The sampling effort required for effectively depicting estuarine biodiversity may be reduced with new metagenomic techniques and knowledge. As recent as the early 2000s, aquatic scientists have been detecting the presence of freshwater species using environmental DNA (eDNA; Ficetola et al.,2007, Deiner et al., 2017; Rees et al., 2014b; Schwartz et al., 2017; Stoeckle et al., 2017). Specific species or compositions of entire communities can potentially be identified in a single water sample (Foote et al., 2012; Pilliod et al., 2013; Rees et al., 2014b; Forsström & Vasemägi, 2016; Keskin et al., 2016; Kelly et al., 2017; Stat et al., 2017; Stoeckle et al., 2017; Boussarie et al., 2018; Li et al., 2018). Such a tool has changed how biodiversity is viewed and recorded in institutions (Sluys, 2013; Padial & De La Riva, 2010). However, the reliability of eDNA surveys depend on the ability to capture and amplify DNA deposited from organisms that occur in the area of question (Goldberg et al., 2016; Stoeckle et al., 2017). This includes validating sequencing pipelines, genetic markers, minimizing contamination, collecting representative samples, amplifying DNA, and building locally trustworthy DNA references (Goldberg et al., 2016).

Environmental DNA surveying has shown to be a promising biological assessment method, outperforming traditional methods in species richness, and having strong correlations with species biomass (Maruyama et al., 2014; Goldberg et al., 2016; Zou et al., 2020; Yates et al.,2020). However, degradation and production of DNA material is found to vary within and between species and amongst water bodies (Goldberg et al., 2016; Seymour et al., 2018; Garlapati et al., 2019; Jo et al., 2019; Harper et al., 2020; Jeunen et al., 2020; Zhang et al., 2020). Thus, the development of environmental DNA methods continues to be an area of research to clarify differences in the behavior of eDNA for various aquatic environments with growing investigation of environmental inhibitors in specific habitats (Hunter et al., 2019) and species dependent and independent relationships related to eDNA shedding (Maruyama et al., 2014; Yates et al.,2020). When used in management, collaborative efforts with local experts and

biologists are needed to discern true or relevant species in the eDNA data set. Such collaboration helps identify certain phyla that may be under sampled or inaccurately represented in the DNA reference library used. Biological review of the species detected is currently a continuing process for natural resource managers when using eDNA as a surveying practice.

Cross method comparisons between traditional and eDNA surveying practices have allowed scientists to validate eDNA as a surveying tool. Method improvements for eDNA will hopefully give practitioners the confidence in using eDNA surveying as a practical tool that is faster, accurate, and cheaper in the long run. Comparative studies have helped validate genetic primers, identify false negatives, and assess species biases between methods (Kelly et al., 2017; Zou et al., 2020; Rees et al., 2014a; Mächler et al., 2019; Evans et al., 2017; Shaw et al., 2016). However, current method comparisons performed in estuaries have relied on surface water eDNA samples, at times a single primer, as well as one traditional gear type for species confirmation and comparison (Kelly et al., 2017; Zou et al., 2020). With taking only surface water samples, these studies did not consider differences in detection of DNA material that may vary due to physiochemical mixing that shifts with depth in estuary water columns as well as organisms that primarily dwell on the benthic floor (Harper et al., 2019). Like the biophysical stratification in lakes and ponds, it is recommended for heterogeneous waterbodies to be sampled for at different depths in order to maximize species detection (Handley et al., 2019; Harper et al., 2019; Hänfling et al., 2016). Currently there is not a standard practice for multi-depth sampling in eDNA sampling for estuaries. In addition, current method comparisons between eDNA sampling and traditional gears are limited by the use of a single traditional gear type which may have also contributed to fewer overlapping species found between methods.

Environmental DNA surveying may provide a more practical means of standardizing rapid biological assessments for large complex systems such as estuaries (Deiner et al.,2017; Schwartz et al., 2017). To effectively assess the strength of eDNA surveying in estuaries, this study compares sampling efforts between a multi-depth and multi-primer eDNA sampling scheme and a manual biological survey sampling scheme using four traditionally used sampling gears, i.e., benthic camera, cast net, minnow traps, and angling. We aimed at sampling multiple water columns across an estuary stream mouth. In doing so, this study (1) examined differences in alpha diversity (Whittaker et al., 2001) between methods, (2) identifies unique contributions for each eDNA and traditional method component, (3) measures the agreement of species in a given survey between methods, (4) and investigates the need to take eDNA samples at multiple depths, i.e., surface, center, and benthic, and positions, i.e., middle column or stream bank, for efficient collection standards. This study aimed at answering the following questions:

1. Does incorporating multiple traditional sampling gears and sampling fish eDNA at multiple depths provide equivalent conclusions of biodiversity? (i.e., Can eDNA surveying detect all the species traditional gears detect when incorporating more detection methods and sampling locations?)

2. Does sampling eDNA at different depths and different positions of a stream channel cross-section offer different compositions of species compared to just collecting surface samples?

2.0 Methods

2.1 Study site

Located on the south shore of O‘ahu , Hawai′i, the Pearl Harbor estuary receives an estimated 40% of O`ahu’s surface water from drainage of approximately 285 km^2 of watershed (Coles et al., 2009). This 20-km^2 coastal plain estuary has three main lochs which are surrounded by four cities, as well as Joint Base Pearl Harbor Hickam (JBPHH; Figure1). For purposes of this study, Waikele, E’o, Waiawa, Kalauao, and Hālawa streams were surveyed seven times between October 2018-July 2020 (Table 1). This consisted of three wet and four dry survey events (Figure 2). Sites were selected using diversity data from an original baseline report (Englund et al.,2000) and needs of JBPHH. Each downstream channel where surveying took place is characterized as box shaped with a mean (±SD) width of 3.0 m ± 0.53 m, depth of 2.20 ± 0.92 m, and width depth ratio of 1.64 m, similar to the width-depth ratio metric used to categorize stream type categories based on Rosgen stream classification (Rosgen,1994). The average depth, temperature, salinity, dissolved oxygen, and conductivity sampled for each stream in this survey can be seen in Table 2. Invasive red mangrove (*Rhizophora mangle*) lined stream banks in thickets. Sediments sampled within 10-cm depth at downstream sites in year one sampling events were fine, resembling silt in character with mangrove detritus or mixed with crushed calcium carbonate deposits from mollusks.

2.2 Sample collection

For each survey event, four traditional sampling gears were deployed, and seven eDNA water samples were collected about a fixed transect line extending the total width of each stream-mouth (Figure 3-5). Underwater cameras, cast netting, angling, and minnow trapping gears were selected based on their ability to capture fish and invertebrates of various sizes, behaviors, and habitat usages within a water column (Steele et al., 2006; Lowry et al., 2011; Stein et al., 2014; Kuriyama et al., 2019; Schemmel et al., 2019;). At each transect site on each sampling date, all sampling methods were performed within the same 24-hour period. Methods were conducted in sequence to avoid fish deterrence. For instance, cast netting was prioritized approaching a stream site before other field methods or time spent at a site disturbed waters and fish presence. A Sony© Digital HDR-AS50 Action Camera (Sony Corp., Tokyo Japan) attached to a ruler and baited with chunks of raw market Tuna was placed within 100 m of the transect line on the benthic floor (Table 3, Figure 6). Cameras faced downstream and recorded two hours of underwater video footage. Turbidity, depth, and cloud cover were recorded at each fixed camera location. Video footage was later transcribed for each site. The number of species observed in videos and their size range was estimated to the nearest inch. Cast net sampling along each transect was performed within a 100 m range from each site’s fixed transect line using a pre-sanitized monofilament cast net with a 4.87-m diameter and a 0.64-cm mesh size. Six throws per site were deemed efficient to maximize species richness given time restraints in the field through species accumulation estimates from preliminary data obtained in survey 1 (PH1) (Schemmel et al., 2019). At least three throws were made on each left and right streambank. Blind throws were made when fish activity was absent during surveys, or turbidity prevented any visual indication of present fish. For each throw, the opening of the net was recorded in a category of either being >%75 or <%75 of the total opening of the cast net. Species caught, count per species, and size range estimated to the nearest 1 cm was also recorded for each survey. Cast net deployment standardization (Stein et al.,2014) was pre-calculated using SketchAndCalc™ (ICalc Inc., Flordia, USA). The cast net operator had a mean (±SD) throw of 9.50 ± 2.60 m2.

Environmental DNA water samples were then collected using equipment pre-sterilized with 10% bleach solution. To compare eDNA sampling locations in an estuarine water column, three 500-ml water samples and four 250-ml water samples were taken using a horizontal Niskin bottle. At the right and left bank, a single 250 ml of water was sampled for 30.5 cm (1 ft) below the surface and 30.5 cm (1 ft) above the benthic floor. Surface and benthic samples taken from the left bank were later combined with samples taken on the right bank to create a single 500-ml surface bank and a 500-ml benthic bank sample, respectively. At the middle of the transect line, three 500 ml of water was collected; one 500-ml sample at 30.5 cm (1 ft) below the surface, one 500-ml sample at half the total depth, and one 500-ml sample 30.5 cm (1 ft) above the benthic floor (Figure 5). Water samples were retaken if water was turbid or contained any avoidable debris or substrate. Stream sites with insufficient sampling depth may incorporate water samples collected at or near the same location. Only after being sanitized with 10% bleach solution and wiped with clean paper towels were sample bottles promptly placed on ice until filtering later the same evening. A field control or Nalgene bottle containing 500-ml of deionized water was kept in the same cooler throughout field sampling for each eDNA collection day.

At the middle of the transect line, flow rate, temperature, salinity, PH, dissolved oxygen, and turbidity were recorded at three different depths (i.e., surface, center, and benthic) using a freshly calibrated Marsh McBirney flow meter and YSI-Pro DSS. Flow and water chemistry measurements were taken at the same depths and location middle column environmental DNA samples were taken (Figure 5). Physiochemical characteristics corresponding to the surface, center, and benthic water columns in which eDNA samples were collected were averaged across all quarterly sampling events for each stream site. Before leaving the transect, an 80-cm long cylindrical minnow trap with a 6 cm opening was placed on the left and right bank of the transect while a 45 cm x 41 cm x 20 cm square minnow trap with a 4 cm opening was placed at center (Figure 5). Traps laid flat on the benthic floor with trap openings facing downstream. Traps were left overnight and retrieved the following day roughly 20 hours after deployment. Species caught, length of species, and trap location was recorded for captured species.

The last gear type deployed at each site included 60 minutes of angling within 100 meters of the horizontal transect line. Two fishers used a hook and line to angle fish with live fish caught from cast netting or frozen store-bought shrimp. Fish species used for live bait primarily included Goldspot Herring (*Herklotsichthys quadrimaculatus*), Kanda Mullet (*Moolgarda engeli),* and or the Hawaiian Anchovy (*Encrasicholina purpurea*). Live bait ranged from 7 cm-14 cm in size. J-hooks or circle hooks between 8-10 in size were typically used. Number of fishers and poles, fishing duration, species caught, catch length, and time caught was recorded.

2.3 eDNA Filtering and Sample Preparation

At the end of each eDNA collection day, field controls and each stream site's 500 ml aliquot eDNA samples were separately vacuum filtered through a 300-ml polypropylene filtering cup attached to a single 0.45-µm membrane disc filter (Figure 7; Turner et al., 2014). The 250-ml surface bank and benthic bank samples collected from the left and right bank of each stream site were homogeneously combined onto a single filtering cup. Once filtered, the membrane disc filters containing DNA were placed into a 2-ml microcentrifuge tube containing 700 µl of

Longmire's buffer solution and stored at room temperature until further processing (Renshaw et al., 2015; Longmire et al., 1997).

2.4 Positive and Negative Controls

A positive control and two types of negative controls were included to monitor contamination (Olds et al., 2016). A mock community sample which includes a 500-ml water sample collected from aquaria species that do not occur in Hawai'i was processed alongside Pearl Harbor eDNA samples to monitor contamination as a control for lab handling steps. A list of species in the mock community sample and the primer (assay) used to indicate contaminant presence is listed in Table 4. Two negative quality control procedures included 500 ml of sterile water (i.e. field blanks; usually 3 or 4 each survey event) and a PCR negative control containing sterile water in place of template DNA. Field blanks were vacuum filtered and prepped for Illumina sequencing alongside Pearl Harbor eDNA samples to cover field and lab contamination. However, DNA extraction for field controls were performed independently of Pearl Harbor samples.

The presence of reads from mock community species in the Pearl Harbor eDNA samples or reads from Pearl Harbor species in the mock community library was evaluated through assay-specific contamination incidents (Olds et al., 2016). The incidence rate of contamination was calculated by dividing the greatest number of contaminant reads in a single sample by the total number of reads produced for that contaminant species in the mock community sample, to produce a threshold. This incidence level was then used as a threshold with all read numbers detected for a given sample. Species detected in a sample with read numbers ≤ contamination incidence rates were removed from individual sample libraries. For the negative controls, assay-specific OTU detections were removed from all corresponding eDNA samples. For example, OTUs detected in a negative PCR control led to the removal of that OTU from the entire sequencing run, while detections from negative field controls led to the removal of OTUs from the eDNA samples tied to the individual field blank.

2.5 DNA Extraction, PCR, and Library Preparation

DNA extraction, primer amplification (PCR), and library preparation were performed in the OLC Annex at the Oceanic Institute of Hawai'i Pacific University (OI-HPU) and followed eDNA processing methods of Renshaw et al. (2015) and Li et al. (2018; Figures 8- 9). First, DNA was extracted from filters using Phenol-Chloroform-Isoamyl alcohol (PCI, 25:24:1) according to Renshaw et al.,2015. Potential inhibitors in the DNA extractions were removed using OneStep™ PCR Inhibitor Removal Kits (Zymo Research, California). Then, each site's five samples, their field controls, as well as a mock community sample and a single PCR negative control (described above) were PCR amplified for four gene regions (i.e., mitochondrial and nuclear) (Table 5). The first round of PCR was run for each sample and assay combination to amplify targeted fragments from eDNA filter extracts and add part of the Illumina adaptor sequence. PCR products from the first round of PCR were cleaned with Mag-Bind® TotalPure NGS (Omega Bio-Tek Inc, Georgia) magnetic beads at a ratio of 1(beads):1(DNA) and following the manufacturer's recommendations. A second round of PCR used the PCR products from the first round of PCR as a template to add library-specific dual-indexes to uniquely identify libraries for Illumina sequencing (Li et al., 2018; Olds et al., 2016). PCR products from the second round of PCR were cleaned with Mag-Bind® TotalPure NGS (Omega Bio-Tek Inc, Georgia) magnetic beads at a ratio of 0.8(beads):1(DNA) and following the manufacturer's recommendations. DNA concentrations for each library were then quantified with the Qubit dsDNA HS Assay (Life

Technologies, California). Libraries were pooled to equal molar concentrations along with 25% PhiX DNA (v3, Illumina, California). Then 300bp paired-ends were sequenced on a single Illumina MiSeq run for each pair of Pearl Harbor sampling events (i.e. 2 quarterly surveys) at the University of Notre Dame's Genomics and Bioinformatics Core Facility (http://genomics.nd.edu) with a MiSeq Reagent Kit v3 (600-cycle, Illumina, California).

2.6 Bioinformatics Pipeline

Trimmomatic version 0.32 (Bolger et al., 2014) was used to remove raw reads with >6bp of the read matching a MiSeq sequencing adaptor, raw reads with a Phred score lower than 20 within a 10bp sliding window, and raw reads shorter than 50bp in total length (Figure 9). A custom Perl script Demultiplex_primer _v1. 4.pl (available at https://github.com/pfrender-laboratory/epps), was used to remove reads without an exact match to either a forward or reverse primer sequence from either assay, trim primer sequences from the surviving reads, and finally demultiplex the reads into the four different assays (Figure 9). Following demultiplexing, the bioinformatics pipeline used steps outlined in Li et al., (2018) (Figure 9). USEARCH version 8.0.1623 (Edgar, 2010) was used to merge overlapping paired-end reads, discarding reads with expected errors >0.5 or ambiguous base pairs at any nucleotide site. Using the custom Perl script unique.pl (available at https://github.com/pfrender-laboratory/epps), identical merged reads were collapsed into a single unique read and the number of identical dereplicated reads was associated with the unique read (Table 6, Figure 9). USEARCH version 8.0.1623 (Edgar, 2010) was then used to remove singleton reads, denoise the data (remove chimera sequences), and cluster reads into Operational Taxonomic Units (OTUs) (Blaxter et al., 2005) based on 97% similarity. The abundance of reads in each OTU, as a combination of the number of unique and identical reads clustered within each OTU, was associated with each OTU. A reads table (number of reads per OTU per sample) and a fasta file of centroid sequences from each OTU was output.

Using the BLAST+ toolkit (Camacho et al., 2009), megablast was run locally to match OTU sequences to records in the nt_v5 database (Downloaded July 2020). The custom script taxid2lineage.py (Sarah, T.K., 2015) was used to match lineages (i.e. Kingdom, Phylum, Class, Order, Family, Genus, and Species) to taxid output from BLAST searches. All taxa assignments for OTUs were based on Query% and Identity% BLAST matches. A ≥ 99% Query% was used for all assays. The Identity% match varied by assay with the COI assays requiring a ≥97% match, while a ≥99% match was required for the 12S, 16S and 18S assays. OTUs were then subset for animal, invertebrate, and fish taxon for further analyses (Table 6). Metazoan taxa excluding class aves, amphibia, reptilia, and mammalia were retained for COI analyses. The 18s assay only included invertebrate taxa detected. Lastly, only class Actinopterygii and Chondrichthyes was retained for both the 12s and 16s assays.

3.0 Data Analysis

A total of eight surveys (Table 1) to include the use of four eDNA primers (Table 5) were conducted in this study. However, method comparisons between eDNA and traditional survey methods exclusively analyzed fish taxa detected at downstream sites using the 12s and COI primers (for eDNA methods) for surveys PH2-PH6. This is due to un-finalized sequencing results for surveys PH7 and PH8 as well as un-finalized sequencing results from the 16s and 18s assays across all surveys. The PH1 survey was exploratory and limited to surface water (vs.

multiple depths) eDNA sampling. Examinations of sampling effort, as well as generalizations of overall biodiversity in Pearl Harbor, used fish detections from all downstream surveys within quarters PH1-PH8 for traditional methods, and surveys PH2-PH6 for eDNA methods.

3.1 Sampling Effort

Examinations of sampling effort used fish detections from all downstream surveys PH1-PH8 for traditional methods, and surveys PH2-PH6 for eDNA methods. Sampling effort was calculated for traditional surveys using catch per unit effort (CPUE) for the number of fish species detected as well as individuals caught for each traditional gear type. Gears were compared using average CPUE values calculated from individual deployments of benthic cameras (i.e., 2 hrs per site), cast net (i.e., six casts per site), minnow traps (i.e., three per site), and angling (i.e., 90 mins per site) along downstream transects from each site visit in surveys PH1-PH8. Time spent for each gear given the realities of field work were used to generate the average time gears were deployed and calculate CPUE values.

The total number of reads surviving the bioinformatic pipeline and used to indicate species detections are summarized for each assay and quarterly sampling event in Table 6. The amount of sequenced DNA material which led to the detection of species, or number of reads per sample bottle and sequencing run, was used to generalize eDNA sampling efforts. The number of reads (i.e., after using Trimmomatic and before OTU generation using USEARCH) collected in each 500-ml eDNA water sample was combined for each assay (COI, 12s) and averaged across all sample bottles in a sequencing run. Average number of reads per sample bottle for a given sequencing run was then averaged across all sequencing runs to provide a final estimate of number of reads per sample bottle. In addition, proportions of reads per sample bottle to the total number of reads in a quarterly sequencing run were averaged across all sequencing runs to estimate the contribution of reads per sample bottle to the total number of reads analyzed across the study. The percent of reads that were detected from the COI and 12s primers were calculated for each sequencing run and averaged across all sequencing runs.

Species accumulation curves were used to evaluate the total sampling effort of the study as well as between eDNA sampling and each traditional surveying component (‘vegan’ package (Oksanen et al.2020) in R version 4.0.3 (2020; R Core Team, 2018). Curves illustrated the accumulation of newly detected fish species in five downstream transects (i.e., Waikele, E’o, Waiawa, Kalauao, Hālawa) over multiple quarterly survey events. Total sampling effort when eDNA and traditional methods are combined are analyzed using surveys PH2-PH6. Environmental DNA detections from surveys PH2-PH6 were used in an eDNA specific species accumulation curve. Four species accumulation curves were calculated for each traditional gear using detections in surveys PH1-PH8.

3.2 Overall Biodiversity Trends

Two Principal Coordinates Analysis (PCoA) using the ‘vegan’ package (Oksanen et al.2020; R Core Team, 2018; Kruskl & Wish, 1978; Whittaker et al., 2001, McCune & Grace, 2002, Knight 2016) were used to compare species compositions for individual survey events (i.e., sites, and

sampling dates) between traditional and eDNA sampling schemes. One PCoA ordinated differences of species composition through detections from traditional methods, another used eDNA data alone. Ordinations were used to indicate influences of sampling location, date, and sampling method on species composition. Positive and negative correlations between eDNA and traditional methods for alpha diversity values as well as richness and evenness components were assessed in scatter plots. Correlations for Shannon diversity (Shannon et al., 1950; Whittaker et al., 2001), richness, evenness, McNaughton's Index of Dominance, and precent index of singletons values for stream sites (i.e., Waikele, E'o, Waiawa, Kalauao and Halāwa stream) were assessed as a function of sampling method to establish relationships ('vegan' package, Oksanen et al.2020; R version 4.0.3, 2020, R Core Team, 2018).

3.3 Method Contribution and Species Agreement

Venn Diagrams were used to illustrate the number of unique and overlapping species detected between eDNA and traditional methods and each of their individual components over the entire study using the 'eulerr' package (Larson, 2019) in R version 4.0.3 (2020; R Core Team, 2018, Kelly et al., 2017). This included data from surveys PH1-PH8 for traditional methods and surveys PH2-PH6 for eDNA methods. Species agreement between eDNA and traditional sampling schemes for a given survey event was calculated using interrater Cohen's Kappa values (Cohen, 1960, 'irr' package (Gamer & Lemon, 2019) in R version 4.0.3 (2020; R Core Team 2018;)) for further resolution of whether eDNA sampling can detect the same species as traditional methods in situ (Harper et al., 2018, Schneider et al., 2016, McHugh, 2012). This 1:1 method comparison used 24 paired instances or surveys from surveys PH2-PH6 as some samples were lost due to field or lab inhibitions. Kappa values ≤ 0 indicate no agreement and 0.01–0.20 as none to slight, 0.21–0.40 as fair, 0.41– 0.60 as moderate, 0.61–0.80 as substantial, and 0.81–1.00 as almost perfect agreement (Cohen, 1960). An average Kappa value as well as an average percent agreement was calculated.

3.4 Multi-Depth Analyses

Hydrochemistry characteristics between surface, center, and benthic samples were averaged for each stream site to note any differences in environmental variables among water columns. Beta diversity was calculated between sampling depths using Sørensen similarity values (Dice, 1945, Sørensen 1948, Bray & Curtis, 1957) in 'adespatial' version 0.3-8 (Dray et al., 2020) and 'ade4' version 1.7-16 (Thioulouse el al., 2018) packages in R version 4.0.3 (2020, R Studio Core Team 2018; Knight, 2016; Krebs, 1999; Jacobs, 2008). Three beta diversity indices were used to ordinate differences of species compositions between surface, center, and benthic eDNA sampling depths. Chi-square (Pearson, K. 1900), Bray Curtis (Bray & Curtis,1957), and Sørensen dissimilarity distances (Dice 1945, Sørensen 1948, Bray & Curtis, 1957, 'adespatial' r package, Dray et al., 2020, and 'ade4' r package, Thioulouse el al., 2018) were explored in three separate PCoA plots (Whittaker et al., 2001) (Knight 2016, 'vegan' package (Oksanen et al.2018) in R version 4.0.3 (2020; R Core Team, 2018). Ordination of the three beta diversity metrics were explored further by filtering out outliers as well as separating combinations of surface, center, and benthic samples from middle and bank sampling positions as well as upstream and downstream sampling transects, yet the results are not shown.

Relative abundance of reads for species were explored to assess differences in the amount of DNA material detected or not detected for a species based on eDNA sampling depth. Species were ranked according to the number of reads respectively found in surface, center, and benthic sample bottles. Relative abundance curves were exclusively calculated for samples bottles that were vertically collected at the middle of transect lines (e.g. middle of transect vs. left and right (streambank)). Comparisons of relative abundance of reads for species between stream bank and middle column eDNA sampling locations helped indicate species unique to stream bank samples but are not shown in tables or figures of this study because of the imposition of unequal sampling volumes in comparisons between surface, center, and benthic sampling depths (i.e., 500-ml of middle center water, and 1000-ml of surface water & 1000-ml of benthic water (to include 500-ml aliquots of surface bank and benthic bank samples)). Relative abundance curves were calculated using the 'BiodiversityR' package (Kindt and Coe, 2005) in R version 4.0.3 (2020; R Core Team, 2018).

The number of unique and overlapping species between sampling depths were configured in Venn Diagrams. Lastly, species agreements between surface and benthic samples were evaluated through interrater agreement calculations using Cohen's Kappa and percent agreement values ('irr' package (Gamer & Lemon, 2019) in R version 4.0.3 (2020; R Core Team 2018;)). Values across each downstream survey were averaged for each survey in PH2-PH6. This was done for two different comparisons. One interrater agreement compared only surface and benthic samples in the middle positions of the downstream transect. Comparisons of surface and benthic eDNA samples in middle positions of stream transects consisted of 74 fish species. The other interrater agreement comparisons included surface bank and benthic bank sample bottles and groups with middle surface and middle benthic sample bottles. Comparisons including stream bank position samples consisted of 84 fish species.

4.0 Results

We had high confidence in the quality of our eDNA detections with contributions of locally referenced fish species. Mock community incident thresholds indicated by the 12s and COI primers for surveys PH2-PH6 made up less than 0.01% of the maximum number of reads occurring per sequencing run (Table 6). The assay-specific incident thresholds allowed detections with reads at least 0.01% of the total number of reads to be considered in a sample library. This optimized potential to detect fish species in the NCBI reference library. Contaminant species identified by mock community control samples which were removed from sequencing runs for each primer can be seen in Table 7. There were ten contaminant fish species identified through PCR negative controls and/or field blanks (Table 8). Contamination incidences primarily occurred in the field or lab during the fifth and sixth survey (Table 8). Examples of these removals include, 'o'opu akupa *Eleotris sandwicensis* and Hawaiian Ladyfish *Elops hawaiensis* (Table 8).

4.1 Sampling Effort

CPUE calculations indicated that on average, benthic cameras detected six species, cast net detected three, minnow traps detected two, and angling detected one species per deployment (Table 9). When looking at the average contribution of species and number of individuals (i.e.,

potential biomass) per amount of time spent in the field, cast netting reveals larger CPUE values for both unique species and number of individuals (Table 9). However, benthic cameras detect more species of fish with an average of six species observed every two-hour deployment at a site. We also know that stationing camera equipment takes less physical effort than throwing a net. Yet, with a throw net you can target species and maneuver around habitat, where camera footage relies visibility, positioning of camera equipment, and post-survey review.

A total of 216 500-ml eDNA water samples were processed between quarters PH2-PH6 (Table 1). A sample bottle contributed an average of 70,226 reads per sample bottle. This was roughly 0.01864 of the total number of reads detected in the biodiversity study. A sequencing run outputs an average of 3,084,195 reads. The 12s assay accounted for an average of 61.46% of the total number of reads per sequencing run. The COI assay accounted for an average of 38.54% of total number of reads per sequencing run. This includes metazoan groups to exclude birds and mammals.

Environmental DNA and traditional methods combined reached a plateau effect of species richness with 110 fish species detected from data in surveys PH2-PH6 (Figure 10). When separating methods, eDNA surveys reached a species richness plateau sooner than traditional gears; after three surveys or 15 sampling events, reaching a total of 84 unique fish species at 25 events (Figure 11). In the same sampling quarters, individual traditional gear accumulation curves show to be steeper than eDNA curves (Figure 12). This is especially seen in the deployment of minnow traps which caught the least number of different fish species; indicating the additional sampling effort needed if only employing this method (Figure 12). When comparing the effort of each traditional gear independently to that of eDNA sampling effort, gears did not fully capture maximum fish diversity within twenty-five sampling events nearly as well as eDNA surveys (Figures 11 and 12).

4.2 Overall Biodiversity Trends

The PCoA plots using Sørensen's dissimilarity values for sampling date and stream site indicated different and similar views of species composition between eDNA and traditional sampling methods. E'o, Waiawa and Kalauao stream surveys are ordinated to show similarities amongst one another in both eDNA and traditional method ordination plots (Figure 13). Species composition amongst surveys PH2-PH3 and surveys PH4-PH6 are also seen to be more similar amongst both ordinations (Figure 13). Although, similarities were seen for the same stream sites and sampling dates between ordinations, the strength of similarities for species composition varies depending on sampling method. For instance, both eDNA and traditional sampling methodologies identified similar patterns of seasonal diversity amongst Pearl Harbor streams; although they were poorly explained with >20% of variation in species composition being explained between sampling points in PCoA plots (Figure 13). Surveys taken in wet seasons (sampling dates of October 2018, February 2019, and January 2020) were more similar in species composition for both eDNA and traditional methods. However, stronger similarities between sampling events such as in October and February are seen via eDNA sampling.

Differences between rare and dominant species and species richness between methods can present different views of diversity for sampling sites. Such a difference in the values and rankings of sites based on diversity metrics between methods illustrates differences in

perceptions of study sites that can be made based on the exclusivity of using one sampling method over another (Table 10). Mean diversity, richness, and evenness values across sites using eDNA methods was greater than mean values using traditional methods. Negative correlations of Shannon diversity values between sampling methods were seen amongst individual survey events in Figure 14, while positive and negative relationships were seen depending on individual stream site's Shannon diversity values (Figure 15). Both positive and negative correlations were found for alpha diversity values between sampling methods depending on stream site (Figure 15). For instance, Waiawa, E'o, and Kalauoa stream had positive correlations of Shannon diversity between methods while Waikele and Halawa stream were in disagreement of Shannon diversity values with negative relationships (Figure 15). Inverse relationships of diversity indices can be attributed to dominance, species richness, and evenness being negatively correlated between methods for Halawa and Waikele stream (Figure 15). Shannon diversity, richness, and evenness values for Waikele, E'o, Waiawa, Kalauao, and Hālawa streams calculated from using traditional and eDNA methods are indicated in Table 10.

4.3 Method Contribution and Species Agreement

There was a total of 113 fish species detected at downstream sites of Pearl Harbor by combining both methods (i.e., surveys PH1-PH8 for traditional methods and surveys PH2-PH6 for eDNA methods). Of the 113 fish species observed in this study, the use of eDNA surveying accounted for 84 fish species, and traditional methods accounted for 55 (Figure 16). There were 29 species missed by eDNA sampling and exclusively found using traditional surveying gears. Twenty-six species were detected by both eDNA sampling and traditional methods and seen in the overlap in Figure 16. Amongst the 84 fish species observed through eDNA surveys, 46 species were exclusively found using the 12s primer while 13 were exclusively found with the COI primer (Figure 17). Of the 55 species found through traditional methods, cast netting, minnow trapping, angling, and underwater video footage contributed 11, 0, 2, and 19 unique species respectively (Figure 18).

Species agreements between eDNA sampling and traditional methods across all sites and surveys in PH2-PH6 had an average Cohen's Kappa value of 0.091 and a percent agreement of 73.6%. According to Cohen (1960), a 0.09 agreement suggests that in a single survey, traditional and eDNA sampling methods are more in disagreement with one another than agreement; despite being performed at the same time and covering all water columns.

4.4 Multi-Depth Analyses

Average salinity, DO, Conductivity, and temperature taken from each quarterly stream site visit indicated gradients between surface, center, and benthic sampling locations at all stream sites (Table 12). Average salinity and conductivity were highest on the benthic floor at all sites. Average dissolved oxygen and temperature was generally higher for surface water than at the benthic floor. Sørensen's similarity values for binary (absence/presence) comparisons between surface, center, and benthic samples had a value range of 0.40-0.47, revealing relatively weak differences in species composition between eDNA sampling depth locations (Table 13). Sampling depth and species compositions using Chi-square, Bray-Curtis, and Sørensen dissimilarity values did not show any distinct clustering of samples within a PCoA ordination

(Figure 17A, B, and C). A table of the first four axes for Chi-square, Bray-Curtis, and Sørensen's dissimilarity matrices are displayed in Table 14.

Bray-Curtis and Sørensen ordinations had almost identical patterns for samples. The Chi-square ordination was most unique with a tight central cluster of samples suggesting symmetry along axis 1, as well as a clear triangular inequality amongst outliers from the surface, center, and benthic samples in surveys 5HAD, 4KAD, and 4EOD, respectively. A benthic and center outlier from the centroid cluster is at direct opposite ends of axis 1 while a few surface water samples are positioned above and on either side of the centroid cluster that is positioned around 0 in axis 1 (Figure 18). Outliers in the Chi-square ordination suggest that the most unique composition of species detected occurred in a center and benthic sample. Ordinations of the three metrics after filtering out outliers as well as exploring variations of samples using middle and bank sampling positions, as well as upstream and downstream sampling transects, did not reveal any further distinctions between eDNA sampling depths.

When exclusively examining eDNA samples sampled in the middle position of downstream transects, we found that surface water detected the greatest number of fish species while center column eDNA samples detected the greatest number of reads. Thus, DNA quantity did not reflect species richness at these sampling depths (Figures 19-21). Abundance of DNA material (i.e., reads) found for each species was similar despite sampling depth location (Figures 19-21, Appendix A). Out of 74 total fish species, surface samples detected 62 species over 559,714 reads, center samples detected 59 species over 586,610 reads, and benthic samples detected 55 species over 62,563 reads (Figures 19-21, Appendix A). The overall number of reads observed between the surface and center water columns gathered cumulatively over five stream sites in Pearl Harbor was more similar in total numbers. Larger quantities of DNA material may therefore be indicative to surface and center water columns. This could be an indication of DNA materials gathered over drainage carried down by streamflow to river mouths.

Benthic dwelling species such as *Awaous stamineus* was within the top 15th ranked species for surface, center, and benthic samples (Appendix A). Other benthic dwelling species that are found in lower reaches of stream networks and estuaries such as *Eleotris sandwicensis*, *Stenogobius hawaiiensis,* and *Synodus ulae* were also detected across surface, center, and benthic sampling locations. The freshwater invasive *Clarias fuscus* was ranked higher in number of reads detected in surface samples than center and benthic samples yet shared similar ranking orders at 15,18, and 19 respectively. Other exclusively freshwater catfish like *Hypostomous watwata* were also found throughout all sampling depths with the highest rank found in surface samples. Surface dwelling species such as *Sphyraena barracuda* and *Encrasicholina purpurea* had abundance rankings from sampling depths that were not indicative of their swimming behavior. *E.purpurea* was ranked in the top three read abundance positions in all instances of sampling depths. Nekton such as *Caranx melampygus* and *C.ingnobilis,* had lower read abundance rankings in center depth.

There were also rare species, like the Tigershark *galeocerdo cuvier*, which was detected in one survey at a single sight (i.e., 5WKD) and was exclusively detected in a single center column eDNA water sample with a rank of 53 out of the total 59 center sample fish species found. Other uncommon detections of shark species, such as the Scalloped Hammerhead *Sphyrna lewini*,

which was detected in two surveys (i.e., 3HAD and 4WKD) ranked highest for center column eDNA samples yet detected in all three sampling depths.

In addition, there were ten species that were uniquely found from eDNA sampling along stream banks, including Fryer's False Moray *Xenoconger fryeri*m, Spider-eye Puffer *Canthigaster amboinensis*, Lutke's Halfbeak *Hemiramphus lutkei*, Rockmover wrasse *Novaculichthys taeniourus*, Redlip Parrotfish *Scarus rubroviolaceus*, Cocoos Frillgoby *Bathygobius cocosensis*, Common Carp *Cyprinus.carpio*, Blacktail Snapper *Lutjanus fulvus*, Bright-eye Damselfish *Plectroglyphidodon imparipennis*, and the Blacktip Shark *Carcharhinus limbatus.* This was found by visually comparing rank abundance curves for samples that included and excluded surface bank and benthic bank sample bottles.

The number of species exclusively detected in surface, center, and benthic samples from middle column eDNA sampling positions can be seen in the Figure 22 Venn Diagram. There were ten fish species uniquely detected in surface water samples, six uniquely detected in center water samples, and four fish species uniquely detected in benthic water samples (Figure 22). A majority of forty-eight fish species were detected in middle column sampling locations from all three eDNA sampling depths. The Venn Diagram indicated that 12 fish species would have not been included in overall biodiversity estimates if center and benthic sampling did not occur. Species that were detected in benthic samples but were not detected in surface samples include Chub species *Kyphosus vaigiensis*, and *Kyphosus elegans*, the Shortbill Spearfish *Tetrapturus angustirostris*, the Hawaiian Silverside *Atherinomorus insularum*, the Hawaiian Shrimp Goby *Psilogobius mainlandi*, and the reef dwelling Saddleback Butterflyfish *Chaetodon ephippium*. There were eight fish species that were found in center column eDNA samples but not detected in surface samples. This includes: the Hawaiian Cleaner Wrasse *Labroides phthirophagus*, the endemic Bay Cardinalfish *Foa brachygramma*, the Tiger Shark *Galeocerdo cuvier*, a *Gobiosoma* sp., *Psilogobius mainlandi*, the Indo-Pacific Sergeant Major *Abudefduf vaigiensis*, *Atherinomorus insularum*, and the freshwater demersal *Amphilophus citrinellus*.

In a comparison of 50 fish species across 19 paired instances, surface and benthic samples have an average Cohen's Kappa value of 0.22 and a percent agreement of 67.8% for a given survey (Table 15A). When including bank samples, values increased to a Cohen's Kappa value of 0.51 and a percent agreement of 80.3% (Table 15B).

5.0 Discussion

This study carried out extensive sampling efforts to identify whether eDNA sampling and traditional methods reveal the same conclusions of diversity in estuaries and to assess if multi-depth eDNA sampling is necessary to capture total species richness and assess species compositions in estuaries. Various assumptions made regarding the use of eDNA sampling which were acknowledged in this study can be seen in Table 16. Environmental DNA surveying revealed to be powerful for inventorying aquatic species. The COI and 12s primers captured 84 out of 113 fish species detected in Pearl Harbor. Fifty-eight species of which were unique to eDNA sampling, compared to twenty-nine uniquely detected using traditional gears. Due to each traditional gear, apart from minnow traps, and each eDNA primer detecting unique species, suggest the need to implement both traditional and eDNA methods in diversity surveys. These

results parallel findings made by Kelley et. al., 2018 and Zou et. at., 2020. By measuring whether eDNA and traditional sampling methods agree on the detection and non-detection of species in a single sampling event, this study confirmed that traditional and eDNA sampling schemes have poor levels of agreement. These effects along with differences in evenness and richness detected between methods lead to different perspectives of species compositions and estimates of alpha diversity between sites. Exclusively using traditional or eDNA methods can therefore alter perceptions of diversity at sites, in turn affecting management perspectives. By minimizing the number of false negative detections for eDNA sampling methods could improve the reliability in exclusively using eDNA sampling for diversity assessments. Nevertheless, spatial, and temporal patterns observed using eDNA sampling were clearer to interpret and agreed with spatial and temporal patterns observed using traditional methods in Pearl Harbor.

Although this study shows that eDNA sampling cannot detect all the species found in traditional sampling efforts, multi-depth and streambank eDNA sampling increased the detection of species and total species richness in Pearl Harbor. The influence on sampling volume compared to sampling depth or location on the detection of species however was not further explored in this study. For instance, comparisons of sampling volumes and or including true replicates for each eDNA sampling position may have provided further clarity on the influence of water quantity and the ability to detect rare eDNA. Regardless, multi depth eDNA sampling in Pearl Harbor may offer a more efficient means of capturing rare species than extending sampling efforts when sampling exclusively at surface waters. The majority of fish species were observed regardless of sampling position in the water column and this study did not find distinct differences in species compositions or quantities of DNA material for species between multiple eDNA sampling depths. However, unique fish species were found in streambank, surface, center, and benthic water samples. Twelve species would have been missed without the inclusion of multi-depth sampling as well as ten species that would have been missed if streambank sampling did not occur. Handley et al., 2019 found similar conclusions, that eDNA is to an extent, spatially structured within stratified water columns. In a lake system, the majority of fish species were homogenously found with the exception of a few benthic and littoral species (Handley et al., 2019). Strong spatial structuring was observed for the detection of species when environments are known to be heterogenous in water chemistry or physical structure. Offshore and multi-depth sampling improved detections in summer months when lakes are more stratified, while nearshore sampling was sufficient in capturing species in winter months when lake waters were more homogenous. These dependencies were only important for a few species. Zhang et al., 2020 conducted multi-depth eDNA sampling in a marine system and found that 50% of species were found regardless of sampling depth, while the other 50% of species were exclusively found at particular water sampling depths.

We recommend implementing both metabarcoding eDNA sampling and traditional gears with emphasis that eDNA sampling should be incorporated in best practices for conducting aquatic biological surveys due to increase species richness in surveys and smoother seasonal signals between sites. A combined methods approach uncovers a greater diversity of taxa and a more holistic view of species compositions. This supports the conclusions of Kelly et al., 2017 that environmental DNA sampling and traditional gears provide unique and complementary views of estuarine biodiversity. This study also found that facets of multi-depth environmental DNA sampling and traditional gears can be used in efficient and inefficient means. For instance, multi-depth eDNA sampling could improve species detections if the number of sampling events are

limited. When using traditional gears, we observed that benthic cameras and cast netting were the most efficient methods among all four traditional survey gears, having greater species overlap, as well as higher levels of species diversity and individuals observed.

5.1 Sampling Effort

We obtained optimum and a near-optimum number of fish species that could be observed between methods and by combining methods in Pearl Harbor (Figures 10, 11, and 12). This allowed for a proper comparison between eDNA and traditional surveying methods. Benthic cameras and cast netting were the most efficient traditional gears for diversity and biomass CPUE values (Table 19). Benthic cameras detected more unique species as well as had a wider range of detecting the same species across the other three traditionally used gears (Figure 18). What is not noted in this study is whether the removal of inefficient methods, such as angling, from the sampling scheme would have decreased the number of unique fish species for traditional methods or even in a given survey, contributing to the observed Kappa values between methods. For example, Figure 18 illustrates the number of unique and overlapping species within the traditional sampling scheme, but unique species observed may or may not be the unique or overlapping species in Figure 16 between eDNA and traditional methods. Cameras are limited by visibility underwater (i.e., turbidity); with more opportunities of zero detections and hindrance of taxonomic identifications in the video review. Despite limitations of visibility as well as being the least deployed gear due to field complications, benthic cameras detected the most fish species (Figure 12). Camera detections may further be improved by deploying cameras in center water columns (vs. benthic) to capture predatory nekton and or with the deployment of more cameras in a survey. Opportunities for minnow traps to capture unique species might also have been influenced by the tampering of organisms in traps by the public during the study and or the loss of traps in the field.

A metabarcoding eDNA sampling method for inventorying estuarine species was more efficient in comparison to traditional gears. Environmental DNA surveys achieved species saturation sooner than that of traditional gears. Species richness obtained from eDNA surveys was maximized with at least twenty-five surveys (PH2-PH6) (Figure 11) using the COI and 12s primer. This information can be used to anticipate sampling efforts needed for estuarine biodiversity surveys. Four quarterly surveys using eDNA surveying, or a single year of quarterly sampling may be sufficient for inventorying estuarine biodiversity. Traditional gears provide unique species but require more sampling effort to reach species saturation. Multiple primers could have been attributed to the efficiency of eDNA sampling. The 12s primer for example was more useful in detecting fish species than the universal COI primer. Detecting 71 out of 84 of all the fish detected in eDNA surveys and attributing 61.46% of the total number of reads per sequencing run. Universal primers, e.g., COI, provide lower taxonomic resolution. Yet, targeting multiple genetic regions is necessary as multiple assays provide higher levels of confidence for species detection when they are detected by multiple primers.

5.2 Overall Biodiversity Trends

Environmental DNA and traditional sampling schemes can reveal similar and misleading conclusions about estuarine surveys. Although similar seasonal patterns can be represented in either collection method, alpha diversity values between streams can be viewed differently depending on sampling method used. Differences amongst diversity estimates for survey events and stream sites between collection methods as seen in figure 13 and figure 14a, can be

attributed to inverse relationships between species richness and or evenness for a few stream sites. Alpha diversity generated between methods show to be sensitive to the presence or absence of a given species which is highly dependent on sampling effort and biases in the range of species detected from the sampling method chosen, such as eDNA reference libraries and fishing expertise. Traditional sampling methods require a greater amount of sampling effort to maximize species richness. This introduces greater variability in evenness estimates between surveys. Having a wider array of species detected such as with eDNA surveying, and differences in the number of singletons and dominant species found between methods provides different and opposing views of diversity estimates at sites as thus only improve biodiversity estimates for estuaries when using both methods.

5.2 Method Contributions and Species Agreement

With poor agreement (i.e., K= 0.09) between eDNA and traditional methods for a given survey, it is evident that eDNA sampling and traditional surveying do not detect the same species in situ. When listing most frequently found species between sampling methods we see that traditional methods detect more species within genera of fish such as Mullet, Surgeonfish, Puffer fish, Porcupine fish, and Flagtail than that of eDNA. However, more Goatfish and four out of the five endemic Hawaiian goby species were detected in eDNA methods as opposed to being missed using traditional methods (Appendix B). We also observe higher values of rare and dominant species amongst traditional methods (Figure 14) although cryptic species are better observed via eDNA (Appendix B).

Assumptions regarding the presence of DNA material in the water may influence differences in detection between methods (Table 16). When using eDNA the possibility of species shedding DNA material at different rates is disregarded. DNA detections are also assumed to reveal species that occupy the area but are not necessarily present in a single point in time, as opposed to traditional methods which are more limited because true positive detections are physically observed. For instance, unknown transient DNA is included in sampling if that species is known to be found within the sampling area. In additional there were several species that were not detected in eDNA methods but observed in traditional gears. Because false negatives were found amongst eDNA surveys, suggests influences of the behavior of DNA material in the environment on detection of a species.

Out of the 113 unique fish species detected at downstream transects, eDNA provided roughly half of the unique fish species while traditional methods contributed roughly a quarter of the unique fish species observed (Figure 17). Pairing the broad range COI primer and the 12s fish primer together almost doubled the number of species detected in Pearl Harbor from eDNA surveys. Only about a quarter of the total number of fish species detected overlapped between eDNA sampling and traditional survey methods in Pearl Harbor. So, although eDNA surveying detected 84 out of 113 fish species, there were still roughly 29 aquatic species that would not have been accounted for if a multi-method traditional sampling scheme weren't performed. This is of course smaller than the 58 fish species that would not have been documented if multi-depth metagenomic eDNA surveying did not occur (Figure 17). Therefore, both traditional and eDNA methods should be implemented in aquatic bioassessments as they supplement species detection and provide the most optimized documentation of species compositions.

5.4 Multi-Depth eDNA sampling

Sampling eDNA at multiple sampling depths (i.e., surface, center, benthic) and positions (i.e., middle and bank) along estuary transects revealed that unique sampling locations provided unique fish species. Center and benthic eDNA sample locations offered twelve unique fish species (Figure 23). When incorporating stream bank sampling, compared to that of sampling eDNA in the middle of stream mouth transects, bank samples offered ten unique fish species (Table 15). However, the species observed between different sampling depths were still found to be a random process. This is because ordinations of species compositions between sampling depths did not show any relevant clustering, DNA material for common species were homogeneously available within estuarine water columns (Figure 19, Appendix A), and species agreement was found to be fair and increase with the inclusion of more samples from streambanks.

Regardless of the behavior or habitat preferences of species, ordinations of species compositions were not different amongst various eDNA sampling locations. Variation or differences in species compositional arrangement between eDNA sampling depths may therefore be primarily influenced by small differences between a few commonly detected species, while rare species or grouping of rare species are observed in only a few samples. Also, the amount of DNA material for particular species wasn't drastically different between the surface, center, and benthic sampling depths. Rank abundance (of reads) for a species shared similar ranks in the surface, center, and benthic sampling depths. The chances of detection are thought to be influenced by sampling effort as species agreement increased with sample size (Table 15).

Differences between eDNA sampling depths may be due to greater sampling water volumes rather than the physical location. When incorporating more sample bottles from stream bank locations, species agreements between sampling depths improved from 0.2 to 0.5 (Table 15). Multi-depth eDNA surveys may therefore not be a definite need for recording species when sampling volume is increased in a survey event. Multi-depth eDNA sampling might improve species detections if the number of surveys or sampling effort is limited. This is seen with the large number of overlapping species in Figure 23, the resemblance between Sørensen diversity values for the surface, center, and benthic sampling locations (Table 13), and the similar abundance rankings of species between sampling depth locations (Appendix A). It may be that the biomass of species or the shedding of DNA dominates eDNA detections to a point, and sampling various areas of an estuary with different hydrologic properties influence the detection of rare species or enhance the ability to detect species that shed DNA conservatively.

In addition, data exclusively from middle transect eDNA samples at downstream surveys suggests that the total number of reads detected from sampling depth locations did not necessarily correspond with species richness (Figures 20-22). Differences between total read numbers for the surface, center, and benthic samples were not compared for individual surveys or sites. Doing so would reveal clearer differences in the amount of DNA material found for species between water columns at a single point in time as well as the range in the number of reads detected for a given species for each positive detection.

6.0 Conclusion

Method comparisons between eDNA and traditional methods should examine the extent of which sampling multiple water columns with a broader selection of multiple traditional gears to

confidently compare the potential for both methods to detect species. We found that with extensive detection efforts, eDNA surpasses traditional gears in detecting species. Environmental DNA should be prioritized in estuarine biodiversity monitoring for its ability to efficiently capture a broader selection of taxa than traditional gears and capture smoother patterns of seasonal variation and differences between estuary stream sites. This is consistent with previous results to include observations between detections for multi-depth eDNA sampling. Multi-depth and multi-position eDNA sampling are effective to an extent. It increased species detections; however, this effect may have been a random process influenced by sampling volume in this study. Greater sampling volumes at surface water may have the ability to capture unique eDNA that was exclusively found in center, benthic, and streambank sampling of this study. This was not tested and is suggested for future studies in estuaries that resemble the chemical stratification of Pearl Harbor water columns and should be compared with estuaries that have stronger physiochemical stratification structures than Pearl Harbor. Sampling multiple columns in estuaries may be advantageous if sampling funds and time are limited. If physiochemical stratification is known to be strong, eDNA sampling should be implemented to follow those unique characteristics.

Environmental DNA may demonstrate a more cost-effective means to detect fish compositions and discriminate seasonal variations yet, the results of this study highlight the need to implement both traditional and eDNA sampling methods. Each method observes rare and abundant species differently as well as detects different species (species disagreements) in a single point in time. The low level of species agreements between methods can result in inverse conclusions for diversity estimates between estuary stream sites and patterns of presence for some taxa. This is consistent to other method comparisons in estuaries. The use of a single method may consequently reflect a misleading ecological view of the presence of species and diversity.

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Tables

Table 1: Date and season of survey events. Survey events (Survey ID) are categorized as being in a wet or dry season (Season). Eight surveys were conducted between August 2018 to July 2020 (Date). Each survey included sampling at an upstream and downstream transect for five streams entering the Pearl Harbor estuary.

Survey ID	Date	Season
PH1	8/13-15/2018	Dry
PH2	10/25-28/2018	Wet
PH3	2/14-17/2019	Wet
PH4	5/14-18/2019	Dry
PH5	7/16-20/2019	Dry
PH6	1/3-9/2020	Wet
PH7	6/16-30/2020	Dry
PH8	7/13-17/2020	Dry

Table 2: Physical and chemical stream characteristics. Mean stream depth, water temperature, salinity, dissolved oxygen, and conductivity for downstream sampling sites. Depth measurements were taken on the left, middle, and right sections of sampling transect for five streams, Waikele (WKD), EO (EOD), Waiawa (WAD), Kalauao (KAD) and Hālawa (HAD). Depths for each location were averaged across all eight survey events. Water chemistry data was obtained by season (wet or dry) from surface water (30.5 cm depth) sampling locations with an YSI DSS Pro. Values for each season were averaged by season across three wet surveys (10/2018,2/2019,1/2020) and four dry surveys (5/2019,7/2019,6/2020,7/2020)

	Stream depth (m)			Water Chemistry				
Site ID	Left	Middle	Right	Season	Temp (C)	Salinity (ppt)	DO%	C (us/-cm)
WKD	2.01168	1.92024	1.3716	Wet	21.6	7.19	76.6	11440.1
				Dry	28.3	24.5	85.2	41526.0
EOD	1.79832	2.34696	1.64592	Wet	25.2	27.4	82.6	43030.6
				Dry	28.8	30.5	81.7	50657.5
WAD	1.95072	2.92608	2.80416	Wet	23.3	12.8	84.8	21149.3
				Dry	27.8	27.0	66.9	44675.0
KAD	1.58496	1.55448	1.18872	Wet	22.9	18.5	84.1	29718.5
				Dry	28.0	26.0	66.1	36317.3
HAD	1.95072	2.37744	0.9144	Wet	24.9	23.45	76.5	35040.0
				Dry	28.0	33.3	65.6	53716.2

Table 3: Locations of baited benthic cameras for each downstream transect stream site (WKD=Waikele, EOD=E`O, WAD=Waiawa, KAD=Kalauao, HAD=Hālawa) These locations are repeatedly used throughout the study and placed near structural habitat.

Site	Lat.	Long.
WKD	21°22'22.29"N	158° 0'47.34"W
EOD	21°23'0.44"N	157°59'32.79"W
WAD	21°22'45.20"N	157°58'44.74"W
KAD	21°22'45.79"N	157°56'37.17"W
HAD	21°21'56.58"N	157°56'17.42"W

Table 4: List of aquatic species detected in mock community samples by assay. These represent all taxa that were evaluated (by assay) as potential mock community contaminants in the Pearl Harbor eDNA samples.

12s	COI	
Alosa fallax	*Ammodytes marinus*	*Lucilia cuprina*
Ammodytes marinus	*Amphiprion frenatus*	*Luidia sarsi*
Amphiprion frenatus	*Amphiprion ocellaris*	*Meganyctiphanes norvegica*
Amphiprion ocellaris	*Calanus finmarchicus*	*Mysis diluviana*
Clupea harengus	*Caligus elongatus*	*Obelia dichotoma*
Merlangius merlangus	*Clupea harengus*	*Ophiocten affinis*
Pollachius virens	*Clytia sp. 2 SL-2013*	*Oxyporhamphus micropterus*
Polydactylus sexfilis	*Cyanea capillata*	*Parupeneus multifasciatus*
Salmo salar	*Doryteuthis opalescens*	*Pollachius virens*
Sebastes mentella	*Echinocardium cordatum*	*Polydactylus sexfilis*
	Echinocardium flavescens	*Rathkea octopunctata*
	Echinus esculentus	*Salmo salar*
	Felis silvestris	*Sargocentron xantherythrum*
	Gempylus serpens	*Selar crumenophthalmus*
	Katsuwonus pelamis	*Stylophora pistillata*

Table 5: Forward and reverse primers (Primer sequences) of four assays (Gene) previously used in eDNA literature (Citation) that were targeted for in all samples collected in Pearl Harbor. Primers were used to detect specific targeted groups of taxa (Target taxa). Each primer had different PCR amplification protocols (PCR Protocol).

Citation	Gene (organelle)	Target taxa	Primer sequences	PCR Protocol
Leray et al. 2013	COI mitochondrial	Animals	GGWACWGGWTGAACW GTWTAYCCYCC TAIACYTCIGGRTGICCR AARAAYCA	62°C (-1°C/cycle) - 16 cycles; 46°C - 25 cycles
Machida and Knowlton 2012	18S nuclear	Invertebrates	AACTTAAAGRAATTGAC GGA CKRAGGGCATYACWGA CCTGTTAT	55°C - 30 cycles
Berry et al.2017 Deagle et al.,2007	16S mitochondrial	Fish	GACCCTATGGAGCTTTA GAC CGCTGTTATCCCTADRG TAACT	65°C - 7 cycles; 62°C - 7 cycles; 60°C - 35 cycles
Evans et al.,2016	12S mitochondrial	Fish	AGCCACCGCGGTTATAC G CAAGTCCTTTGGGTTTT AAGC	65°C - 7 cycles; 62°C - 7 cycles; 60°C - 35 cycles

Table 6: Summary of eDNA sequencing pipeline. Summary includes the number of sequencing records at different stages of the Operational Taxonomic Unit (OTU) generation pipeline for each of the four assays used in each Pearl Harbor sampling event (PH1-PH7). Reads indicate the number of reads after removing poor quality reads and demultiplexing adapter sequences in Trimmomatic version 0.32 (Bolger et al., 2014). These reads were input into USEARCH version 8.0.1623 (Edgar, 2010) for OTU generation. Filtering Merging indicates the number of single reads resulting after overlapping similar sequences or removing heterogenous sequences. Pipe Out includes the number of unassigned taxa as represented as an OTU, created from single reads with 97% OTU similarity. Controls include the assay specific incidence level of contamination identified by mock community libraries. Final indicates the number of OTUs for specific taxonomic groups retained and ultimately assigned a species ID.

Survey	Assay	Reads	Filtering Merging	Pipe Out	Controls	final
PH2	COI	1,317,896	1,121,582	11,096	0.008697	274
	18S					
	16S					
	12S	2,030,599	1,752,370	123	0.003364	52
PH3	COI	1,679,943	1,417,535	13,219	0.000000	283
	18S					
	16S					
	12S	2,083,202	1,798,198	133	0.000885	58
PH4	COI	1,470,592	1,921,267	5,023	0.000000	151
	18S					
	16S					
	12S	2,236,077	1,246,880	87	0.001496	48
PH5	COI	774,215	688,884	3,455	0.000000	125
	18S					
	16S					
	12S	1,617,560	1,312,700	80	0.028713	49
PH6	COI	810,297	730,846	6,610	0.000132	238
	18S					
	16S					
	12S	1,400,592	1,135,046	65	0.004536	45

Table 7: Contaminant species identified by mock community control samples and removed (X) from quarterly sequencing runs (PH2, PH3, PH4, PH5, PH6). Mock community species were identified by either the 12s or COI primer, or both. Their incident level of contamination (<0.01) was used to remove OTUs that were greater than or equal to contaminant incidence rates.

Species	PH2	PH3	PH4	PH5	PH6
		12s			
Alosa fallax	X	X	X		
Ammodytes marinus	X	X			
Amphiprion frenatus	X	X	X	X	X
Amphiprion ocellaris	X	X	X	X	X
Clupea harengus	X	X	X		
Merlangius merlangus	X				
Pollachius virens	X	X	X		
Polydactylus sexfilis	X	X			
Salmo salar	X				
Sebastes mentella	X		X		
		COI			
Ammodytes marinus	X	X	X		
Amphiprion frenatus	X	X		X	X
Amphiprion ocellaris	X	X	X	X	X
Calanus finmarchicus	X	X	X		
Caligus elongatus		X			
Clupea harengus	X	X			
Clytia sp. 2 SL-2013	X	X			
Cyanea capillata	X	X			
Doryteuthis opalescens	X				
Echinocardium cordatum	X				
Echinocardium flavescens	X	X	X		
Echinus esculentus	X	X			
Felis silvestris					X
Gempylus serpens			X		
Katsuwonus pelamis			X		
Lucilia cuprina			X		
Luidia sarsi		X			
Meganyctiphanes norvegica	X				
Mysis diluviana	X	X	X		
Obelia dichotoma	X	X	X		
Ophiocten affinis	X				
Oxyporhamphus micropterus			X		
Parupeneus multifasciatus	X		X		
Pollachius virens		X			
Polydactylus sexfilis		X			
Rathkea octopunctata	X	X			
Salmo salar	X	X			
Sargocentron xantherythrum			X		

Selar crumenophthalmus	X	
Stylophora pistillata		X

Table 8: Contaminant species identified in negative control samples (i.e. PCR or field blanks) and removed from Pearl Harbor sample sets. Samples effected are indicated by stream location (WK=Waikele, EO= E'o, WA=Waiawa, KA=Kalauao, and HA=Hālawa), survey event (quarters 2-6), and weather downstream=D or upstream=U samples were affected. Contaminant species were detected independently from the 12s and COI primer.

Common Name	Species	WK	EO	WA	KA	HA
	12s					
Brown Bullhead	*Ameiurus nebulosus*	2D				
Yellowfin Sureonfish	*Acanthurus xanthopterus*		5D			5D
Longjaw Bonefish	*Albula virgata*	5D	5D	5D	6D	5D,6D
Moonfish	*Lampris incognitus*	5D	5D	5D	5D	5D
Hawaiian Ladyfish	*Elops hawaiensis*	6D	5D	6D		5D
Hawaiian Lizardfish	*Synodus ulae*		5D,6U		6U	5D,6U
Kanda Mullet	*Moolgarda engeli*		5D		6D	5D,6U
	COI					
Great Barracuda	*Sphyraena barracuda*		4U	4U	4U	4U
'O'opu Akupa	*Eleotris sandwicensis*	5U	5U	5U	5U	5U
Bluefin Trevally	*Caranx melampygus*	6U	6D	6U		

Table 9: Rank of catch per unit effort (CPUE) for individuals and species richness for four traditional sampling methods. The time spent, the number of species detected, and the number of individuals caught deploying each gear was averaged across all site visits and used to generate CPUE's for species and for individuals. A single gear deployment is represented by the average duration a gear was used within a sampling scheme. Sampling schemes include one benthic camera, at least six throws of cast net, three minnow traps, and two fishers angling.

Traditional Gears	Benthic Camera	Cast Net	Minnow Traps	Angling
Duration (minutes)	125	68.19	1220	87.17
Species Detected	6.13	3.46	2.1	1.58
Individuals Detected	70.72	86.35	8.4	2.63

	CPUE for Species		CPUE for Individuals	
	Rank	Value	Rank	Value
	Cast Net	0.060	Cast Net	1.490
	Benthic Camera	0.056	Benthic Camera	0.574
	Angling	0.018	Angling	0.028
	Minnow Traps	0.001	Minnow Traps	0.007

Table 10: Stream site Shannon diversity (Shannon 1948), species richness, and species evenness by sampling method. Stream sites are listed by location, WK=Waikele, EO= E'o, WA=Waiawa, KA=Kalauao, and HA=Hālawa, between traditional and eDNA sampling methods. Values were generated from species presence data obtained from downstream surveys PH2-PH6.

	Diversity		Richness		Evenness	
	eDNA	Traditional	eDNA	Traditional	eDNA	Traditional
WKD	3.691182	3.033831	49	26	0.9484463	0.9311666
EOD	3.793972	2.816397	56	21	0.9425194	0.9250703
WAD	3.961835	3.136882	61	29	0.9637452	0.9607894
KAD	3.544236	2.436097	40	14	0.9607894	0.9230936
HAD	3.911522	2.506026	56	15	0.9717218	0.925398

Table 11: Species agreement between eDNA and traditional sampling methods. Mean Cohen's Kappa value and percent agreement generated from the agreement of a given species from surveys PH2-PH6 between eDNA and traditional sampling methods (2 raters). There were 110 fish species or observations that were compared across 24 site visits or paired instances (surveys PH2-PH6).

Cohen's Kappa for 2 Raters (Weights: equal)
0.091415
Percentage agreement (Tolerance=0)
73.63%

Table 12: Mean water chemistry characteristics for stream sites by depth. Mean salinity, dissolved oxygen (DO), conductivity, and temperature for YSI PRO DSS measurements collected at middle surface (MS), middle center (MS), and middle benthic (MB) water columns for each downstream field site (WKD=Waikele, EOD= E'o, WAD=Waiawa, KAD=Kalauao, HAD=Hālawa).

Site ID	Stream Position	Salinity ppt	DO mg/L	Conductivity us/cm	Temperature C
WKD	MB	27.45222	5.326667	45721.48	27.68889
WKD	MC	25.75375	6.05625	43488.09	27.775
WKD	MS	15.525	6.392	26161.03	25.56
EOD	MB	33.41125	4.96875	55127.25	27.7
EOD	MC	32.31	5.18125	53662.38	27.75
EOD	MS	29.66375	5.2575	35663.13	26.2875
WAD	MB	33.47	4.8025	53914.63	27.8875
WAD	MC	33.36375	6.07375	52043.5	27.57875
WAD	MS	21.52375	5.47625	48277.13	27.575
HAD	MB	34.03	3.9425	53646.13	26.8125
HAD	MC	33.87875	4.54375	53564	26.875
HAD	MS	30.4675	5.03625	48534.63	26.95
KAD	MB	34.13714	3.775714	46317.04	27.12857
KAD	MC	34.02286	4.201429	46428.14	26.14286
KAD	MS	23.46571	5.067143	34946.59	26.67143

Table 13: Sorenson's similarity values (1948) for surface, center and benthic eDNA water samples. Sorenson's similarity values were generated using the 'adespatial' version 0.3-8 (Dray et al.,2020) and 'ade4' package (Dray et al.,2020) in R version 4.0.3 (2020; R Studio Core Team 2018).

	Benthic	Center	Surface
Benthic	0.0000000	0.4354942	0.4067771
Center	0.4354942	0.0000000	0.4778561
Surface	0.4067771	0.4778561	0.0000000

Table 14: Percent variation in principal component (PCoA) axis of three beta diversity metrics for eDNA sampling depths. PCoA was ran using Chi-square, Bray-Curtis, and Sorensen dissimilarity values between surface, center, and benthic eDNA water samples collected in Pearl Harbor surveys PH2-PH6.

	Axis 1	Axis 2	Axis 3	Axis 4
Chi-square	67.2	32.8	0.0	0.0
Bray Curtis	24.8	17.6	10.7	8.6
Sørensens	13.7	9.6	6.5	5.7

Table 15: Species agreement between eDNA sampling depths. Mean species agreement between surface and benthic eDNA samples (2 raters) as expressed using Cohen's Kappa values and a precent agreement with different number of surface and benthic sample bottles (i.e., A., B.). A. excludes stream bank samples and only compares a 500-ml middle surface sample and a 500-ml middle benthic sample for 19 surveys across 50 fish species. B. incorporates stream bank samples to provide a comparison of 84 fish species across 23 surveys between 1000 ml of surface water and 1000 ml of benthic water. Data from A. and B. comparisons were obtained from downstream eDNA surveys PH2-PH6.

A. Middle surface and middle benthic sample comparison
Cohen's Kappa for 2 Raters (Surface and Benthic eDNA samples) (Weights: equal)
0.211633
Percentage agreement (Tolerance=0)
67.89474
B. Surface and benthic samples to include surface bank and benthic bank samples
Cohen's Kappa for 2 Raters (Surface and Benthic eDNA samples) (Weights: equal)
0.513677
Percentage agreement (Tolerance=0)
80.3%

Table 16: Assumptions of eDNA sampling methods. Assumptions addressed in the study were tested through an analysis and either verified or unverified.

Method	Assumptions	Test	Findings
eDNA	eDNA is homogenized4 in estuary water columns	1. Comparison of alpha and beta diversity amongst multi-depth samples 2. Rank Abundance Curves	Verified: There were similar conclusions of alpha and beta diversity metrics for multi-depth samples Verified: Proportions of DNA material is similar for species regardless of sampling depth
eDNA	eDNA material found is true to species presence	1.Species Agreement	Unverified: There were low levels of agreement of species detected between eDNA and traditional methods
eDNA	Sample volumes are adequate to assess diversity	1.Species Accumulation Curves	Verified: Sample collectively reached maximum levels of species richness in the study.
eDNA	eDNA material shed by an individual is the same amongst all fish species	1.Rank Abundance Curves	Unverified: Similar quantities of DNA material are found for species throughout water samples

Figures

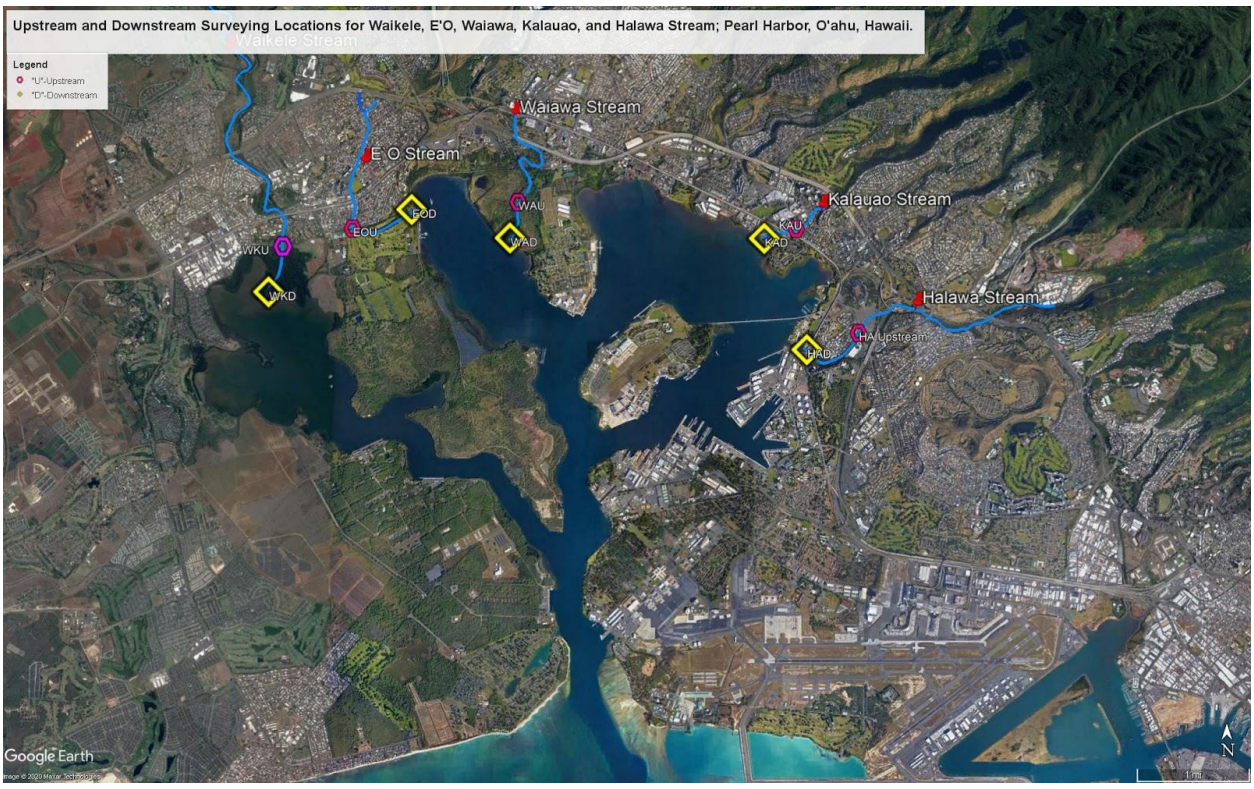


Figure 1: Map of Pearl Harbor study sites located on the south shore of O`ahu, Hawai'i. Five streams (Waikele (WK), E'o (EO), Waiawa (WA), Kalauao (KA) and Hālawa (HA) stream are represented in blue line and labeled with a red thimble. Yellow squares indicate downstream surveying locations for each stream. Pink hexagons represent upstream surveying locations.

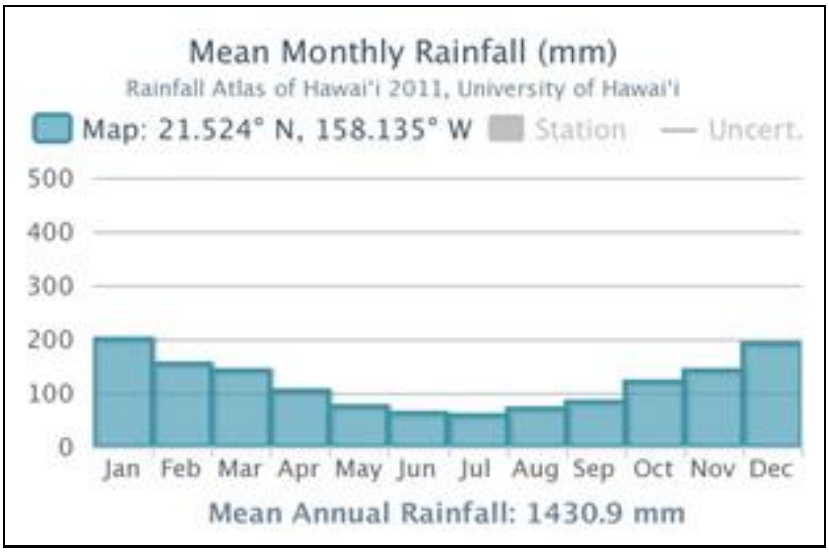


Figure 2: Mean monthly rainfall for O`ahu. Rainfall data generated from Rainfall Atlas of Hawai'i (Frazier et al., 2016)

Figure 3: Positioning of downstream transect lines (marked in white) as indicated for Waikele (WK) steam. Downstream transects for all streams are marked at the point in which the stream begins to widen to the ocean.

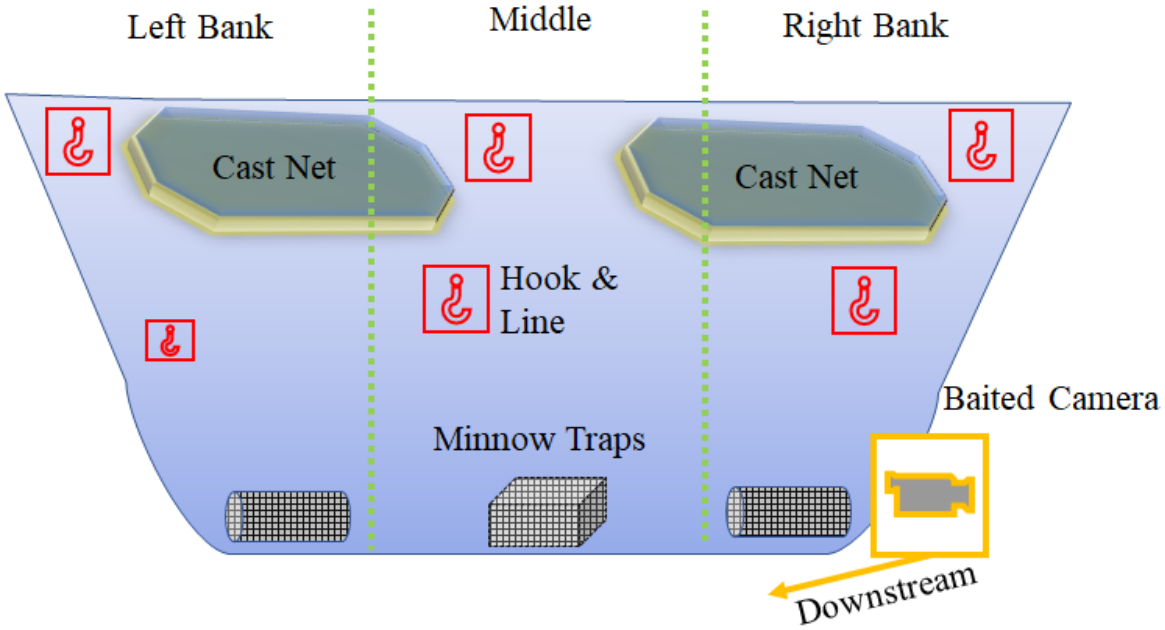


Figure 4: Deployment of traditional method sampling gears along downstream transect lines for five stream mouths of Pearl Harbor. Water column sampling locations for each gear is depicted. A baited benthic camera (Figure 7) is placed within 100 m downstream of the transect line, facing downstream along the benthic floor. Cast net fishing occurs within 100 m of the transect line, capturing fish at surface and subsurface waters. A minimum of 6 throws are made (ideally 3 throws on each left and right bank). Three stream flow measurements are taken at three different depths (30.5 cm below the surface, ½ of total depth, and 30.5 cm above the benthic floor). PH, DO, salinity, conductivity, and turbidity are recorded at three different depths (30.5 cm below the surface, ½ of total depth, and 30.5 cm above the benthic floor). Three baited minnow traps are placed along the transect line and left soaking for 12-24 hours. One 80 cm long cylindrical minnow trap with a 6 cm opening is placed on the left and right bank of the transect while a 45 cm x 41 cm x 20 cm square minnow trap with a 4 cm opening is placed at center. Lastly angling occurs for 60 minutes within 100 m of the transect line, capturing riskier nekton (predatory) in multiple columns of the estuarine area.

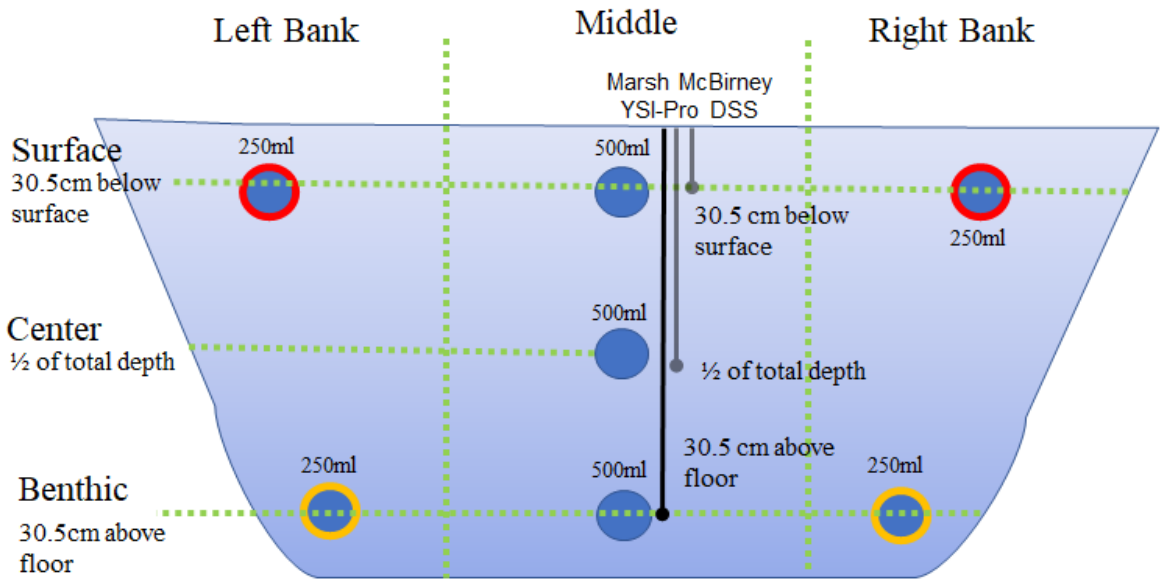


Figure 5: Collection of eDNA water samples along downstream transect lines. Sample locations taken at multiple depths within the water column. Combined samples are marked in red (surface bank) and yellow (benthic bank). A total of seven water samples are collected from each site. 250 ml of water is collected 30.5 cm (1 ft) below the surface at the left and right bank and combined in lab during filtering to comprise a single 500-ml of surface bank material.,250 ml of water is collected 30.5 cm above the benthic floor at the left and right bank and combined in lab during filtering to comprise a single 500-ml of benthic bank material.,At the middle of the transect line, which is delineated by ½ of the stream width, three 500-ml water samples are taken through the water column. One 500 ml sample is taken 30.5 cm below the surface, at ½ of the streams center depth, and 30.5 cm above the benthic floor.

Figure 6: Photograph of the baited benthic camera design. The Sony Digital HDR-AS50 Action Camera Recorder is fastened on top of a 5lbs diving weight. Placed directly 30.48 cm (12 in) at center of the camera lens is a 37.1 cm3 fluorescent orange wiffle ball which is filled with chunks of raw Ahi Tuna through a small slit. A 30.48 cm ruler is fixed horizontally below the baited ball to measure attracted fauna.

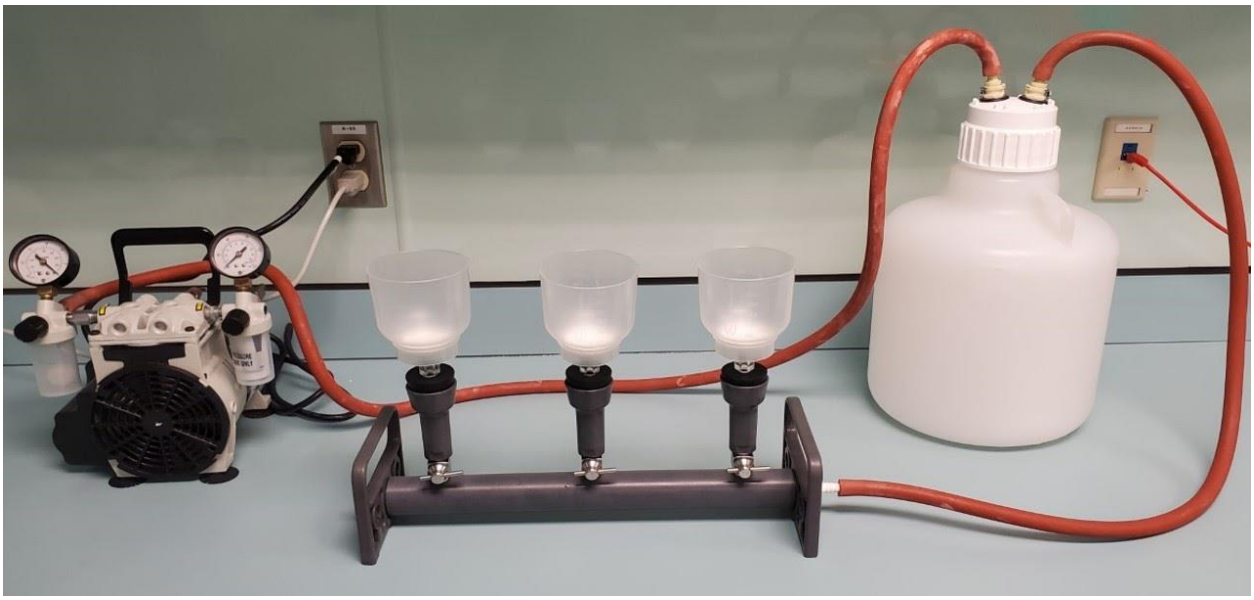


Figure 7: Vacuum filtering equipment for eDNA samples. Equipment vacuum filtered each 500-ml water sample onto a single 0.45 µm membrane disc filter inside polypropylene filtering cups. Collected water is poured into polypropylene filtering cups (300 ml) in which water and DNA material is separated by vacuuming water through a 0.45 µm membrane disc filter which is attached to filtering cups.

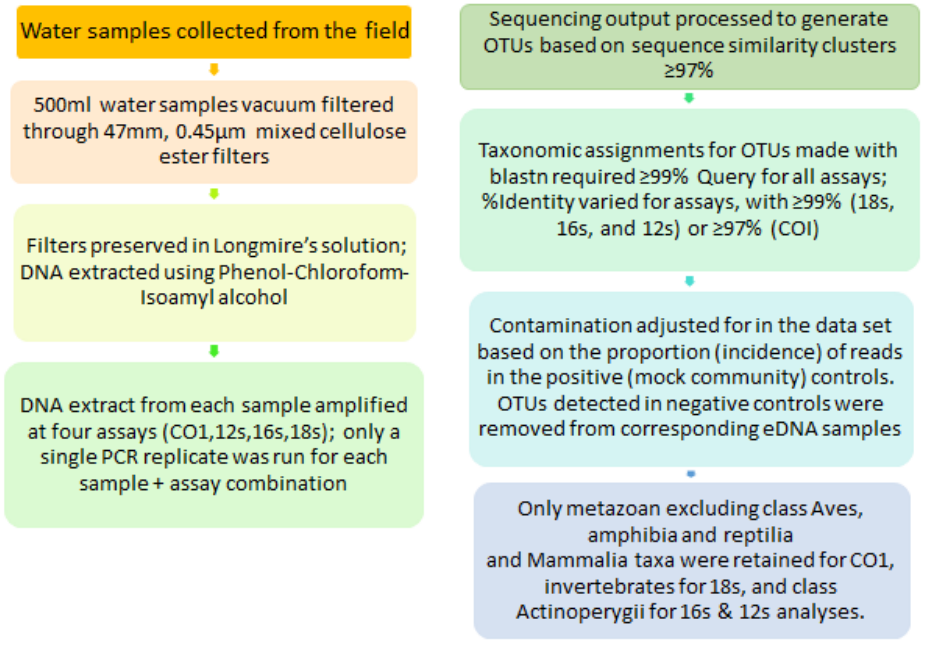


Figure 8: Processing steps for sequencing eDNA samples into Operational Taxonomic Units (OTUs) to generate unique species as indicated by four targeted loci (COI, 18s, 16s, and 12s primers). Steps include filtering water samples, storing DNA material, amplifying DNA material at four loci, generating OTUs, accounting for contamination, and taxa retention.

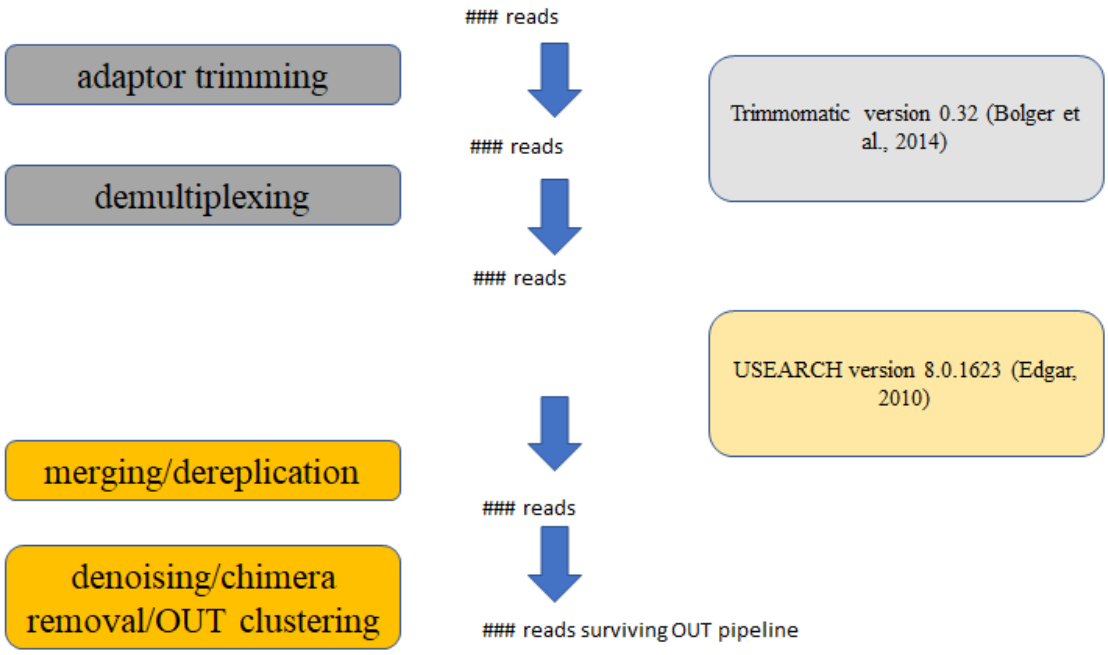


Figure 9: Number of reads (DNA material) minimized after surviving steps in the bioinformatic pipeline. Number or reads are sequencing output processed to generate OTUs using Trimmomatic version 0.32 (Bolger et al., 2014), custom Perl scripts, and USEARCH version 8.0.1623 (Edgar, 2010). Trimmomatic was used to remove raw reads, trim adaptors, and remove low quality sections at the ends of reads; a custom Perl script was used to demultiplex reads into four assays. USEARCH was used to merge paired-end reads (read1 and read2) into a single read and cluster single reads into OTUs.

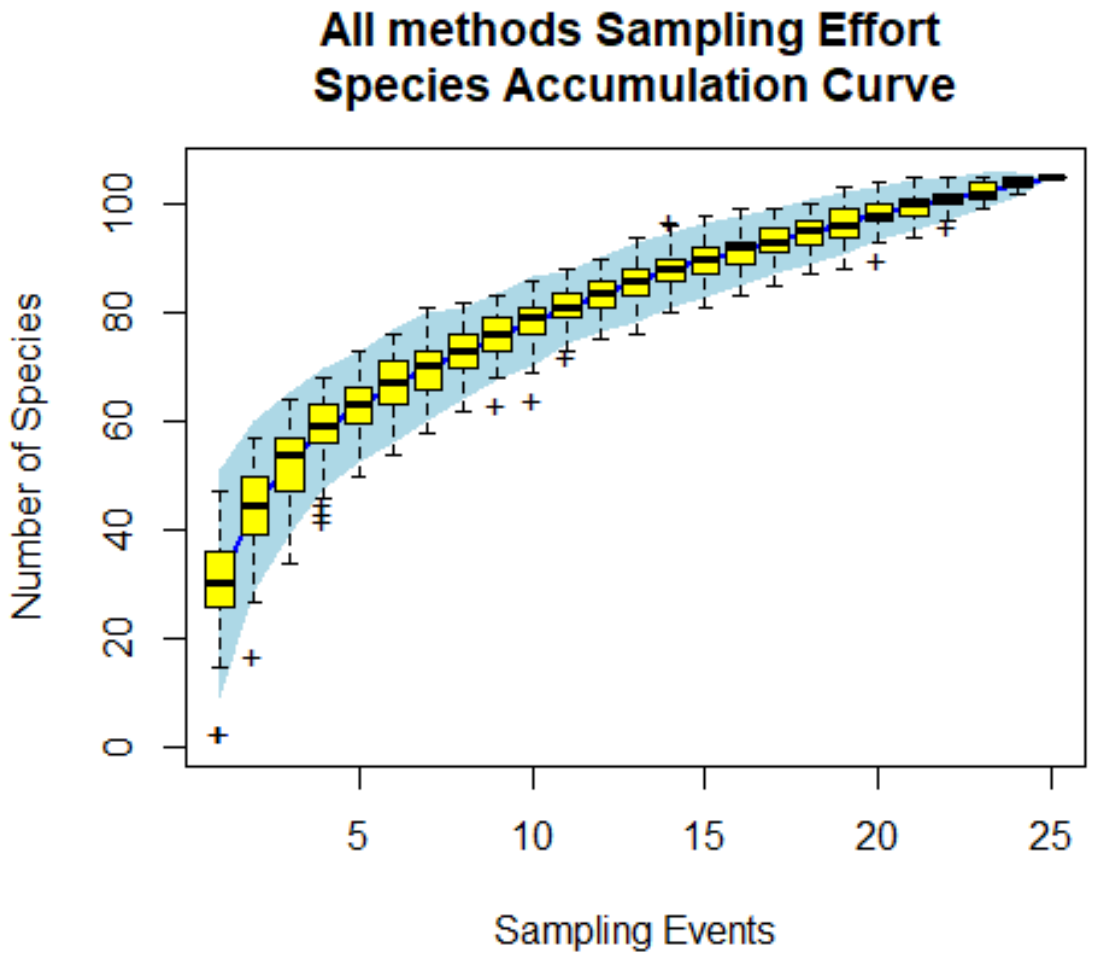


Figure 10: Species accumulation curve using eDNA and traditional sampling methods over 25 downstream sampling events in PH2-PH6 (i.e., covering five streams) through combining environmental DNA and traditional surveying detections per event. Curves were generated using the 'vegan' (Oksanen et al.,2007) and 'labdsv' (David W. Roberts, 2019) packages in R version 4.0.3 (2020; The R Core Team 2018). Detections for fish taxa through eDNA surveying was obtained from the 12s and COI primer. Fish species detected from traditional gears were obtained from benthic cameras, cast netting, minnow trapping, and angling. A total of 110 fish species were observed.

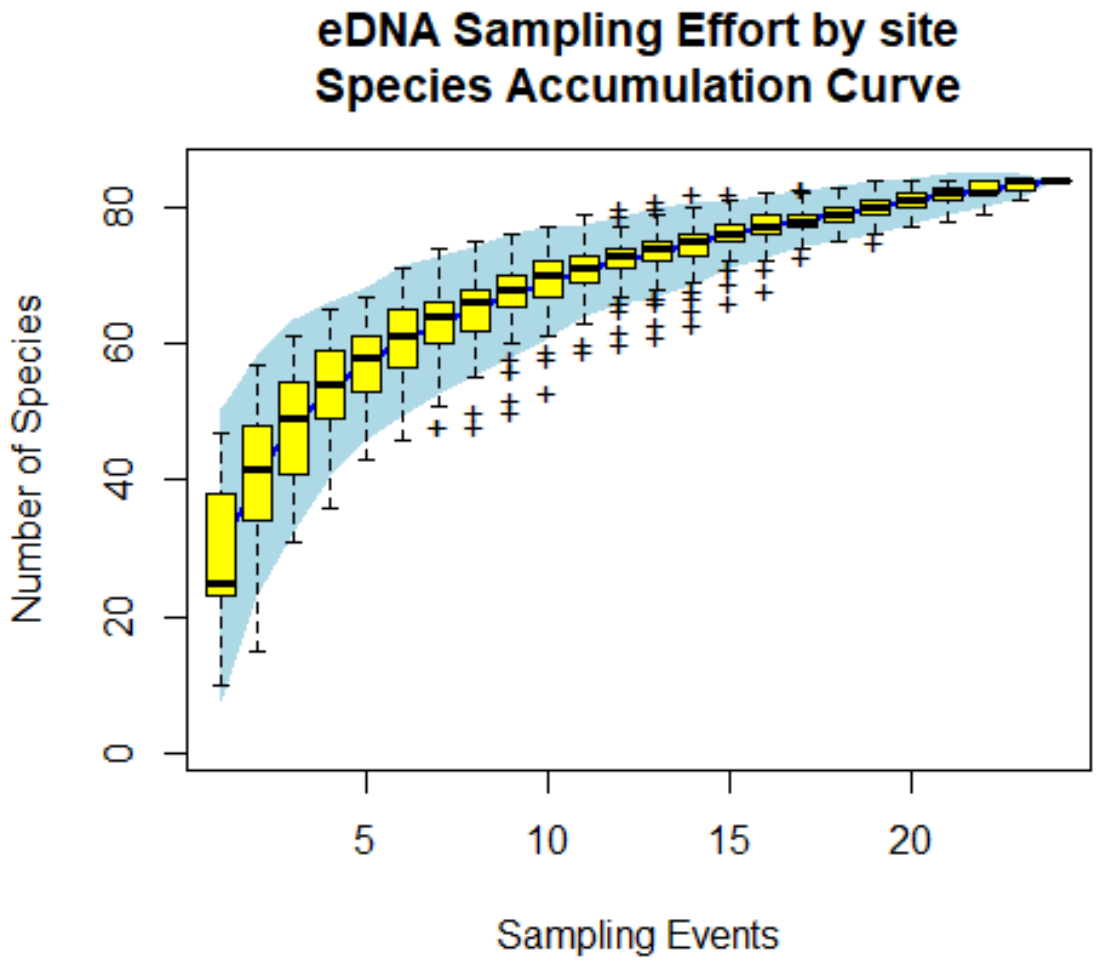


Figure 11: Species accumulation curve using eDNA sampling methods. The accumulation of the number of newly found fish species over each eDNA survey were conducted using the 12s and COI primer. Individual eDNA surveys include downstream eDNA collections across five streams over five survey events (PH2-PH6).

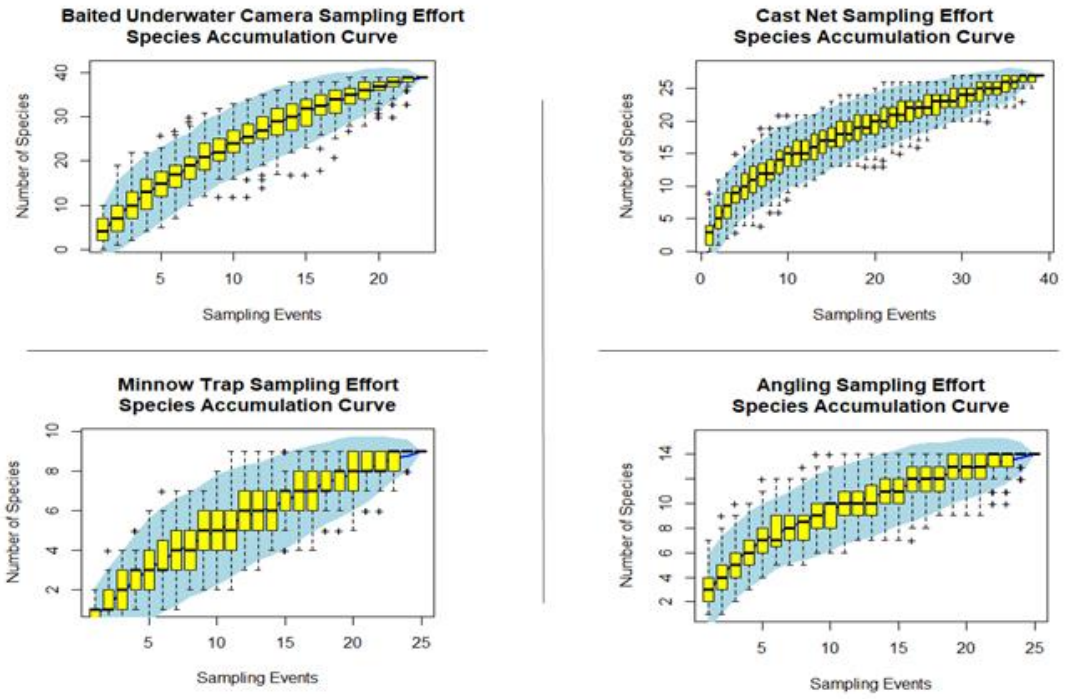


Figure 12: Species accumulation curves using traditional sampling methods. Each curve represents individual calculations from deployments of the benthic camera, cast netting, minnow trapping, and angling in five stream sites from quarterly surveys (PH1-PH8). Curves indicate the number of unique species found cumulatively over each downstream site visit across five survey events. Top left: baited benthic camera, top right: cast net, bottom left: minnow traps, bottom right: angling. Due to field complications, the number of gear deployments were uneven across sampling events. In addition, illustrations constructed using the 'vegan' package (Oksanen et al.,2007) in R version 4.0.3 (2020; R Core Team, 2018) did not count deployments with no species found. Thus, x axis values are not true. Baited benthic cameras had a total of 25 deployments, cast net was deployed over 40 surveys, minnow traps had a total of 36 deployments, and there were 30 angling deployments with two poles.

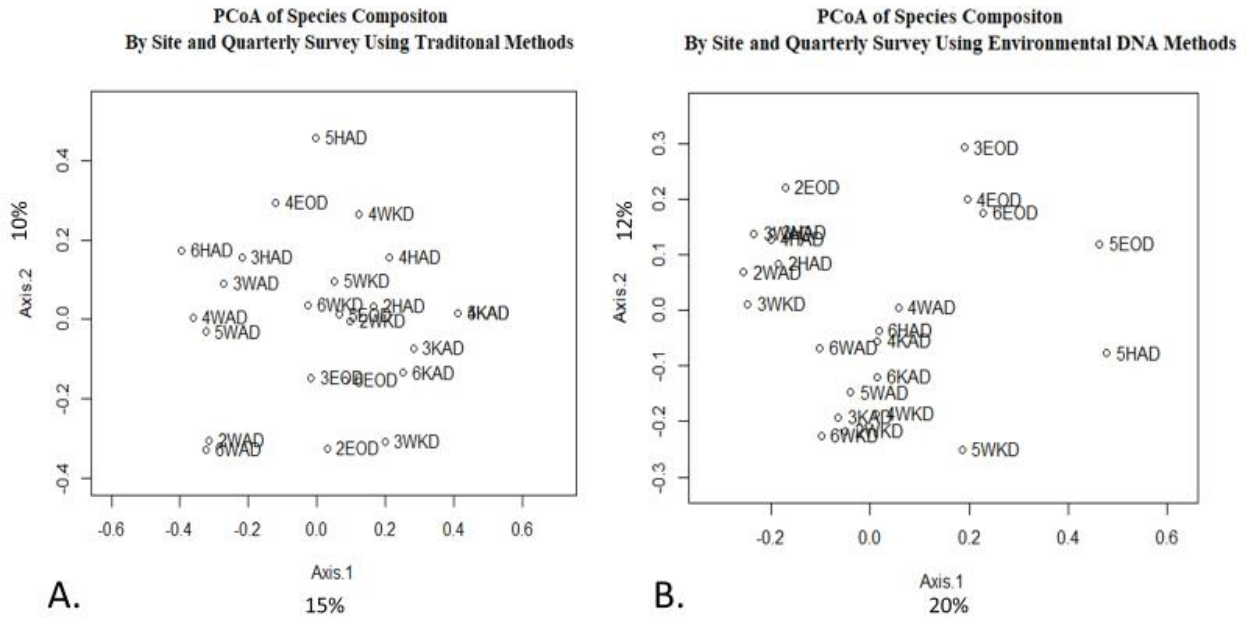


Figure 13: Principal Coordinate Analysis (PCoA) (McCune and Grace, 2002) of stream site species compositions between eDNA and traditional method sampling schemes. Points represent sampling events from surveys PH2-PH6 (Table 1) collected at downstream sites (WKD=Waikele, EOD= E'o, WAD=Waiawa, KAD=Kalauao, HAD=Hālawa). Species composition generated at sights via traditional methods is seen in ordination A with axis 1 explaining 15% of the variation and axis 2 explaining 10% of the variation. Species composition at sights via eDNA methods is seen in ordination B with axis 1 explaining 20% of the variation and axis 2 explaining 12% of the variation. Species composition ordination models were constructed using Sorensen's dissimilarity values (Sorensen et al., 1950) using the 'ape' Package (Paradis et al., 2020) in R version 4.0.3 (2020; R Core Team, 2018) over a total of 55 fish species in A. and 84 fish species in B.

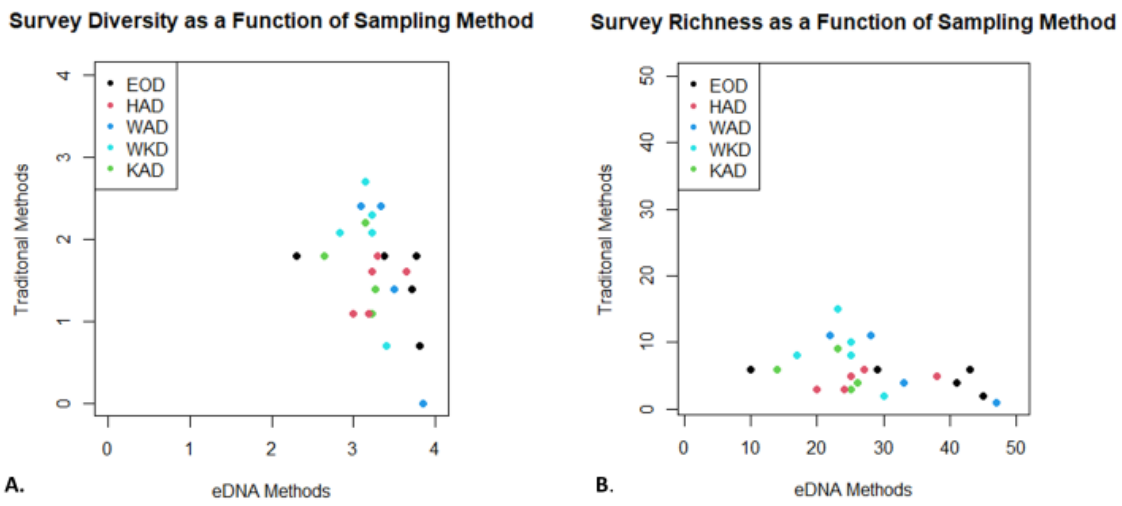


Figure 14: Correlations between eDNA and traditional sampling methods for estimates of Shannon Diversity (A.) and species richness (B.) between surveys for each downstream site (WKD=Waikele, EOD= E'o, WAD=Waiawa, KAD=Kalauao, HAD=Hālawa).

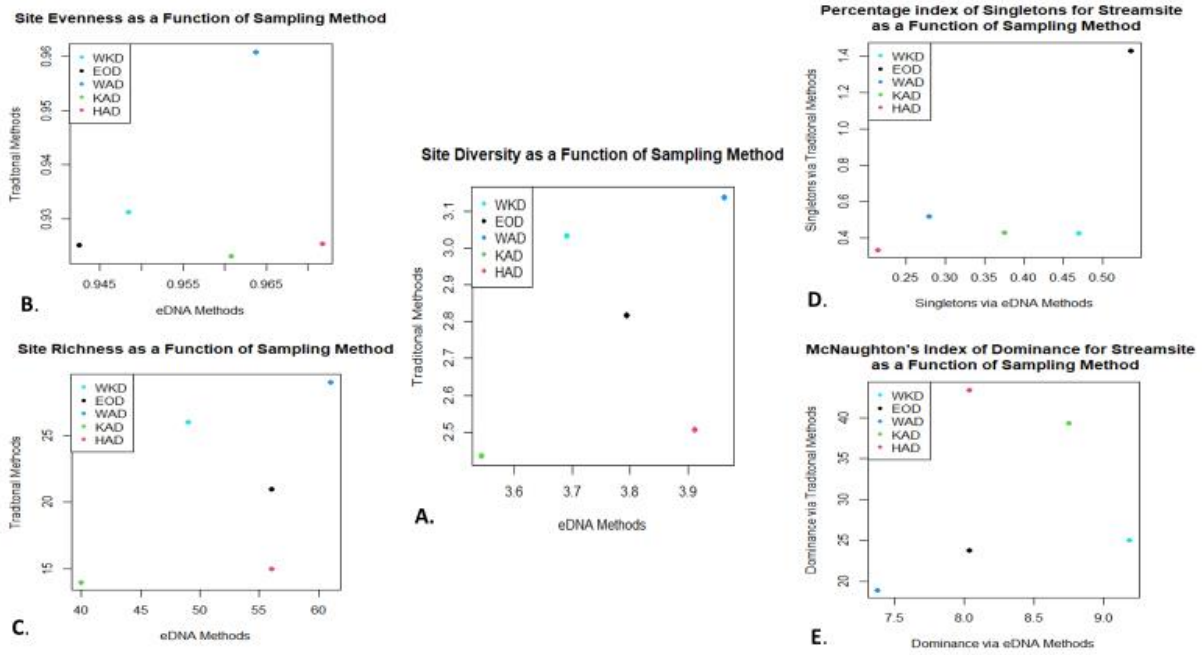


Figure 15: Correlations between sampling methods for estimates of diversity, richness, evenness, percent singletons, and dominance for stream sites. Shannon Diversity (A.), evenness (B.), species richness (C.), percent index of singletons (D.), and McNaughton's index of dominance (E.; McNaughton and Wolf; 1970) between each downstream transect site (WKD=Waikele, EOD= E'o, WAD=Waiawa, KAD=Kalauao, HAD=Hālawa) are compared for eDNA and traditional sampling methods.

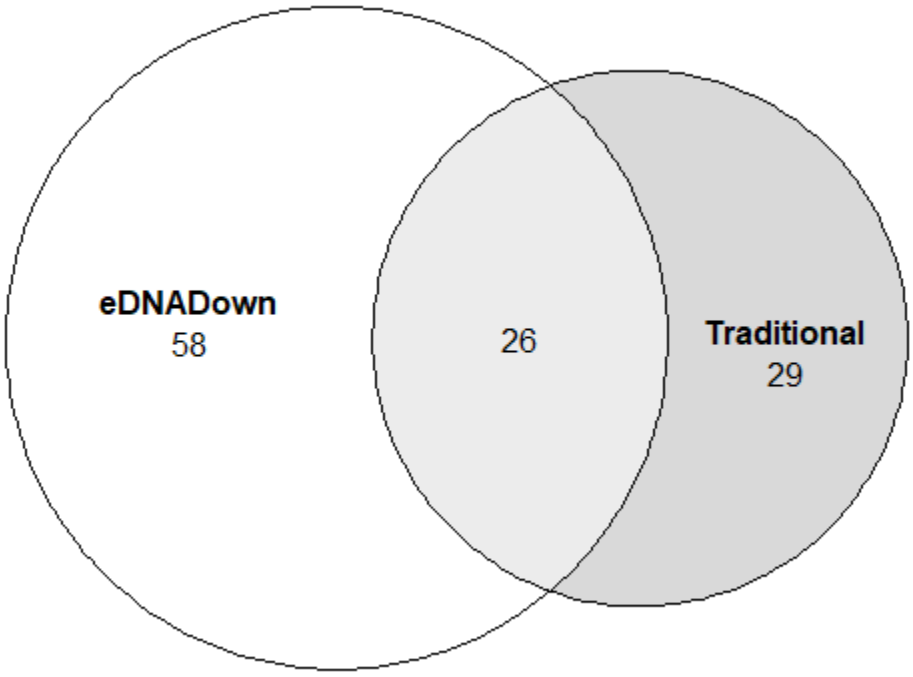


Figure 16: Venn Diagram of number of unique and overlapping fish species detected between eDNA sampling and traditional sampling methods. Venn Diagram was made using 'eulerr' package (Larson, 2019) in R version 4.0.3 for downstream field site for five stream mouths of the Pearl Harbor estuary. A total of 113 fish species were detected. Environmental DNA (eDNA) surveying detected 84 fish species from surveys (PH2-PH6) using the 12s and COI animal primers. Of those 84 species, 58 fish species were uniquely detected using eDNA surveying which traditional methods did not detect. Traditional methods detected a total of 55 fish species; obtained from baited benthic cameras, cast netting, minnow trapping, and angling across all sampling periods (PH1-PH8). Of the 55 fish species identified in traditional methods, 29 fish species were uniquely identified with manual surveying gears and not detected by eDNA sampling.

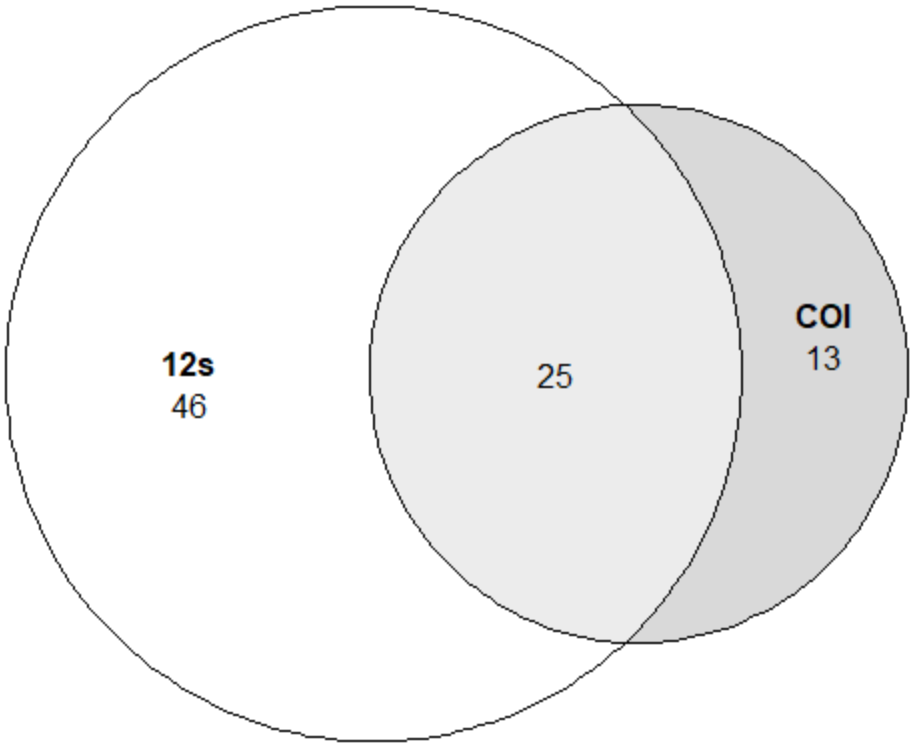


Figure 17: Venn Diagram of number of unique and overlapping fish species detected between eDNA 12S and COI primers. Venn Diagram was made using 'eulerr' package (Larson, 2019) in R version 4.0.3 for the number of unique and overlapping species within classes Actinopterygii and Chondrichthyes detected from OTUs of the 12s and CO1 primers, from five sequencing runs across downstream eDNA surveys (PH2-PH6). A total of 84 fish species were detected using eDNA surveying methods. The 12s fish primer contributed 46 unique fish species which were not detected by the COI animal primer. The COI animal primer contributed 13 unique fish species which were not detected by the 12s fish primer. There were 25 fish species that both primers detected over all sequencing runs.

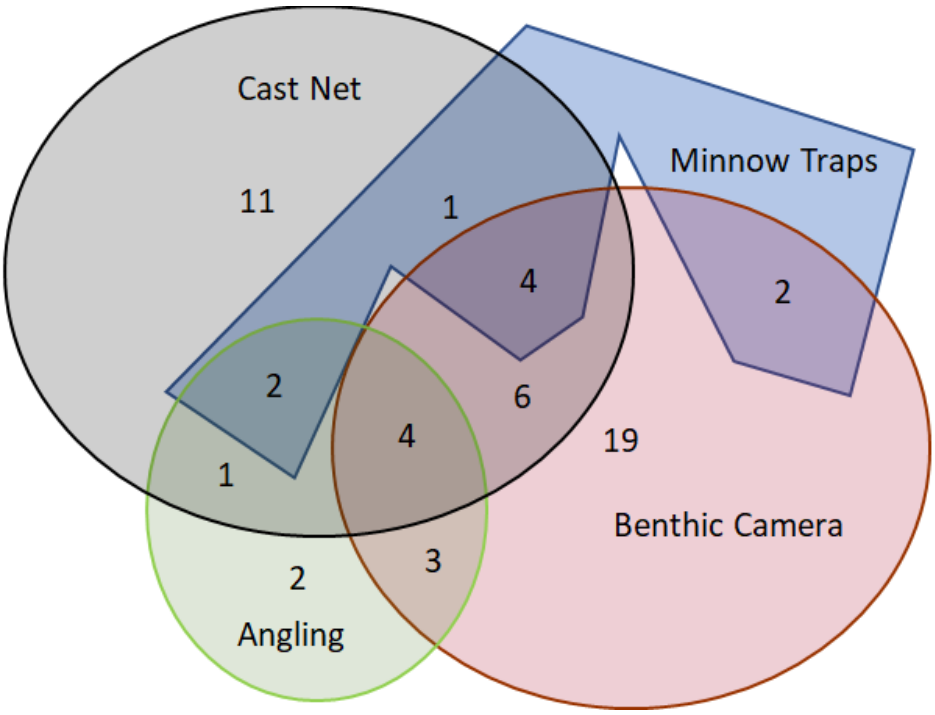
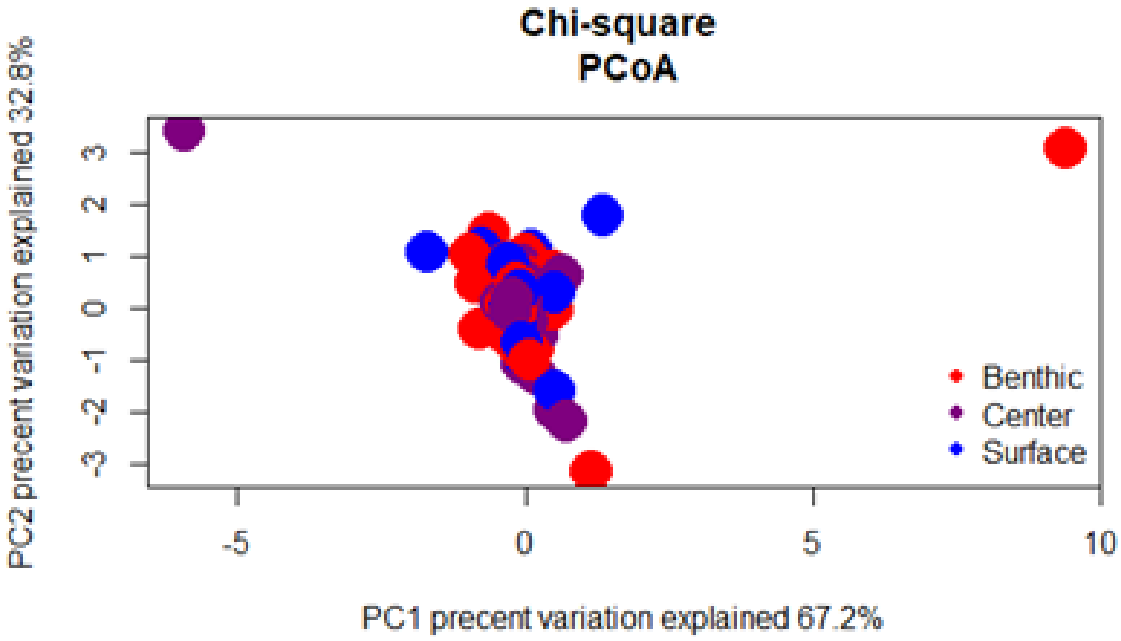
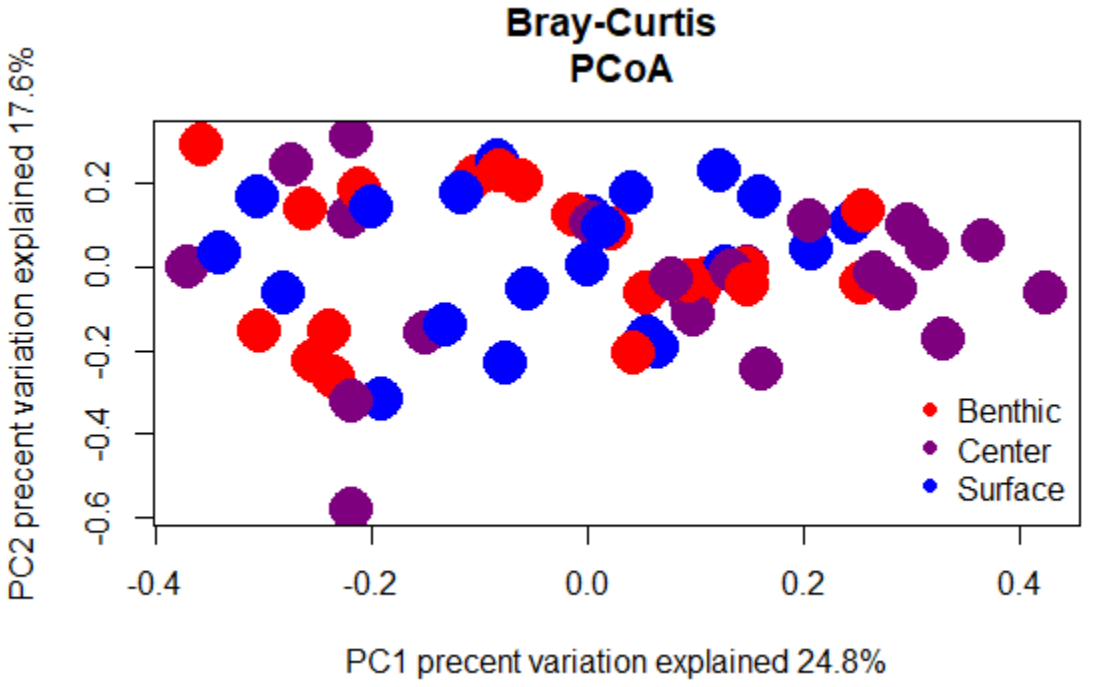


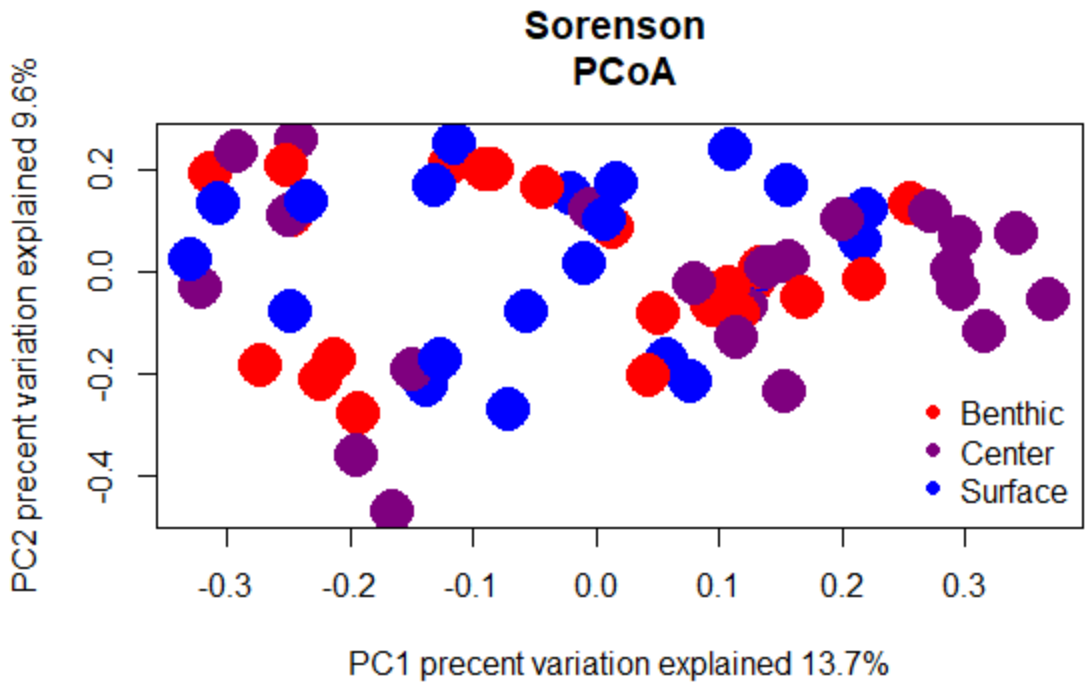
Figure 18: Venn Diagram of number of unique and overlapping fish species detected between four traditional surveying gears (i.e., Cast net, Minnow Traps, Benthic Camera, & Angling). Venn Diagram was made using 'eulerr' package (Larson, 2019) in R version 4.0.3 for the number of unique and overlapping species between traditional sampling methods in surveys PH1-PH8. A total of 55 fish species were detected by traditional gears. Benthic cameras detected 38 fish species, 19 of which were uniquely found in that method. Cast net detected 29 fish species, 11 of which were unique to the method. Minnow traps detected 9 species, with no unique species detected from that method. Angling detected 12 species, 2 of which were only found from angling. Sampling effort for each method can be seen in Table 9.


Chi-square
PCoA
PC2 precent variation explained 32.8%
PC1 precent variation explained 67.2%
Benthic
Center
Surface
-3
-2
-1
0
1
2
3
-5
0
5
10

A.


Bray-Curtis
PCoA
PC2 precent variation explained 17.6%
PC1 precent variation explained 24.8%
Benthic
Center
Surface
-0.6
-0.4
-0.2
0.0
0.2
-0.4
-0.2
0.0
0.2
0.4

B.



C.

Figure 19: Principal Coordinate Analysis (PCoA) (McCune and Grace, 2002) of Chi-squared (A.), Bray-Curtis (B.), and Sorensen dissimilarity (C.) between surface, center, and benthic sampling locations (red=benthic, purple=center, blue=surface) from downstream samples in surveys PH2-PH6. x and y axis labels of each plot note the percent of variation explained in each principal component amongst dissimilarity values.

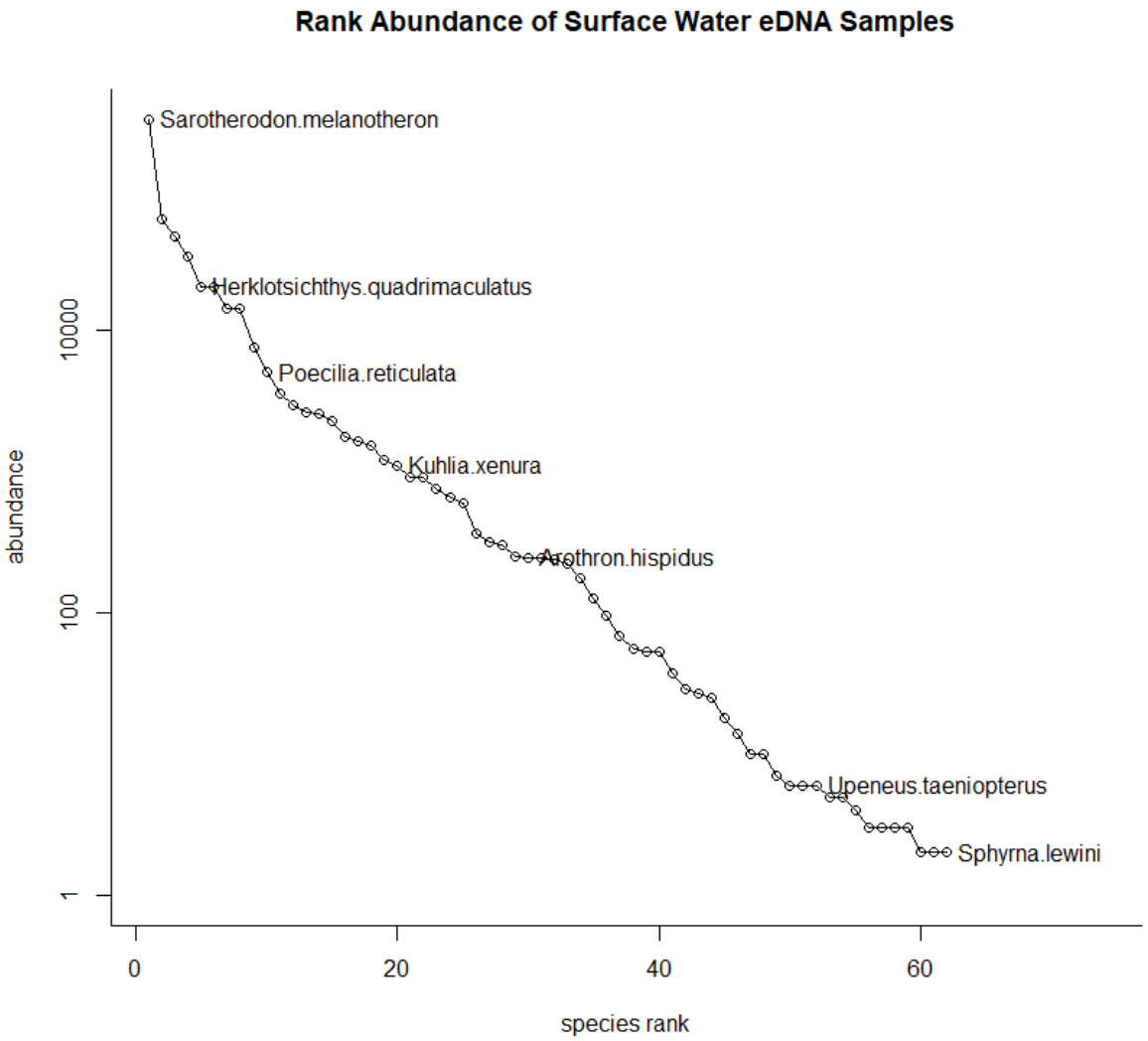


Figure 20: Relative abundance of reads detected for fish species observed in surface column eDNA samples from all downstream transects in surveys PH2-PH6. Surface samples had a total number of 559,714 reads detected and reported as a logarithmic function on the y axis. Species rank on the x axis is according to the total number of reads detected per species found. A total of 62 fish species were found across surface samples. The 1st, 5th, 10th, 20th, 30th, last and 10th to the last ranked species is listed.

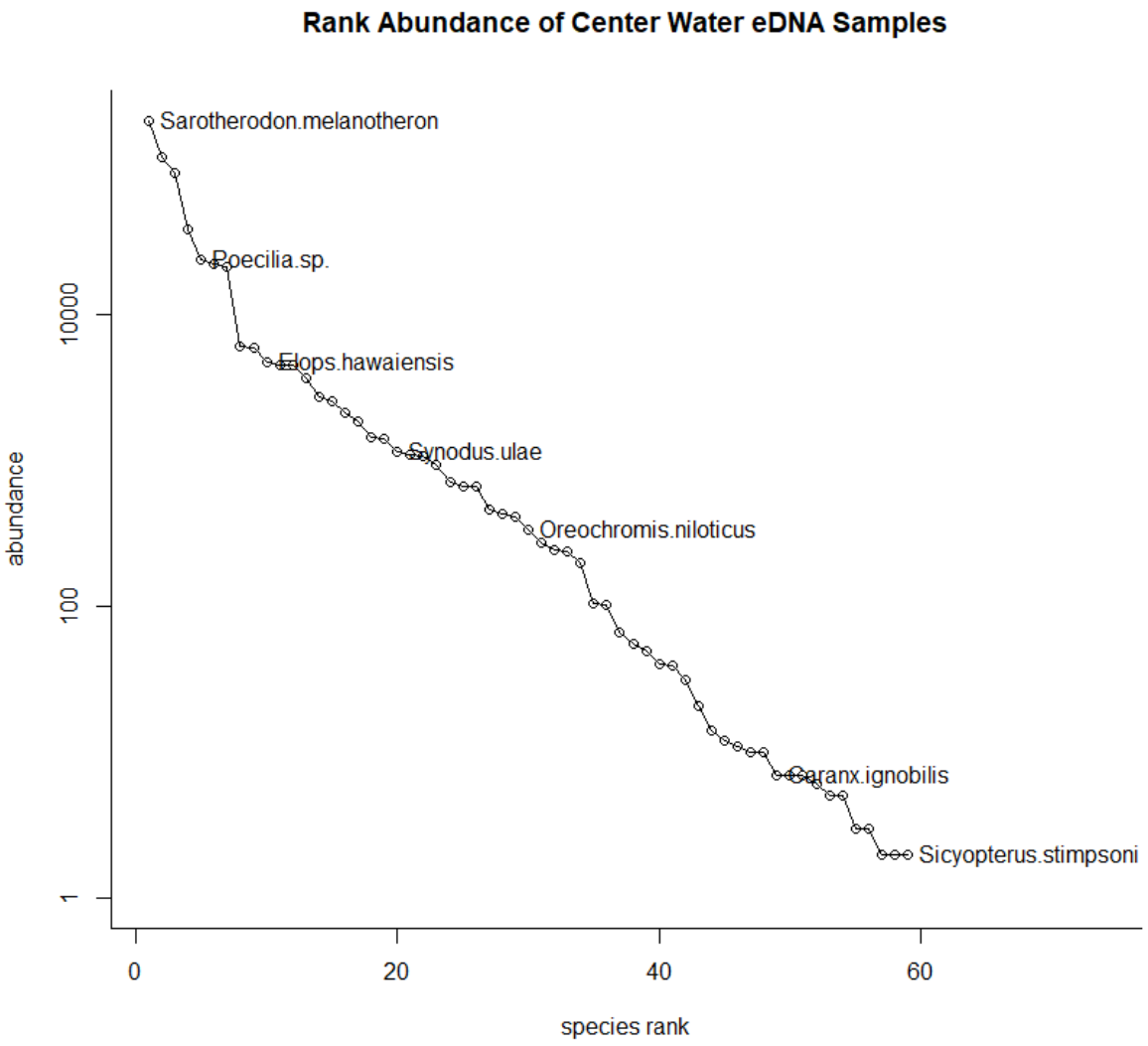


Figure 21: Relative abundance of reads detected for fish species observed in center column eDNA samples from all downstream transects in surveys PH2-PH6. Center samples had a total number of 586,610 reads detected and reported as a logarithmic function on the y axis. Species rank on the x axis is according to the total number of reads detected per species found. A total of 59 fish species were found across center samples. The 1st, 5th, 10th, 20th, 30th, last and 10th to the last ranked species is listed.

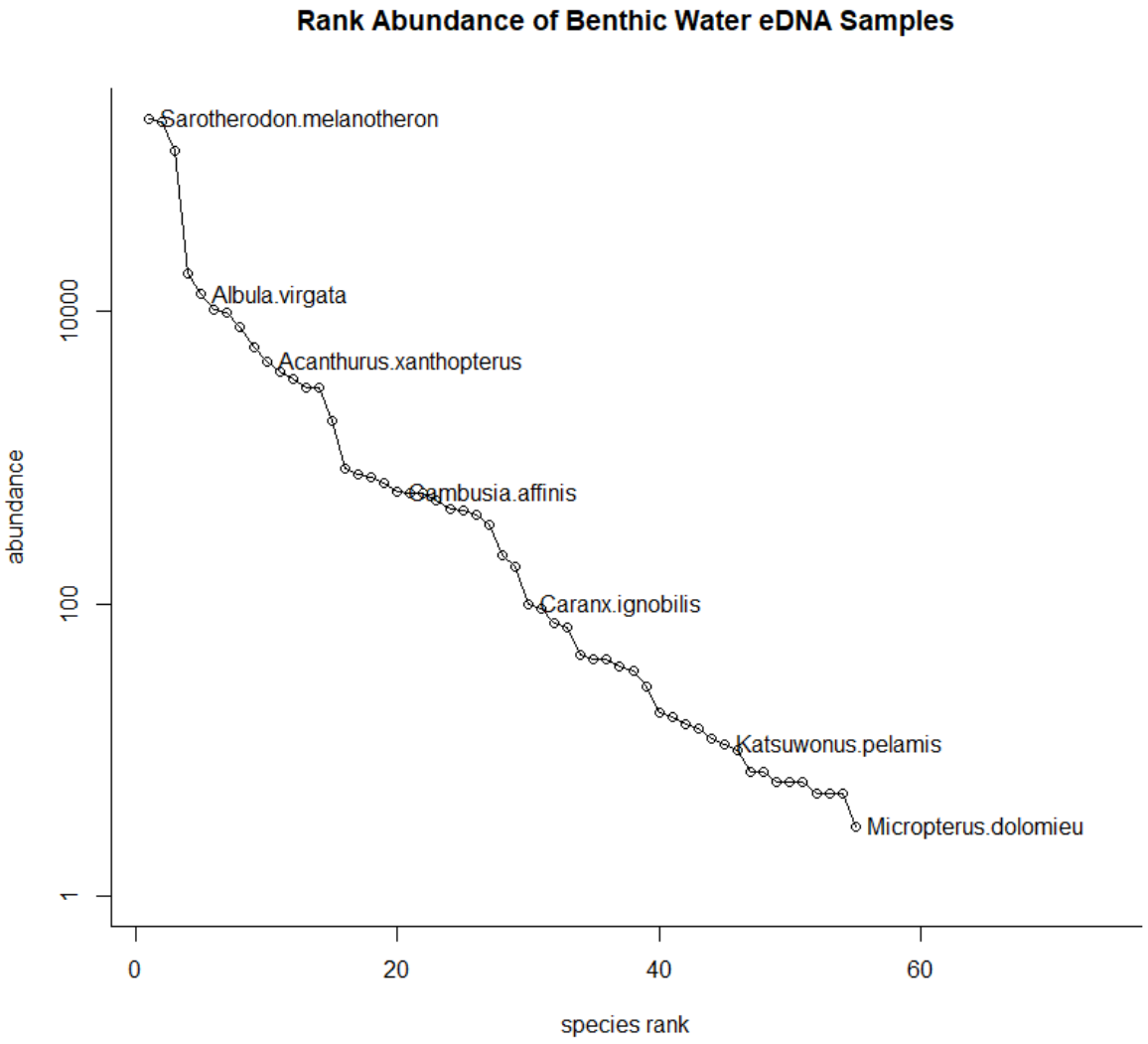


Figure 22: Relative abundance of reads detected for fish species observed in benthic column eDNA samples from all downstream transects in surveys PH2-PH6. Benthic samples had a total number of 62,563 reads detected and reported as a logarithmic function on the y axis. Species rank on the x axis is according to the total number of reads detected per species found. A total of 55 fish species were found across benthic samples. The 1st, 5th, 10th, 20th, 30th, last and 10th to the last ranked species is listed.

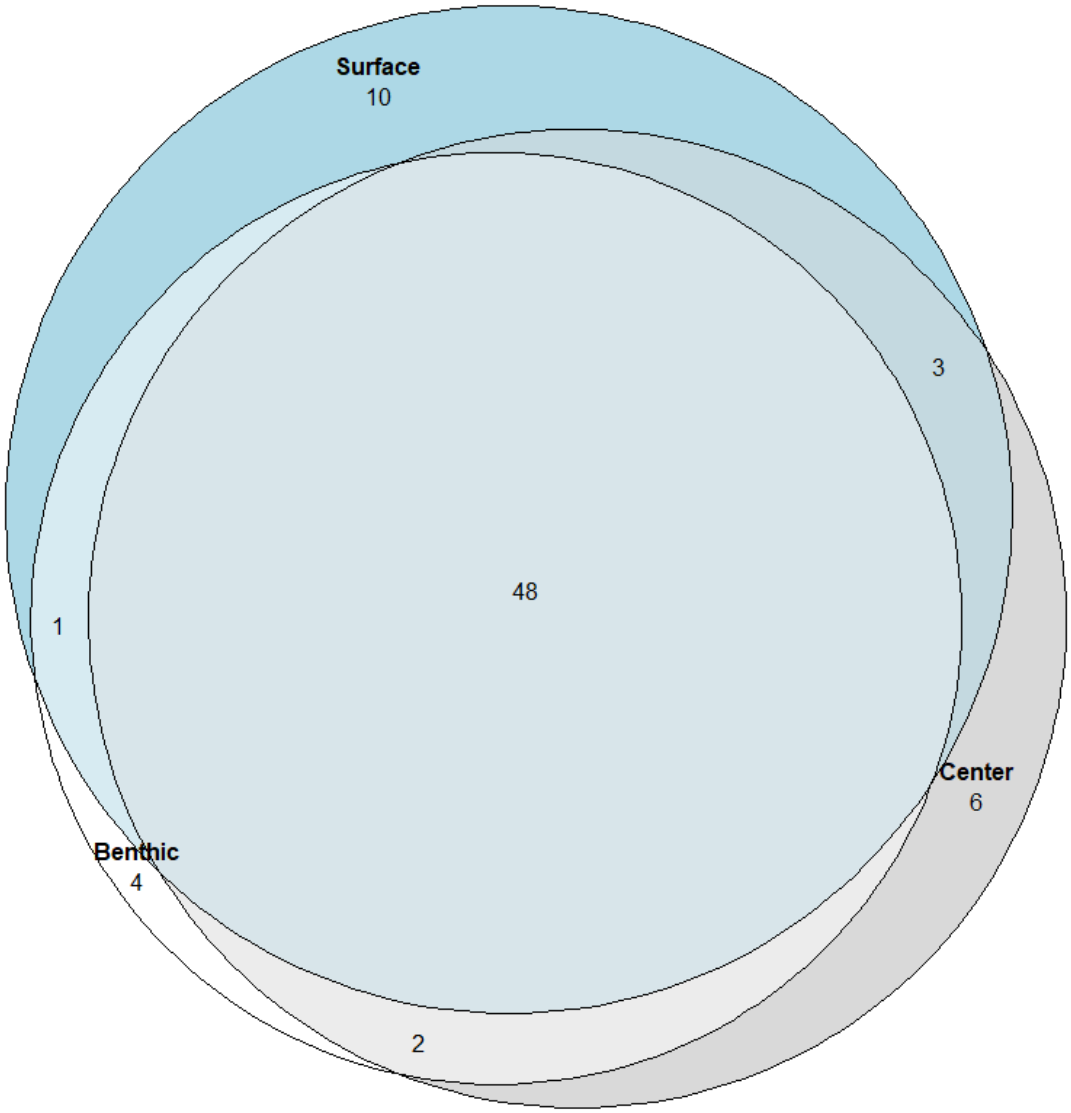


Figure 23: Venn Diagram of the number of unique and overlapping fish species between surface, center, and benthic eDNA water samples collected in the middle of downstream transects (i.e., excludes bank surface and benthic samples) from surveys PH2-PH6.

Appendices

Appendix A: Rank abundance and richness of fish species detected from middle surface, middle center, and middle benthic water eDNA samples collected at downstream transects in surveys PH2-PH6. Sampling depth location, species found, species rank based on the largest numbers of total reads (DNA material sequenced) within a total of 74 fish species, and proportion of DNA material sequenced for that species over the total number of reads sequenced for each sampling location is listed. Species with 0 read abundance values were not detected in those sampling locations although ranked out of 74.

Surface Species	rank	reads	proportion
Sarotherodon.melanotheron	1	309639	55.3
Encrasicholina.purpurea	2	61133	10.9
Hypostomus.watwata	3	45817	8.2
Poecilia.sp.	4	33118	5.9
Herklotsichthys.quadrimaculatus	5	20377	3.6
Oxyurichthys.lonchotus	6	20120	3.6
Ancistrus sp.	7	14416	2.6
Awaous.stamineus	8	14387	2.6
Albula.virgata	9	7547	1.3
Poecilia.reticulata	10	5014	0.9
Amatitlania.nigrofasciata	11	3589	0.6
Ameiurus.nebulosus	12	2940	0.5
Gambusia.affinis	13	2641	0.5
Mugil.cephalus	14	2591	0.5
Clarias.fuscus	15	2295	0.4
Hemichromis.elongatus	16	1755	0.3
Oreochromis.niloticus	17	1645	0.3
Stenogobius.hawaiiensis	18	1528	0.3
Mugilogobius.cavifrons	19	1213	0.2
Kuhlia.xenura	20	1095	0.2
Elops.hawaiensis	21	915	0.2
Xiphophorus.hellerii	22	905	0.2
Acanthurus.xanthopterus	23	750	0.1
Eleotris.sandwicensis	24	651	0.1
Sardinella.marquesensis	25	597	0.1
Synodus.ulae	26	366	0.1
Amphilophus.sp.	27	316	0.1
Scomberoides.lysan	28	304	0.1
Acanthurus.nigrofuscus	29	249	0
Arothron.hispidus	30	244	0
Moolgarda.engeli	31	242	0
Caranx.sexfasciatus	32	239	0

Thunnus.albacares.obesus	33	220	0
Acanthurus.olivaceus	34	177	0
Mulloidichthys.vanicolensis	35	127	0
Chanos.chanos	36	95	0
Mulloidichthys.flavolineatus	37	68	0
Caranx.melampygus	38	56	0
Abudefduf.sordidus	39	53	0
Parachromis.managuensis	40	53	0
Asterropteryx.semipunctata	41	37	0
Sphyraena.barracuda	42	29	0
Caranx.ignobilis	43	27	0
Zebrasoma.flavescens	44	25	0
Chromis.vanderbilti	45	18	0
Zanclus.cornutus	46	14	0
Chromis.agilis	47	10	0
Diodon.hystrix	48	10	0
Saurida.nebulosa	49	7	0
Hyporhamphus.yuri	50	6	0
Sargocentron.punctatissimum	51	6	0
Upeneus.taeniopterus	52	6	0
Rhinecanthus.rectangulus	53	5	0
Sicyopterus.stimpsoni	54	5	0
Micropterus.dolomieu	55	4	0
Monopterus.albus	56	3	0
Parupeneus.chrysonemus	57	3	0
Pethia.ticto	58	3	0
Triaenodon.obesus	59	3	0
Gymnothorax.undulatus	60	2	0
Katsuwonus.pelamis	61	2	0
Sphyrna.lewini	62	2	0
Abudefduf.vaigiensis	63	0	0
Amphilophus.citrinellus	64	0	0
Atherinomorus.insularum	65	0	0
Chaetodon.ephippium	66	0	0
Foa.brachygramma	67	0	0
Galeocerdo.cuvier	68	0	0
Gobiosoma.sp.(LT.2015)	69	0	0
Kyphosus.elegans	70	0	0
Kyphosus.vaigiensis	71	0	0
Labroides.phthirophagus	72	0	0
Psilogobius.mainlandi	73	0	0
Tetrapturus.angustirostris	74	0	0

Center Species	rank	reads	proportion
Sarotherodon.melanotheron	1	213909	36.5
Oxyurichthys.lonchotus	2	120892	20.6
Encrasicholina.purpurea	3	94211	16.1
Albula.virgata	4	38646	6.6
Poecilia.sp.	5	23801	4.1
Hypostomus.watwata	6	22527	3.8
Herklotsichthys.quadrimaculatus	7	21485	3.7
Acanthurus.xanthopterus	8	6033	1
Ancistrus sp.	9	5967	1
Elops.hawaiensis	10	4717	0.8
Mugil.cephalus	11	4541	0.8
Chanos.chanos	12	4486	0.8
Poecilia.reticulata	13	3658	0.6
Ameiurus.nebulosus	14	2741	0.5
Awaous.stamineus	15	2588	0.4
Asterropteryx.semipunctata	16	2144	0.4
Xiphophorus.hellerii	17	1877	0.3
Clarias.fuscus	18	1459	0.2
Mugilogobius.cavifrons	19	1417	0.2
Synodus.ulae	20	1153	0.2
Gambusia.affinis	21	1090	0.2
Kuhlia.xenura	22	1073	0.2
Thunnus.albacares.obesus	23	936	0.2
Arothron.hispidus	24	708	0.1
Amatitlania.nigrofasciata	25	667	0.1
Mulloidichthys.flavolineatus	26	661	0.1
Sardinella.marquesensis	27	465	0.1
Stenogobius.hawaiiensis	28	435	0.1
Scomberoides.lysan	29	411	0.1
Oreochromis.niloticus	30	337	0.1
Diodon.hystrix	31	274	0
Acanthurus.nigrofuscus	32	245	0
Moolgarda.engeli	33	238	0
Eleotris.sandwicensis	34	201	0
Abudefduf.sordidus	35	105	0
Mulloidichthys.vanicolensis	36	102	0
Hemichromis.elongatus	37	67	0
Abudefduf.vaigiensis	38	56	0
Amphilophus.sp.	39	50	0
Atherinomorus.insularum	40	40	0
Caranx.melampygus	41	39	0
Sargocentron.punctatissimum	42	31	0
Sphyraena.barracuda	43	21	0

Labroides.phthirophagus	44	14	0
Sphyrna.lewini	45	12	0
Chromis.agilis	46	11	0
Foa.brachygramma	47	10	0
Upeneus.taeniopterus	48	10	0
Caranx.ignobilis	49	7	0
Gymnothorax.undulatus	50	7	0
Katsuwonus.pelamis	51	7	0
Micropterus.dolomieu	52	6	0
Galeocerdo.cuvier	53	5	0
Gobiosoma sp.	54	5	0
Psilogobius.mainlandi	55	3	0
Saurida.nebulosa	56	3	0
Amphilophus.citrinellus	57	2	0
Caranx.sexfasciatus	58	2	0
Sicyopterus.stimpsoni	59	2	0
Acanthurus.olivaceus	60	0	0
Chaetodon.ephippium	61	0	0
Chromis.vanderbilti	62	0	0
Hyporhamphus.yuri	63	0	0
Kyphosus.elegans	64	0	0
Kyphosus.vaigiensis	65	0	0
Monopterus.albus	66	0	0
Parachromis.managuensis	67	0	0
Parupeneus.chrysonemus	68	0	0
Pethia.ticto	69	0	0
Rhinecanthus.rectangulus	70	0	0
Tetrapturus.angustirostris	71	0	0
Triaenodon.obesus	72	0	0
Zanclus.cornutus	73	0	0
Zebrasoma.flavescens	74	0	0

Benthic	rank	reads	proportion
Sarotherodon.melanotheron	1	208123	33.3
Oxyurichthys.lonchotus	2	198417	31.7
Encrasicholina.purpurea	3	126408	20.2
Herklotsichthys.quadrimaculatus	4	18108	2.9
Albula.virgata	5	13152	2.1
Hypostomus.watwata	6	10409	1.7
Poecilia.sp.	7	9761	1.6
Chanos.chanos	8	7860	1.3
Mugil.cephalus	9	5659	0.9
Acanthurus.xanthopterus	10	4563	0.7
Elops.hawaiensis	11	3889	0.6

Ancistrus sp.	12	3437	0.5
Poecilia.reticulata	13	3035	0.5
Awaous.stamineus	14	3012	0.5
Moolgarda.engeli	15	1806	0.3
Xiphophorus.hellerii	16	848	0.1
Mugilogobius.cavifrons	17	765	0.1
Asterropteryx.semipunctata	18	732	0.1
Clarias.fuscus	19	678	0.1
Gambusia.affinis	20	580	0.1
Sardinella.marquesensis	21	576	0.1
Hemichromis.elongatus	22	568	0.1
Kuhlia.xenura	23	508	0.1
Oreochromis.niloticus	24	449	0.1
Scomberoides.lysan	25	435	0.1
Thunnus.albacares.obesus	26	404	0.1
Synodus.ulae	27	349	0.1
Eleotris.sandwicensis	28	215	0
Acanthurus.nigrofuscus	29	179	0
Caranx.ignobilis	30	100	0
Amatitlania.nigrofasciata	31	93	0
Arothron.hispidus	32	74	0
Sphyraena.barracuda	33	69	0
Kyphosus.vaigiensis	34	45	0
Mulloidichthys.flavolineatus	35	42	0
Mulloidichthys.vanicolensis	36	42	0
Caranx.melampygus	37	37	0
Tetrapturus.angustirostris	38	35	0
Diodon.hystrix	39	27	0
Sargocentron.punctatissimum	40	18	0
Saurida.nebulosa	41	17	0
Gymnothorax.undulatus	42	15	0
Ameiurus.nebulosus	43	14	0
Stenogobius.hawaiiensis	44	12	0
Katsuwonus.pelamis	45	11	0
Caranx.sexfasciatus	46	10	0
Amphilophus.sp.	47	7	0
Atherinomorus.insularum	48	7	0
Abudefduf.sordidus	49	6	0
Chaetodon.ephippium	50	6	0
Sphyrna.lewini	51	6	0
Chromis.vanderbilti	52	5	0
Kyphosus.elegans	53	5	0
Psilogobius.mainlandi	54	5	0
Micropterus.dolomieu	55	3	0

Abudefduf.vaigiensis	56	0	0
Acanthurus.olivaceus	57	0	0
Amphilophus.citrinellus	58	0	0
Chromis.agilis	59	0	0
Foa.brachygramma	60	0	0
Galeocerdo.cuvier	61	0	0
Gobiosoma sp.	62	0	0
Hyporhamphus.yuri	63	0	0
Labroides.phthirophagus	64	0	0
Monopterus.albus	65	0	0
Parachromis.managuensis	66	0	0
Parupeneus.chrysonemus	67	0	0
Pethia.ticto	68	0	0
Rhinecanthus.rectangulus	69	0	0
Sicyopterus.stimpsoni	70	0	0
Triaenodon.obesus	71	0	0
Upeneus.taeniopterus	72	0	0
Zanclus.cornutus	73	0	0
Zebrasoma.flavescens	74	0	0

Appendix B: Ranked detection of species between eDNA and traditional sampling schemes. Presence of species observed most frequently across surveys are ranked highest in separate comparisons of species found via eDNA and traditional methods. The proportion of detections for species as well as the abundance of each species found is listed along side ranked values.

eDNA Detections	proportion	abundance	rank
Sarotherodon.melanotheron	6.1	106	1
Oxyurichthys lonchotus	5.7	99	2
Encrasicholina purpurea	5.3	92	3
Poecilia.sp.	4.9	85	4
Gambusia.affinis	4.2	74	5
Mugilogobius.cavifrons	4.1	71	6
Mugil.cephalus	3.8	66	7
Eleotris.sandwicensis	3.7	65	8
Albula.virgata	3	53	9
Awaous.stamineus	3	52	10
Kuhlia.xenura	2.8	48	11
Sardinella.marquesensis	2.8	48	12
Herklotsichthys.quadrimaculatus	2.6	46	13
Thunnus.albacares.obesus	2.5	44	14
Acanthurus.xanthopterus	2.4	42	15
Hypostomus.watwata	2.4	41	16
Oreochromis.niloticus	2.3	40	17
Clarias.fuscus	2.2	38	18
Poecilia.reticulata	2.1	36	19
Moolgarda.engeli	1.9	33	20
Stenogobius.hawaiiensis	1.9	33	21
Xiphophorus.hellerii	1.9	33	22
Elops.hawaiensis	1.8	31	23
Scomberoides.lysan	1.8	31	24
Ancistrus.cf..temminckii.MR.2020	1.7	29	25
Amatitlania.nigrofasciata	1.6	28	26
Hemichromis.elongatus	1.6	28	27
Arothron.hispidus	1.4	24	28
Chanos.chanos	1.4	24	29
Asterropteryx.semipunctata	1.1	20	30
Amphilophus.sp.	1.1	19	31
Caranx.melampygus	1.1	19	32
Caranx.ignobilis	1	18	33
Mulloidichthys.flavolineatus	0.9	15	34
Acanthurus.nigrofuscus	0.7	12	35
Mulloidichthys.vanicolensis	0.7	12	36
Diodon.hystrix	0.6	11	37

Micropterus.dolomieu	0.6	11	38
Sphyraena.barracuda	0.6	11	39
Synodus.ulae	0.6	10	40
Saurida.nebulosa	0.5	9	42
Caranx.sexfasciatus	0.5	8	43
Abudefduf.sordidus	0.4	7	45
Gymnothorax.undulatus	0.3	6	46
Katsuwonus.pelamis	0.3	6	47
Sargocentron.punctatissimum	0.3	6	48
Ameiurus.nebulosus	0.3	5	49
Chromis.vanderbilti	0.3	5	50
Upeneus.taeniopterus	0.3	5	51
Zebrasoma.flavescens	0.3	5	52
Atherinomorus.insularum	0.2	4	53
Kyphosus.elegans	0.2	4	54
Parachromis.managuensis	0.2	4	55
Psilogobius.mainlandi	0.2	4	56
Sicyopterus.stimpsoni	0.2	4	57
Sphyrna.lewini	0.2	4	58
Zanclus.cornutus	0.2	4	59
Hyporhamphus.yuri	0.2	3	60
Monopterus.albus	0.2	3	61
Abudefduf.vaigiensis	0.1	2	62
Acanthurus.olivaceus	0.1	2	63
Canthigaster.amboinensis	0.1	2	64
Carcharhinus.limbatus	0.1	2	65
Chromis.agilis	0.1	2	66
Kyphosus.vaigiensis	0.1	2	67
Parupeneus.chrysonemus	0.1	2	68
Amphilophus.citrinellus	0.1	1	69
Bathygobius.cocosensis	0.1	1	70
Chaetodon.ephippium	0.1	1	71
Cyprinus.carpio	0.1	1	72
Foa.brachygramma	0.1	1	73
Galeocerdo.cuvier	0.1	1	74
Gobiosoma.sp..LT.2015	0.1	1	75
Hemiramphus.lutkei	0.1	1	76
Labroides.phthirophagus	0.1	1	77
Lutjanus.fulvus	0.1	1	78
Novaculichthys.taeniourus	0.1	1	79
Pethia.ticto	0.1	1	80
Plectroglyphidodon.imparipennis	0.1	1	81
Rhinecanthus.rectangulus	0.1	1	82
Scarus.rubroviolaceus	0.1	1	83

Tetrapturus.angustirostris	0.1	1	84
Triaenodon.obesus	0.1	1	85
Xenoconger.fryeri	0.1	1	86

Traditional Detections	rank	abundance	proportion
Sarotherodon melanotheron	1	21	8.8
Caranx melampygus	2	18	7.5
Scomberoides lysan	3	16	6.7
Sphyraena barracuda	4	16	6.7
Herklotsichthys quadrimaculatus	5	15	6.2
Moolgarda engeli	6	15	6.2
Caranx ignobilis	7	12	5
Synodus ulae	8	11	4.6
Poecilia sphenops	9	10	4.2
Encrasicholina purpurea	10	9	3.8
Mugil cephalus	11	9	3.8
Albula glossodonta	12	8	3.3
Actinopterygii sp.	13	5	2.1
Lutjanus fulvus	14	5	2.1
Acanthurus xanthopterus	15	4	1.7
Eleotris sandwicensis	16	4	1.7
Oxyurichthys lonchotus	17	4	1.7
Upeneus taeniopterus	18	4	1.7
Acanthurus blochii	19	3	1.2
Arothron meleagris	20	3	1.2
Gnatholepis anjerensis	21	3	1.2
Kuhlia xenura	22	3	1.2
Poecilia sp.	23	3	1.2
Acanthurus nigrofuscus	24	2	0.8
Acanthurus sp.	25	2	0.8
Carangoides ferdau	26	2	0.8
Caranx sexfasciatus	27	2	0.8
Enneapterygius atriceps	28	2	0.8
Gobiidae sp.	29	2	0.8
Gymnothorax undulatus	30	2	0.8
Mulloidichthys flavolineatus	31	2	0.8
Poecilia mexicana/salvatoris	32	2	0.8
Abudefduf abdominalis	33	1	0.4
Acanthurus nigroris	34	1	0.4
Anchoa choerostoma	35	1	0.4
Arothron hispidus	36	1	0.4
Caranx sp.	37	1	0.4

Chanos chanos	38	1	0.4
Clupeidae sp.	39	1	0.4
Dasyatis lata	40	1	0.4
Diodon holocanthus	41	1	0.4
Diodon hystrix	42	1	0.4
Elops hawaiensis	43	1	0.4
Gnathanodon speciosus	44	1	0.4
Hippocampus fisheri	45	1	0.4
Hippocampus kuda	46	1	0.4
Kuhlia sandvicensis	47	1	0.4
Lstiblennius zebra	48	1	0.4
Neomyxus leuciscus	49	1	0.4
Sardinella marquesensis	50	1	0.4
Stenogobius hawaiiensis	51	1	0.4
Synodus dermatogenys	52	1	0.4
Tetraodontidae sp.	53	1	0.4