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Water stratification in the marine biome restricts vertical environmental DNA (eDNA) signal dispersal

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Abstract

Background: The utility of environmental DNA (eDNA) metabarcoding surveys to accurately detect species depends on the degree of DNA dispersal. Multiple marine studies have observed only minimal eDNA transport by horizontal water movement across small spatial scales, leading to the conclusion that spatially specific eDNA signals accurately resemble in-field species assemblages along a horizontal axis. Marine communities, however, are also structured vertically according to depth. In marine environments displaying permanent water stratification, vertical zonation patterns may be more apparent and present on smaller spatial scales (i.e., meters) than horizontal community structuring. The scale at which eDNA signals differ along a vertical transect and the accuracy of eDNA metabarcoding in revealing naturally stratified communities have yet to be assessed.

Methods and results: In this study, we determined the ability of eDNA metabarcoding surveys to distinguish vertically localized community assemblages. To test this, we sampled three vertical transects along a steep rock wall at three depths (0 m, 4 m, 15 m), covering two distinct communities that were separated by near-permanent water column stratification in the form of a strong halocline at ~3 m. Using three metabarcoding assays, our eDNA metabarcoding survey detected 54 taxa, across 46 families and 7 phyla, including 19 fish, 15 crustacean, and 8 echinoderm species. Ordination and cluster analyses show distinct eDNA signals across the halocline for all three replicate transects, suggesting that vertical dispersal of eDNA between communities was limited. Furthermore, eDNA signals of individual taxa were only retrieved within their observed vertical distribution, providing biological validation for the obtained results. Our results demonstrate, for the first time, the need to take into consideration oceanographic (e.g. water column stratification) and biological processes (e.g. vertical community structuring) when designing sampling strategies for marine eDNA metabarcoding surveys.

KEYWORDS

biodiversity assessment, marine eDNA, metabarcoding, spatial resolution

1 | INTRODUCTION

Environmental DNA (eDNA) metabarcoding is the process by which high-throughput amplicon sequencing simultaneously identifies multiple organisms from genetic material obtained directly from environmental samples in the absence of biological source material (Taberlet et al. 2012; Eichmiller et al. 2016). By circumventing the need for visual species observation (Goldberg et al., 2016), eDNA metabarcoding has the potential to greatly reduce cost, time, and invasiveness of sampling; while simultaneously increasing the number of taxonomic groups to be surveyed, and thereby aiding ecosystem conservation and management (Thomsen and Willerslev 2015). The recognized potential of eDNA research has led to an exponential increase in eDNA-related publications in recent years (Jeunen et al., 2019a).

Nevertheless, a lack of visual corroboration of species presence/absence in eDNA studies has led to skepticism about the accuracy of eDNA metabarcoding in the highly dynamic marine biome (Roussel et al. 2015). To address the issue of eDNA transport in aquatic environments due to water movement, several studies have investigated the horizontal spatial resolution of eDNA sampling (Port et al. 2016; Bista et al. 2017; O'Donnell et al. 2017; Yamamoto et al. 2017; Jeunen et al., 2019b); Stat et al. 2019). Such studies have concluded that there is a negligible impact of horizontal eDNA transport on species detection accuracy and a high correlation between coastal community composition and obtained eDNA signals at a fine scale (<1 km).

The structuring of marine communities, however, is not limited to horizontal variation. Instead, vertical zonation, defined as the distribution or natural layering of species and communities with shore height or water depth, is considered the most regionally consistent pattern (Chappuis et al. 2014), with distinct communities over scales of meters. Rocky shores, especially, display well-defined universal vertical zonation patterns of animal and plant assemblages when transitioning from the intertidal to subtidal marine environments (Barnes and Hughes 1999; Mariani et al. 2017). To date, however, only three studies have reported differences in vertebrate eDNA signals between surface water samples and samples taken close to the seafloor (Andruszkiewicz et al. 2017; Yamamoto et al. 2017; Lacoursière-Roussel et al. 2018), while a fourth study observed a difference in eDNA abundance for a sea star between surface water and water sampled closely to the seafloor (Uthicke et al. 2018).

Marine community assemblages can transition on finer spatial scales (Underwood 1981) than those previously assessed in marine eDNA metabarcoding studies. The intensity and scale at which marine communities are structured are strongly influenced by abiotic factors (Dutertre et al. 2013). Permanent water column stratification, such as between nonmixing water layers, has the ability to induce abrupt changes in community structures (Grange et al. 1981; Yu et al. 2014). While stratification of ocean water naturally occurs between surface water and deeper water layers (i.e., the deep pycnocline, >200 m) thereby facilitating ocean circulation (Capotondi et al. 2012), certain marine environments (e.g., fiords and hydrothermal vents) display permanent stratification on much smaller spatial scales. To date, no studies have investigated the impact of water stratification and the resulting vertical zonation patterns in the marine environment, on eDNA metabarcoding surveys. Where such structure is significant, we hypothesize that vertical transect sampling would be required to uncover distinct community patterns occurring at different depths.

One place with an extensive vertical zonation is the New Zealand fiords, which include Doubtful Sound, a fiord situated in the southwest region of New Zealand (Figure 1). Doubtful Sound is notable for its steep rock wall dropping down to deep water (>400 m), with a sharp, near-permanent, halocline separating the near-freshwater surface layer (~2–4 m thick) from the underlying full-salinity marine layer (Barker and Russell 2008). This persistent low-salinity layer (LSL) on the surface is the result of high rainfall, fiord morphology, and additional freshwater discharge from a large hydroelectric power scheme (Gibbs et al. 2000). The LSL contains high concentrations of colored dissolved organic matter of terrestrial origin that strongly absorbs light (Lamare et al. 2004). The difference in salinity between the LSL and the lower marine layer, together with the strong light attenuation, creates intense vertical zonation of plants and animals, with low species diversity in the intertidal region and highly diverse assemblages below the LSL that have a distinct vertical zonation with depth (Grange et al. 1981; Boyle et al. 2001; Rutger and Wing 2006).

In this study, we use Doubtful Sound as a test (model) system to determine the influence of water column stratification and

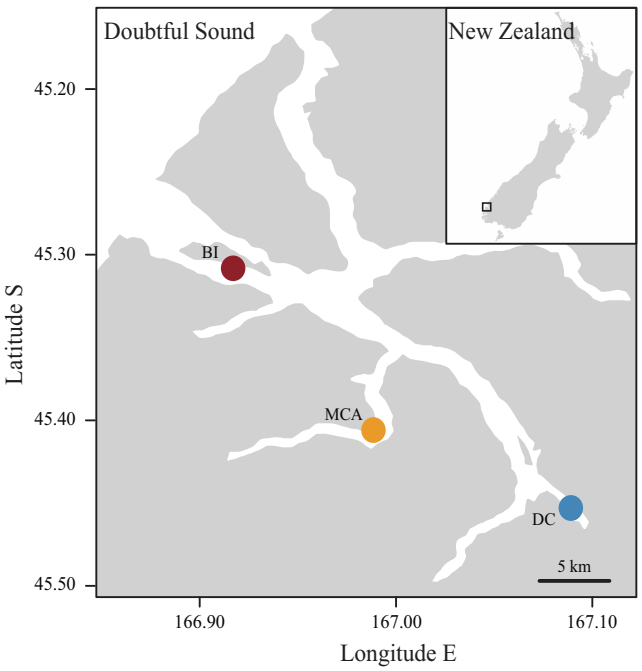

FIGURE 1 Map of Doubtful Sound, New Zealand with three sampling sites. Sampling sites are indicated by colored circles (DC: Deep Cove, blue; MCA: Mid-Crooked-Arm, orange; BI: Bauza Island, red)

associated vertical community zonation on multimarker eDNA metabarcoding surveys. We investigate the need for depth sampling to uncover the biodiversity on a steep-sided rocky shore in a temperate coastal ecosystem. We report environmental DNA metabarcoding data from three established assays, fish (16S), crustacean (16S), and eukaryote (COI) (Jeunen et al., 2019a), for water samples collected at three depths at three different sites within Doubtful Sound and ask the following two questions:

1. Does the biodiversity detected using eDNA metabarcoding discriminate between discrete depth stratified communities down a vertical transect?
2. How do community structure patterns correlate between eDNA-detected and theoretical species distributions?

2 | MATERIALS AND METHODS

2.1 | Sampling sites

The eDNA metabarcoding survey was undertaken at three sites in Doubtful Sound, Fiordland, New Zealand (Figure 1; Appendix S1). The first site, Deep Cove (DC), is situated 35 km from the fiord entrance. Deep Cove has a continuous significant input of freshwater (averaging 400–500 m^{3}/s) from the Manapouri hydroelectricity power scheme (Rutger and Wing 2006). This freshwater input has a major influence on the marine community in the cove (Rutger and Wing 2006). The second site, Mid-Crooked Arm (MCA), is situated in an arm branching off at the midpoint of the fiord, with communities less influenced by either the hydroelectric freshwater discharge or the Tasman Sea, into which Doubtful Sound empties (Boyle et al. 2001). The third site, Bauza Island (BI), is situated at the mouth of Doubtful Sound. The biota of Bauza Island is influenced mostly by the Tasman Sea, with a community assemblage less exposed to low-salinity surface waters and more closely resembling the zonation communities occurring along the adjacent exposed, rocky coastline of Fiordland (Brewin et al. 2008).

2.2 | eDNA sampling

The three fiord sites were sampled for eDNA biodiversity. Prior to sampling, the vertical water column structure at each site was profiled for temperature and salinity by CTD (RBR XR-420 Conductivity, Temperature, Depth Profiler; RBR Ltd, Ottawa, Canada) to determine the thickness of the variable LSL (Figure 2). Subsequently, three depths were sampled for each site: surface water within the LSL, the marine layer directly underneath the LSL (~4 m), and a fully marine sample (~15 m). Samples were taken ~20 min after CTD deployment, which occurred before high tide on 8 December 2016 using a Niskin bottle. Shallow samples were taken first to reduce artificial water mixing and reduce the risks of contamination of water between depths. To further reduce artificial water mixing, samples were taken in a small 3-seater speedboat with an idle engine during sampling. The Niskin bottle was left open at the required depth for ~10 min before samples were taken. Five, 2 L replicate water samples were taken at each sampling site and depth. Samples were transported back to the Marine Sciences Field Station situated at Deep Cove and filtered the same day.

2.3 | eDNA filtration and extraction

Bench space and equipment were sterilized by a 10-min exposure to 10% bleach solution (Prince and Andrus 1992). We decontaminated all sampling bottles (2 L, HDPE Natural, EPI Plastics) by rinsing twice with ultrapure water, submerging in 10% bleach for 10 min, and rinsing twice again with ultrapure water. To test for contamination during sampling, we filled two 2-L bottles with ultrapure water and placed them among the sampling bottles. We added negative filtration controls by filtering 500 ml ultrapure water and extracted two extraction blanks by replacing the filter with 500 µl ultrapure water in our extraction protocol. All negative controls were processed alongside the samples.

Sample processing followed the recommendations in Jeunen et al. (2019a). Briefly, water samples were filtered over a 1.2-µm cellulose nitrate filter (CN, Whatman™). After vacuum filtration (Laboport®, KNF Neuberger, Inc.), filters were rolled up, cut into ~ 1-mm slices, placed in 2-mL Eppendorf tubes, and stored overnight at −20°C. Samples were transported on dry ice to the Department of Zoology, University of Otago, and stored at −20°C until extraction the following day. DNA was extracted from the filter following a modified phenol–chloroform–isoamyl alcohol (PCI) DNA extraction protocol (Renshaw et al. 2015) and stored at −20°C until further processing.

2.4 | 4 eDNA metabarcoding

Library preparation followed the protocol described in Jeunen et al. (2019a). Briefly, samples were analyzed using three metabarcoding assays targeting two fragments of the 16S rRNA gene region and one fragment of the cytochrome c oxidase subunit I (COI) gene region (Appendix S2). Prior to library preparation, input DNA for each sample was optimized using a dilution series (neat, 1/5, 1/10) to identify inhibitors and low-template samples (Murray et al. 2015). Amplification was carried out in 25 µl reactions, prepared with 1× *Taq* Gold buffer (Applied Biosystems [ABI], USA), 2 mmol/L $MgCl_2$ (ABI, USA), 0.4 mg/ml BSA (Fisher Biotec, Australia), 0.25 mmol/L dNTPs (Astral Scientific, Australia), 0.4 µmol/L of each primer (Integrated DNA Technologies, Australia), 0.6 µl of 1/10,000 SYBR Green dye (Life Technologies, USA), 1 U of *Taq* polymerase Gold (ABI, USA), and 2 µl of DNA. qPCR conditions included an initial denaturing step at 95°C for 5 min; then 50 cycles of 30 s at 95°C, 30 s at 51–54°C (see annealing temperatures in Appendix S2), 45 s at 72°C; and a final extension of 10 min at 72°C.

A one-step amplification protocol was used for library building using fusion primers, which contained a modified Illumina sequencing adapter, a barcode tag (6–8 bp in length), and the template-specific primer. Each sample was amplified in duplicate and assigned a

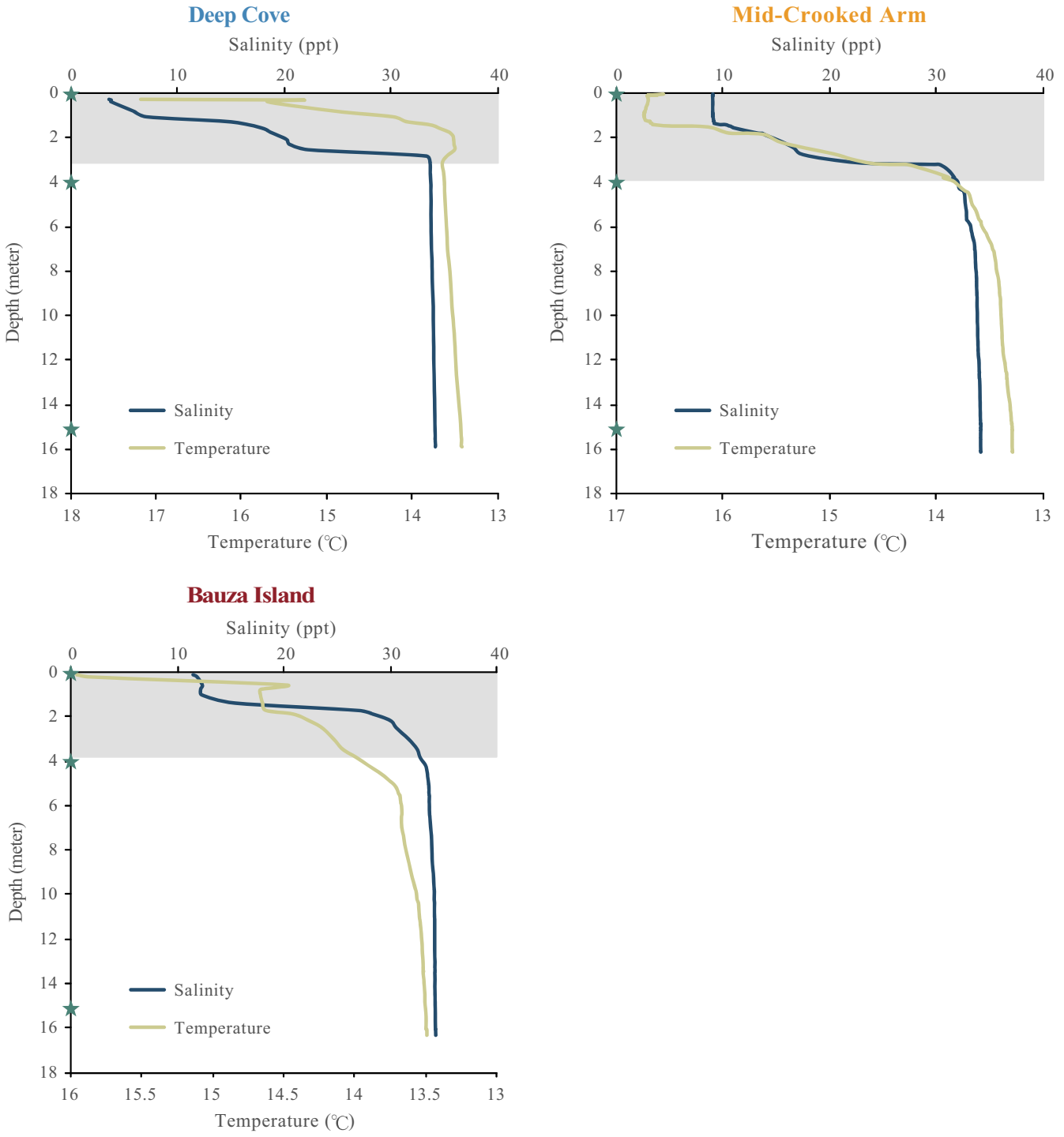

FIGURE 2 Depth profile as measured by CTD profiler for each of the three sampling sites in Doubtful Sound, New Zealand. Y-axis displays depth, while the top and bottom x-axes display salinity (blue line) and temperature (yellow line), respectively. The low-salinity layer (LSL) is indicated by the shaded gray area. Sampling depths (0, 4, and 15 m) are indicated by a green star

unique barcode combination to allow pooling of samples post-qPCR. qPCR conditions followed the amplification protocol described above, with qPCR duplicates of each sample pooled together to reduce stochastic effects from PCR amplification. Samples were then pooled to approximately equal molarity based on end-point qPCR fluorescence and normalized on LabChip GX Touch 24 (PerkinElmer, USA) to produce a single DNA library. The resultant library was size-selected using Pippin Prep (Sage Science, USA) and purified with Qiagen's QIAquick PCR Purification Kit (Qiagen GmbH, Hilden, Germany) prior to final library quantitation on LabChip and Qubit. Sequencing was performed on Illumina MiSeq ® (300 cycle, single-end kits for both 16S assays; and 500 cycle, paired-end kits for the COI assay), following the manufacturer's protocols, with 5% of PhiX to minimize issues associated with low-complexity libraries.

2.5 | Sequence analysis

Paired-end sequencing reads from the COI marker were merged using default settings in PEAR v 0.9.10 (Zhang et al. 2014). Reads were separated by barcode and assigned to samples using OBITools 1.2.11 (Boyer et al. 2016) using default settings. The assigned amplicons were filtered using USEARCH v11.0.667 (Edgar 2010) based on a maximum error of 0.1, minimum length of 100 for single-end reads and 250 for paired-end reads, and removal of singleton sequences and sequences containing ambiguous bases. The success of quality filtering was checked in FastQC v 0.11.7 (Andrews 2010) by comparing reports of FASTQ files before and after the bioinformatic pipeline. Reads passing quality filtering were denoised, and a ZOTU table (zero-radius OTU) was generated according to standard settings in USEARCH. The remaining ZOTUs were queried using BLASTn against the full NCBI database.

Taxonomic assignments from BLAST results were made by an in-house Python script (Appendix S3). Briefly, seven taxonomic ranks (i.e., Kingdom, Phylum, Class, Order, Family, Genus, and Species) were extracted from the NCBI Taxonomy Browser website for each BLAST hit per ZOTU. Assignments were based on the highest taxonomic rank shared between all BLAST hits per ZOTU. The BLAST and taxonomic assignment criteria led to species, genus, or family identification for all three assays. However, due to the lack of voucher specimens or a local reference database at our sampling site, highest taxonomic resolution was set at genus level for all three metabarcoding assays. Based on existing records of species occurrences within a genus in New Zealand (Ayling 1987; de Cook & Archer 2010), a "possible species ID" was added to the taxonomic assignment for BLAST hits achieving 100% similarity and covering 100% of the amplicon size (Appendix S4). BLAST hits resulting in unicellular picoplankton were discarded, as the focus of this study was on multicellular eukaryotes.

2.6 | Statistical analyses

Rarefaction curves were generated to assess sequencing coverage using the "rarecurve" function from the "VEGAN v 2.4–1." Package in R v 3.3.2. (R; http://www.R-project.org). All further statistical analyses were performed on presence–absence data for both eDNA (ZOTU and taxonomy) datasets, as the correlation between true abundance/biomass of a species and eDNA signal strength obtained through metabarcoding is uncertain (Ushio et al., 2017). Also, the one-step amplification approach could cause a loss of efficiency during qPCR or induce an amplification bias (O'Donnell et al. 2016; Alberdi et al. 2018), further reducing the correlation between eDNA signal strength and abundance/biomass of a species.

A permutational multivariate analysis of variance (PERMANOVA) was used to determine whether eDNA signals differed among depths, due to the presence of distinct communities caused by water stratification. A principal coordinate analysis (PCoA) was performed to visualize patterns of sample dissimilarity using the Jaccard index. Hierarchical cluster trees were also constructed using the unweighted pair group method with arithmetic mean (UPGMA) with bootstrap support to examine the robustness of sample clustering. Analyses were performed in R v 3.3.2. using the functions "vegdist," "pvclust," and "adonis" from the "vegan v 2.4–1." package. Indicator values were calculated for each species per sampling site using the R-package "labdsv." Upper limits were set for indicator species, that is, species driving the difference in eDNA signal between depths, to an indicator value index >0.70 and a p-value < .025 (Dufrêne and Legendre 1997). In this study, indicator values were used to determine the taxa driving the partitioning of samples between different sampling depths found in both ordination and cluster analyses. Habitat preference was used as biological validation of the eDNA signal difference found between locations.

3 | RESULTS

3.1 | Sequence analysis

Filtering and quality control returned 5,586,423 reads with 1,339,401, 1,548,054, and 2,698,968 reads for the fish (16S), crustacean (16S), and eukaryote (COI) metabarcoding assays, respectively. Overall, the eDNA samples achieved good sequencing coverage, based on rarefaction curves (Appendix S5) and mean number of reads per sample ± *SD*: fish (16S): 29,764 ± 8,251; crustacean (16S): 34,401 ± 12,751; and eukaryote (COI): 59,977 ± 11,809. No reads were returned after quality control and filtering for negative control samples.

3.2 | Taxonomic diversity

After stringent quality control and ZOTU clustering, we obtained a combined total of 1,658 ZOTUs with 62, 107, and 1,489 ZOTUs for the fish (16S), crustacean (16S), and eukaryote (COI) metabarcoding assays, respectively. BLAST returned a total of 56 taxa with 18, 12, and 26 taxa for the fish (16S), crustacean (16S), and eukaryote (COI) metabarcoding assays, respectively. The majority of ZOTUs for the eukaryote (COI) assay did not achieve taxonomic assignment due to the preferential amplification of unicellular picoplankton, stringent quality filtering steps, and lack of endemic species present in the reference database. Combining the datasets for all three metabarcoding assays, we were able to detect 54 genera covering 46 families and seven phyla (Appendix S4). Taxonomic assignment for the eukaryote (COI) metabarcoding assay identified taxa from the phyla Arthropoda (34.6%), Echinodermata (30.7%), and Miozoa (15.3%). The remaining phyla detected were Chordata (7.7%), Cnidaria (3.8%), Mollusca (3.8%), and Gastrotricha (3.8%).

3.3 | eDNA diversity pattern

The combined results from all metabarcoding assays showed comparable ZOTU/taxon richness patterns for samples taken at Bauza Island (BI) and Mid-Crooked Arm (MCA) (Figure 3; Appendix S4). The number of taxa (BI: 35; MCA: 35) and ZOTUs (BI: 1,168; MCA: 1,211) detected at both sites was highly similar. Furthermore, the highest taxonomic richness was obtained at depths under the halocline at 4

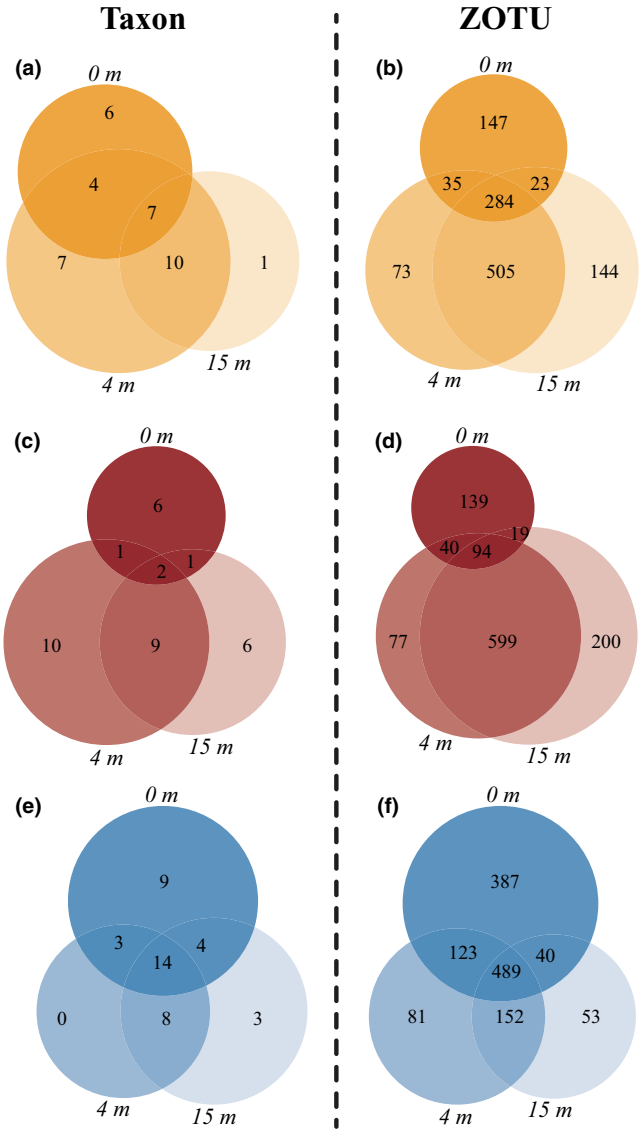

FIGURE 3 Venn diagram showing overlap in taxon (a, c, e) and ZOTU (b, d, f) detection between the three depths at each sampling site. (a, b) Mid-Crooked Arm; (c, d) Bauza Island; and (e, f) Deep Cove. Numbers represent the number of ZOTUs/taxa detected summed over five replicates

and 15 m. eDNA signals within the marine layer, also, showed more taxonomic commonalities, while eDNA signals obtained from the LSL layer were more distinct. The eDNA signals retrieved at Deep Cove (DC), on the other hand, displayed the highest richness in the LSL layer (Figure 3; Appendix S4). Compared to both other sites, the total number of taxa (41) and ZOTUs (1,325) detected across all three depths was higher in Deep Cove. Furthermore, eDNA signals obtained at Deep Cove showed more taxonomic overlap between depths compared to both other sites.

3.4 | eDNA community structure

Overall, the taxonomic and ZOTU dataset from our eDNA survey obtained similar large-scale patterns, with significant differences in community composition across depth according to PERMANOVA (Taxonomic: $F_{8,36} = 5.4193$, $p < .001$; ZOTU: $F_{8,36} = 10.924$, $p < .001$). Further statistical evidence for the partitioning of samples between depths for both the ZOTU and taxonomic dataset was provided by ordination and cluster analyses (Figure 4).

For the taxonomic data, surface water samples in the LSL layer at all three sites clearly differentiated from samples taken within the marine layer in 2D space along the primary axis explaining 19.9% of the variation in community composition using the Jaccard (presence–absence) index (Figure 4a). Samples from the three sites within the LSL layer, on the other hand, separated from one another along the secondary axis explaining 11.4% of the variation. Furthermore, all marine samples clustered together in the 2D space. Similarly, surface water samples taken in the LSL layer clustered separately in a UPGMA tree supported by bootstrap subsampling (Figure 4b) and samples from Deep Cove taken at 4 and 15 m clustered separately from the marine samples at the other two sites.

For the ZOTU data, surface water samples in the LSL layer at Bauza Island and Mid-Crooked Arm clearly separated from all other samples and each other in 2D space along the primary axis explaining 36.2% of the variation, while surface water samples from Deep Cove only separated from the marine samples at the same site in 2D space along the secondary axis explaining 11.2% of the variation (Figure 4c). Furthermore, Deep Cove marine samples separated along the secondary axis from marine samples at both the other sites, while marine samples taken at Bauza Island and Mid-Crooked Arm were structured according to depth along the primary axis. Similarly, surface water samples taken in the LSL layer clustered separately in the UPGMA tree supported by bootstrap subsampling (Figure 4d). As seen in the taxonomic data, the marine samples from Deep Cove clustered separately from the marine samples at the other two sites in the UPGMA tree.

3.5 | Species-specific biological validation for eDNA signal detection

For our taxonomic dataset, we identified a total of sixteen indicator species (fish (16S): 6; crustacean (16S): 1; and eukaryote (COI): 9). Indicator species are organisms whose presence or absence reflect environmental conditions. Due to differences in species detection between sampling sites, the indicator species analysis was performed per site (Figure 5; Appendix S6). At Deep Cove, we identified three freshwater indicator species, including a species of microscopic alga (*Dinobryon* sp.), two fish species (*Galaxias* sp. and *Gobiomorphus* sp.), and three marine indicator species, including a species of krill (*Euphausia* sp.), a copepod (*Acartia* sp.), and a fish species (*Notolabrus* sp.). At Mid-Crooked Arm, we identified one freshwater (*Trachurus* sp.) and six marine indicator species, including a species of krill (*Euphausia* sp.), a copepod (*Paracalanus* sp.), a sea urchin (*Evechinus* sp.), two planktonic algae (*Pseudochattonella* sp. and *Phaeocystis* sp.), and an ascidian (*Ascidia* sp.). At Bauza Island, we identified a single freshwater (*Aplodactylus* sp.) and six marine indicator species, including two copepods (*Paracalanus* sp. and

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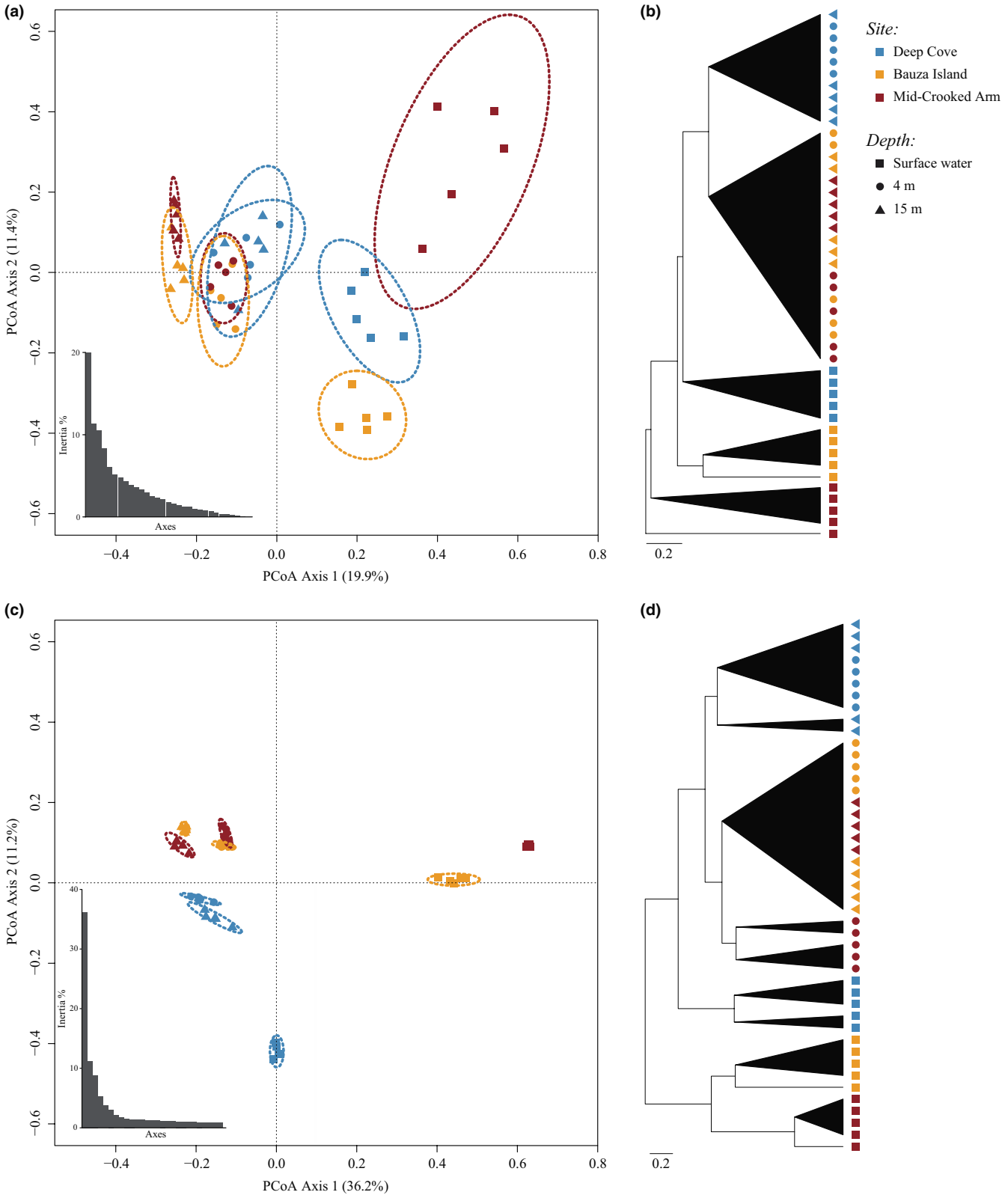

FIGURE 4 Ordination analyses (PCoA; a, c) and cluster analyses (UPGMA; b, d) depicting similarity in community composition based on eDNA taxonomic incidence (Jaccard; a and b) and eDNA ZOTU incidence (Jaccard; c and d). Bar plots indicate eigenvalues representing percentage of variation in dataset explained per axis. UPGMA trees with nodes <98 bootstrap support are collapsed and indicated by black triangles

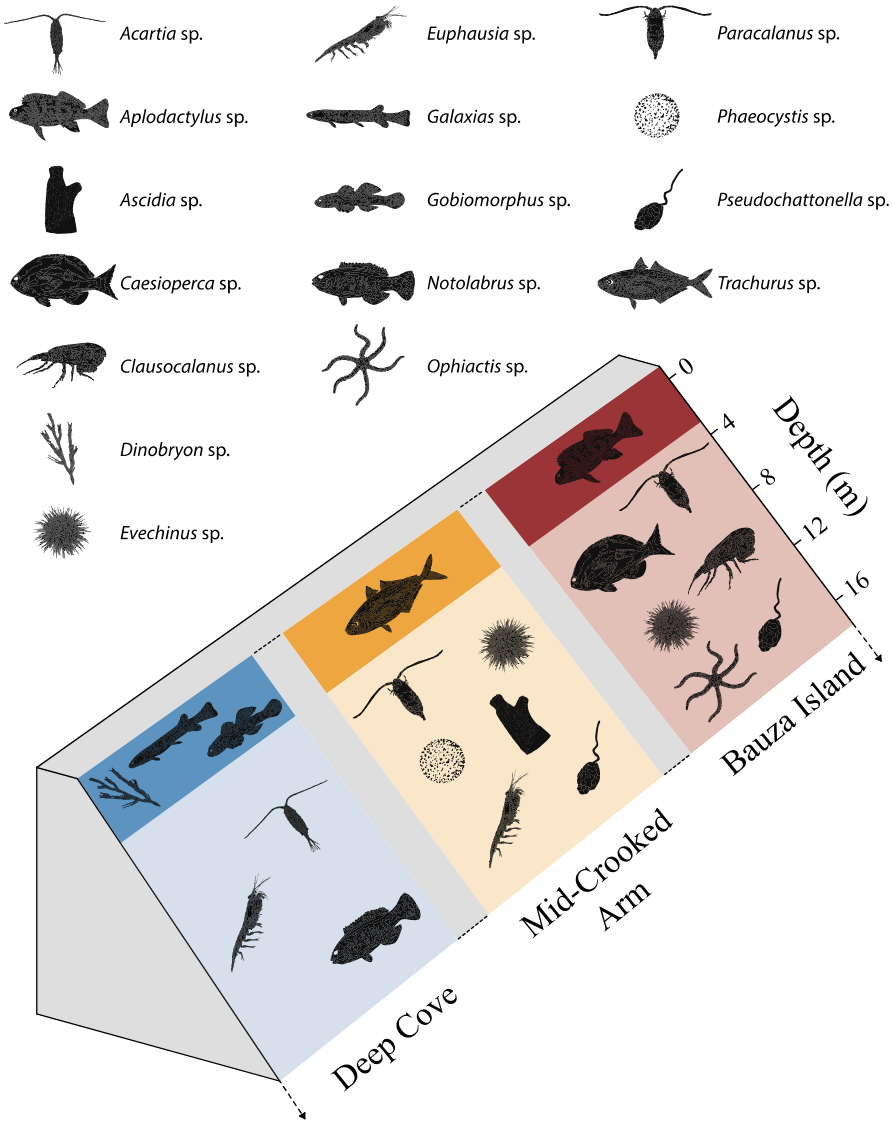

FIGURE 5 Indicator species analysis depicting the indicator species per site, with Deep Cove in blue, Mid-Crooked Arm in orange, and Bauza Island in red. The low-salinity layer (LSL) on top of the marine layer is indicated by a darker color

Clausocalanus sp.), a sea urchin (*Evechinus* sp.), a planktonic alga (*Pseudochattonella* sp.), a fish species (*Caesioperca* sp.), and a brittle star (*Ophiactis* sp.). Ecological descriptions from all indicator species identified in our taxonomic dataset showed strong habitat preference in concordance with the spatial trend of the eDNA signal, with the exception of the two freshwater indicator species identified at Mid-Crooked Arm and Bauza Island.

We also grouped each taxon within a biological community (i.e., freshwater nekton, marine intertidal, marine benthic, marine nekton, marine plankton) based on their habitat preference (Appendix S4). By displaying the number of positive detections for each depth within each biological community, we show the correlation between theoretical occurrence of the biological community and occurrence of the retrieved eDNA signal (Figure 6). Specifically, eDNA signals of freshwater species are restricted to samples taken within the LSL layer, with the exception of two species (*Gobiomorphus* sp. and *Galaxias* sp.) that have a single positive detection at 4 and 15 m in Deep Cove (Appendix S6). Marine intertidal species, such as *Austrominius* sp., *Chamaesipho* sp., and *Hemigrapsus* sp., were only detected within the first four meters in the water column at each site for our eDNA metabarcoding survey, whereas marine benthic, nektonic, and planktonic species were detected in a similar number of samples between the two sampling depths within the marine layer and were detected less frequently in the LSL layer (Figure 6; Appendix S4).

4 | DISCUSSION

The results presented in this study are the first to provide insight into the influence of water column stratification, and the associated fine-scale vertical community structuring, on marine eDNA metabarcoding surveys. Also, our results show the need to establish more sophisticated sampling strategies beyond the standard surface sampling in marine environments displaying permanent water column stratification. We provide evidence for the ability of eDNA metabarcoding surveys to detect spatially specific eDNA signals between samples taken 4 m apart across a strong halocline. Furthermore, these distinct eDNA signals resemble the in-field community

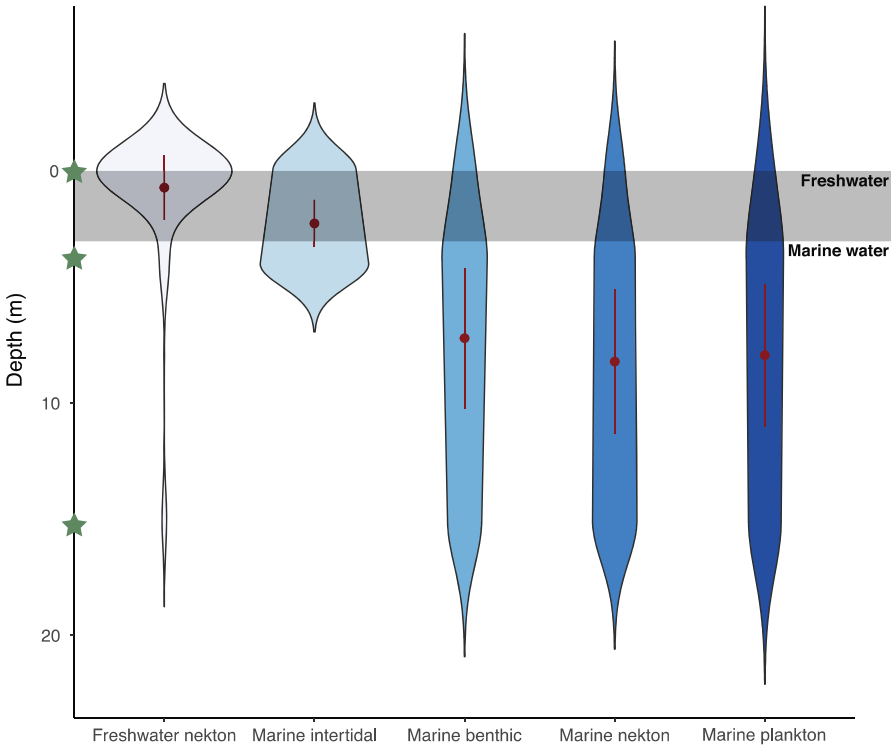

FIGURE 6 Violin plot displaying eDNA signal detection at each depth per biological community. Width of violin plot corresponds to the number of samples with positive detection. Maximum number of positive detections = 3 sites × 5 samples × number of taxa present in the biological community. Red circle represents the mean value where eDNA signal originates, with standard error bars. Green stars depict sampling depths. Gray area indicates the low-salinity layer. Taxa represented in each biological community can be found in Appendix S4

assemblages and concur with community structures obtained from known distribution patterns described for the fiord rock wall communities (Grange et al. 1981).

4.1 | Vertical transect sampling reveals distinct eDNA signals based on oceanographic and biological processes

We obtained distinct eDNA signals between surface water samples from the LSL and samples taken within the underlying marine layer for both the ZOTU (zero-radius OTU) and taxonomic dataset. The distinct eDNA signals we obtained for each water layer were significant when analyzed using both ZOTU/taxon richness and composition. Differentiation of eDNA taxonomic signals was enhanced in the ZOTU dataset compared to the taxonomic dataset, most likely due to the result of the weight given to a higher proportion of rare ZOTUs compared to rare taxa via the implementation of presence/absence analyses.

We observed more overlap in eDNA signals across depth when stronger hydrological processes were occurring. The lowest structuring of eDNA signals across depth was observed at Deep Cove, while eDNA signals across depth at Bauza Island and Mid-Crooked Arm were more distinct. In terms of vertical water mixing, among the three sampling sites within Doubtful Sound, the highest level of mixing occurs at Deep Cove through the turbulent mixing of fast-flowing (up to 7 m^2/s) freshwater input from both the tailrace input from the Manapouri hydroelectric power scheme and the Lyvia River (Witman and Grange 1998; Gibbs et al. 2000) and wind mixing at the fiord head. Additionally, the LSL is deepest on average in Deep Cove year-round, due to the proximity of the freshwater sources (Gibbs et al. 2000). Based on previously conducted traditional surveys (Boyle et al. 2001), the intertidal community of Deep Cove is most severely impacted by the LSL throughout Doubtful Sound with an absence of intertidal invertebrates typical of the region. This turbulent mixing due to freshwater input into Deep Cove is likely to have transported eDNA across both water layers, resulting in less distinct eDNA signal structuring between the intertidal and subtidal communities, in terms of taxon richness compared to both other sites. Furthermore, at Deep Cove the highest richness was recorded in the LSL layer, contrary to patterns obtained from traditional surveys (Grange et al. 1981; Boyle et al. 2001). This diversity most likely originates from eDNA transported by both freshwater sources and the detection of marine eDNA signals through vertical water mixing.

Bauza Island and Mid-Crooked Arm, on the other hand, are less influenced by turbulent and wind mixing than Deep Cove. The Bauza Island site is least influenced by the LSL, as the depth of the LSL decreases with distance from the tailrace input (Gibbs 2001; Kregting and Gibbs 2006), while vertical mixing of water through direct wave action from the Tasman Sea is reduced by its relative sheltered location despite being near the entrance of Doubtful Sound. Mid-Crooked Arm has the most stable water column stratification with minimal turbulent mixing between the LSL and the underlying marine layer (Gibbs et al. 2000; Elliott et al. 2011), being least influenced by wind mixing, wave action, or freshwater discharge, compared to both other sampling sites. The reduced intensity of water mixing in both Mid-Crooked Arm and Bauza Island led to clearly distinct eDNA signals across depth consistent with the community structure patterns observed in previously published descriptions of zonation (Grange et al. 1981; Boyle et al. 2001; Rutger and Wing 2006).

Furthermore, we observed a decrease in eDNA signal diversity in the LSL the farther away from the freshwater source. Traditional

field surveys attributed the low intertidal diversity in Doubtful Sound to the effects of lowered salinity (Grange et al. 1981; Witman and Grange 1998; McLeod and Wing 2008), a pattern intensified with the permanent addition of freshwater from the Manapouri hydroelectric power scheme (Boyle et al. 2001). The lowered salinity led to an algae- and lichen-dominated shore community and the almost total disappearance of invertebrates typical of the region's intertidal community (Boyle et al. 2001). Without incorporating a metabarcoding assay targeting lichen diversity, these taxa could not be detected by our metabarcoding survey, explaining the resulting low diversity retrieved in this water layer at Mid-Crooked Arm and Bauza Island. Deep Cove, on the other hand, obtained higher diversity eDNA signals in the LSL, due to an increased detection of marine species through water mixing and an increased detection of freshwater species, for example, *Galaxias* sp., *Gobiomorphus* sp., *Lepidodermella* sp., *Potamopyrgus* sp., *Anguilla* sp., *Austroperla* sp., and *Dinobryon* sp. The eDNA signals of freshwater species most likely originated from the two freshwater sources near Deep Cove. Predominantly freshwater species, such as cladocerans, are also known to enter the LSL in Doubtful Sound from the tailrace and the Lydia River (Lamare pers. obs.). The reduced detection probability of these species at Mid-Crooked Arm and Bauza Island is attributed to the high horizontal spatial resolution of eDNA metabarcoding surveys in marine and freshwater environments (Deiner and Altermatt 2014; Doi et al. 2017; Sansom and Sassoubre 2017; Port et al. 2016; Bista et al. 2017; O'Donnell et al. 2017; Yamamoto et al. 2017; Jeunen et al., 2019b).

4.2 | Community structure patterns obtained by eDNA surveys accurately resemble in-field assemblages

In addition to ZOTU and taxon richness, taxonomic composition contributed to the difference in eDNA signals found between depths at all three sites. No taxa were detected across all depths and sites by our eDNA metabarcoding survey. The four most ubiquitous taxa according to our eDNA survey were an ascidian (*Ascidia* sp.), a species of krill (*Euphausia* sp.), a copepod (*Paracalanus* sp.), and a planktonic alga (*Phaeocystis* sp.), all known to achieve high abundance in Doubtful Sound.

Environmental DNA signal detection coincided with habitat preference (intertidal vs. subtidal; freshwater vs. marine), providing biological validation for the distinct eDNA signals retrieved between the intertidal and subtidal range. Examples of freshwater species detected in the LSL by eDNA metabarcoding included: two species of galaxid (*Galaxias* spp.), diadromous fish known to occasionally enter the freshwater layer in Doubtful Sound (Ayling 1987); a freshwater eel (*Anguilla* sp.), known to reside in Lake Manapouri (Boubée et al. 2008); an amphidromous native fish (*Gobiomorphus* sp.); and a freshwater snail (Potamopyrgus sp.), known to inhabit the intertidal range in Doubtful Sound (Boyle et al. 2001). All freshwater species were exclusively detected in the LSL, with the exception of two single observations in the marine layer at Deep Cove, the site displaying highest turbulent water mixing. Two of the abovementioned species were identified as indicator species for the Deep Cove low-salinity layer (i.e., *Galaxias* sp. and *Gobiomorphus* sp.), while the third indicator species at Deep Cove (*Dinobryon* sp.) is a microscopic alga most commonly found in freshwater, but known to occur in estuarine habitats (Watson et al., 2015). The indicator species for the LSL at both other sampling sites constituted two marine fish species (i.e., *Aplodactylus* sp. and *Trachurus* sp.). While these eDNA signal detections do not coincide with habitat preference, both fish species are highly mobile and known to enter the LSL at Doubtful Sound.

While intertidal diversity, especially invertebrate taxa, is known to be low in Doubtful Sound due to the permanent low-salinity layer (Grange et al. 1981; Witman and Grange 1998; McLeod and Wing 2008), our eDNA metabarcoding survey was able to detect multiple intertidal taxa within the first two sampling depths. No intertidal organisms were detected at the deepest sampling point. Examples of intertidal organisms detected at the first two sampling depths were as follows: two species of crab (*Hemigrapsus* sp. and *Petrolisthes* sp.) known to tolerate brackish conditions (Hicks 1973); a species of triplefin (*Forsterygion* sp.), native to New Zealand, which resides in rockpools during low tide (Ayling 1987); and two species of barnacle (*Austrominius* sp. and *Chamaesipho* sp.), which occur at the upper-intertidal region in Doubtful Sound (Grange et al. 1981). Conversely, examples of strictly marine species with eDNA signals observed solely in the marine layer included three species of brittlestar (*Ophiactis* sp., *Ophionereis* sp., and *Ophiopteris* sp.), a species of sea cucumber (*Australostichopus* sp.), and a species of perch (*Caesioperca* sp.). Additionally, all eleven indicator species for the marine layer at all three sites were strictly marine taxa.

Several strictly marine taxa were detected in the LSL by our eDNA metabarcoding survey in Deep Cove, including two of seastars (*Coscinasterias* sp. and *Meridiastra* sp.), and a sea urchin (*Evechinus* sp.). These observations could be explained by intensified vertical water mixing at Deep Cove. Interestingly, these species are also often found at the LSL/marine layer interface (Barker and Russell 2008) and migrate into shallow waters (Lamare et al. 2009). The mixing of their DNA between layers may, therefore, be unrelated to intensified hydrological processes. All taxa occurring across the three depths sampled at all three sampling sites were either planktonic taxa occurring throughout Doubtful Sound (e.g., copepods and diatoms) or highly mobile taxa (e.g., fish).

4.3 | Environmental DNA metabarcoding to aid ecosystem conservation and management

From the observations on ZOTU/taxon richness and community composition, we conclude that eDNA metabarcoding surveys are able to distinguish vertically stratified communities on small spatial scales (i.e., meters). Besides biological transport from predator–prey interactions (Barnes and Turner 2016; Sassoubre et al. 2016), eDNA transport might be primarily influenced by physical processes, with certain mechanisms (e.g., water stratification) restricting eDNA dispersal, while others (e.g., intensified wave action) enhance eDNA dispersal. Thus far, two metabarcoding studies have investigated differences in eDNA signals between surface samples and samples taken near the seafloor (Andruszkiewicz et al. 2017; Yamamoto et al. 2017). Both studies reported differences in eDNA signals with samples taken >20 m apart. However, our study is the first to detect eDNA signals on a much finer

spatial scale (4 m). The vertical eDNA resolution obtained in this study is likely influenced and enhanced by the presence of the strong halocline. Haloclines have the ability to restrict the downward transport of suspended particulate matter (Pickrill 1987), a possible source of eDNA, as the origin of eDNA was found to be primarily subcellular in freshwater environments (Moushomi et al., 2019). Further investigation is, therefore, required to determine the vertical structuring of eDNA signals from different coastal systems subject to varying intensities of wave action (Brown 1999). Future eDNA metabarcoding surveys should consider the oceanographic characteristics of the study area, such as water column structure, in the area to be monitored when designing the sampling strategy, as the surface water sampling that has been employed as standard to date may not be able to fully uncover subtidal community assemblages. Additionally, evidence has been provided for the inclusion of different substrates during sampling and multiple primer sets during library preparation (Koziol et al. 2019).

Although entire community characterization might currently be unfeasible, eDNA metabarcoding surveys are particularly useful for detecting difficult-to-classify and difficult-to-observe taxonomic groups. For example, our eDNA metabarcoding survey detected a diverse group of copepods (e.g., *Acartia* sp., *Clausocalanus* sp., *Ctenocalanus* sp., *Oncaea* sp., *Paracalanus* sp.), a group that displays remarkable diversity and a cosmopolitan distribution in freshwater and marine environments (Böttger-Schnack and Machida 2011). Although copepod presence and abundance are a known bio-indicator (Lee et al. 2001), this group of crustaceans is infrequently included in established monitoring programs due to their morphological identification difficulties and specialized sampling requirements (Böttger-Schnack and Machida 2011). Also, both lobsters, *Jasus* sp., and crabs, *Hemigrapsus* sp., *Petrolisthes* sp., of the order Decapoda, a taxonomic group frequently used in monitoring surveys (van Oosterom et al. 2010), were detected by our eDNA metabarcoding survey. Due to their low abundance, elusive occurrence, or cryptic nature in Doubtful Sound (Jack et al. 2009), these taxa can go unnoticed or under-represented in certain traditional survey techniques, such as passive photographic quadrat surveys used in Doubtful Sound for annual monitoring. Our results, therefore, suggest that implementing eDNA metabarcoding in monitoring programs would facilitate the inclusion of known bio-indicators, frequently excluded due to classification difficulties, and improve the detection probability of elusive and low-abundance taxa. However, it should be noted that eDNA metabarcoding surveys, to date, do not have the ability to infer abundance estimates, and this information is required for effective monitoring of commercial species, such as the southern rock lobster (*Jasus edwardsii*) and blue cod (*Parapercis colias*). Based on these considerations, eDNA metabarcoding currently remains complementary to, rather than a complete replacement for, traditional monitoring surveys (Stoeckle et al. 2016; Kelly et al. 2017).

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AUTHOR CONTRIBUTIONS

GJJ, MDL, and NJG have made major contributions to the conception and design of the study. GJJ, MDL, MK, MS, MB, and NJG have made major contributions to the acquisition, analysis, and interpretation of the data. GJJ, MDL, MK, MS, MB, HGS, HRT, and NJG have made major contributions to the writing of the manuscript.

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DATA AVAILABILITY STATEMENT

Sample location coordinates are listed in Appendix S1. Detailed information regarding rtPCR settings is provided in Appendix S2. All bioinformatics scripts used to obtain taxonomic data are provided in Appendix S3. Identified taxa and their presence per site are listed in Appendix S4. Rarefaction curves are provided in Appendix S5. Dereplicated FASTQ files from all assays will be made available on Sequence Read Archive (SRA) upon acceptance of this manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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