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**METHODS**

1. *Sample collection, filtration, and preservation*

Field work for this study occurred in the traditional territories of the Salinan Tribe, Chumash, Tongva, and Acjachemen Nations in central and southern California in February and June 2021. We collected ten replicate seawater samples with 1L Nalgene bottles from mainland rocky intertidal areas at existing long-term biodiversity monitoring sites (n=8; Fig. 1). Seawater collection occurred simultaneously or within 1 hour of long-term biodiversity surveys, which involve real-time visual identification of macroinvertebrates and macrophytes by taxonomic experts using point-contact, quadrat, and swath surveys along parallel transects within an area defined by a 30m along-shore upper baseline. Visual protocol details have been described elsewhere (MARINe 2023). Seawater samples were distributed across upper, middle, and lower habitat zones, discrete tide pools, and adjacent surf-zone edges of the survey area in an effort to represent intertidal microhabitats associated with zonation and account for variable access to seawater at low tide (Shea & Boehm 2023). Collection depth ranged from 0-2 m. In all cases, sampling occurred within established visual transect areas.

Within 1 hour of collection, samples were filtered with peristaltic pumps and 0.45 µm Sterivex capsules then preserved at ambient temperature with Longmire’s buffer until DNA extraction (Renshaw et al. 2015). We included a filtration control of 1L deionized water at each sampling event, which was prepared, transported, and processed alongside seawater samples throughout the experimental workflow. To prevent cross-contamination, we treated sampling and filtration equipment and surfaces with 0.6-0.75% sodium hypochlorite followed by clean-water rinses and storage in clean containers. Sampling bottles, tubing, and Longmire’s reagents were additionally UV-irradiated.

2. *DNA extraction and sequencing*

DNA extractions occurred in a PCR-free laboratory where working surfaces and equipment were treated with 0.6-0.75% sodium hypochlorite followed by 70% ethanol. We followed a DNA isolation protocol developed by Spens et al. (2017) and included 1 negativecontrol per batch of extractions. Extraction products were eluted in 200 ul Buffer AE (Qiagen), quantified using Qubit 2.0 HS dsDNA assays, and stored at -20°C until sequencing preparation.

Based on previous eDNA metabarcoding applications for characterizing marine eukaryote diversity and preliminary testing with extraction products, three primer sets were selected to amplify COI, 18S-V9, and 16S gene regions (Table 1). Sequencing preparation for each marker involved a two-step PCR protocol with initial amplicon PCRs performed in triplicate. These PCR products were pooled by sample, checked for amplification with gel electrophoresis (Agilent D1000 ScreenTape), and cleaned with AMPure XP beads (Beckman Coulter) to size-select for target amplicon length. We quantified cleaned, pooled sample yields with Quantifluor dsDNA fluorometric assays (Promega) and normalized concentrations to 10 ng.

A second PCR was then performed to add unique dual index sequences (IDT for Illumina) to amplicons by sample and marker. Indexed products were cleaned, quantified, and pooled by equal mass, targeting a final concentration of 10-15 nM. Due to differences in target region length, we sequenced COI and 16S+18S amplicons separately using a paired-end 300-bp MiSeq system (100-bp for 16S+18S) with reagent v3 600-cycle kit and 15% PhiX control v3 library (Illumina). Three no-template PCR controls were amplified and sequenced along with samples (n=80), filtration blanks (n=8), and pooled extraction controls (n=5), resulting in 96 samples sequenced per marker. See Supplementary Material for amplicon and indexing PCR chemistry, cycling parameters, and additional protocol details.

Table 1. Metabarcoding primers used in this study.

| Marker | Length | Reference | Forward and reverse primers |
| --- | --- | --- | --- |
| COI - Leray XT | 313 bp | Wangensteen et al. 2018 | mlCOIintF-XT: 5′-GGWACWRGWTGRACWITITAYCCYCC  jgHCO2198: 5′-TAIACYTCIGGRTGICCRAARAAYCA |
| 16S - Metazoa | 114-140 bp | Kelly et al. 2016 | 16s\_Metazoa\_fwd: 5’-AGTTACYYTAGGGATAACAGCG  16s\_Metazoa\_rev: 5’-CCGGTCTGAACTCAGATCAYGT |
| 18S-V9 | 130 bp | Amaral-Zettler et al. 2009 | 1380F: 5’-CCCTGCCHTTTGTACACAC  1510R: 5’-CCTTCYGCAGGTTCACCTAC |

3. *Bioinformatic filtering and taxonomic assignment*

Raw, demultiplexed sequencing files were initially processed to merge paired-end reads then remove primers and adapters with cutadapt (v4.0) using parameters --trim-n, --max-n=0, --minimum\_length=50, and --quality\_cutoff=15 (Martin 2011). Merged, trimmed, quality-filtered reads were passed to DADA2 (v1.22.0) for denoising using parameters --maxEE=12, --min\_ov=10, and –max\_mismatch=1 (Callahan et al. 2016). Chimeras were flagged and removed using vsearch (v2.22.1) with parameters --abskew=16.0 and –fasta\_width=120 (Rognes et al. 2016). Remaining sequences were clustered at 100% identity with cd-hit (v4.8.1) representing unique amplicon sequence variants (ASVs) (Fu et al. 2012, Li & Godzik 2006). We examined negative controls and removed ASVs found only in controls, as well as reads associated with contaminant ASVs by subtracting the number of reads in controls from the read count associated with the same ASVs in biological samples from the same field site. By this method, ASVs with more reads in a control than a sample would be excluded. We additionally removed any ASVs occurring only once (1 read) across ten field replicates per site.

We assigned taxonomy to remaining ASVs using *classify-consensus-vsearch* in QIIME 2 (v2022.8), which performs global pairwise alignment and is ideal when query and reference sequences are similar in length (Bolyen et al. 2019). Taxonomic assignment was implemented by first retaining all hits to amplicon-specific, curated regional reference databases with at least 98% identity (--p-maxaccepts all, --p-perc-identity 0.98, --p-quer-cov 0.8) and finding consensus among top hits (--p-consensus 0.51). Curated regional databases were generated using the CRABS software program (Jeunen et al. 2022). Briefly, we downloaded eukaryote sequence records from NCBI GenBank, BOLD, and MitoFish based on key-term queries for each marker region (Sayers et al. 2022, Ratnasingham & Hebert 2007, Sato et al. 2018). For each marker, we performed *in silico* PCR followed by pairwise global alignment to recover target amplicon regions. We then dereplicated database records to retain all unique sequences assigned to species rank and cleaned records based on length and ambiguous base content. Regional curation was accomplished by filtering records with a list of 2,962 northeast Pacific Coast marine species sourced from field guides, long-term rocky intertidal and kelp forest biodiversity surveys, government agency wildlife and fisheries lists, and species-rank occurrence records for the California Current Large Marine Ecosystem from the Ocean Biodiversity Information System (OBIS 2023) (Sept 2009, Malone et al. 2022, NOAA Fisheries 2022, California Fish & Wildlife 2022). See Supplementary Material for additional details.

Provisional species-level annotations were inspected prior to inclusion in downstream biodiversity analyses and comparisons with visual surveys. First, we identified species for which some reference records are identical in bp-sequence (an expected outcome when retaining all unique sequences per species with CRABS). We then flagged ASV annotations based on ambiguous reference records that cannot distinguish between species and manually reviewed the list of top reference sequence hits for each of those cases. We retained species-rank annotations if top hits with best percent identity and query coverage (closest to 100%) were unambiguous. If best hits corresponded to ambiguous reference records, then we annotated using the least common taxonomic rank among divergent annotations (genus, family, order, etc). Consequently, ASVs reassigned to higher ranks were excluded from downstream analyses.

*Post-hoc:* Sequencing broad eukaryote and metazoan markers, it was not surprising eDNA detected many vertebrates active in nearshore habitats like bottlenose dolphin, northern elephant seal, southern sea otter, harbor seal, white shark, and 26 additional fishes. Since we sampled seawater not only from tide pools but also from adjacent submerged areas and incoming swell, we expected to collect and detect DNA from nearby subtidal zones and possibly further offshore. Interestingly, 97% of the 173 eDNA-only detections came from diverse species with documented intertidal and nearshore habitat associations. A post-hoc survey of habitat information sourced from FishBase and SeaLifeBase revealed 32 species are exclusively intertidal (Froese & Pauly 2010).