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ORIGINAL ARTICLE

Expanding the temporal and spatial scales of environmental DNA research with autonomous sampling

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Abstract

Environmental DNA (eDNA) is an emerging and powerful method for use in marine research, conservation, and management, yet time- and resource-intensive protocols limit the scale of implementation. Long-range autonomous underwater vehicles equipped with autonomous environmental sample processors (LRAUV-ESPs) provide a new means for scaling up marine eDNA sample collection and processing. Here, we used eDNA metabarcoding of four marker genes (mitochondrial 12S rRNA, bacterial and archaeal 16S rRNA, nuclear 18S rRNA, and mitochondrial COI), which encompass the diversity of marine species from microbes to vertebrates, to demonstrate the efficacy of an LRAUV-ESP in sampling eDNA and assessing community structure in the Monterey Bay National Marine Sanctuary. The sequencing results from samples that were autonomously collected were comparable with those collected from a ship at similar locations, times, and depths, supporting previous results that found no significant differences using targeted qPCR. This study demonstrates the potential of equipping autonomous underwater vehicles with ESPs to greatly expand the scale of eDNA sample collection and processing and provide much needed information regarding the changing spatial and temporal patterns of marine biodiversity, especially in many data-poor regions of the world's oceans.

KEYWORDS
biodiversity, biomonitoring, environmental DNA, marine protected areas

1 | INTRODUCTION

Environmental DNA (eDNA) has become a valuable tool for detecting organisms from all domains of life in the environment without visual or auditory observations. Molecular metabarcoding of eDNA is now used routinely to survey community composition and estimate diversity in freshwater and marine habitats (e.g., Berry et al., 2019; Jerde et al., 2019; Stoeckle et al., 2020). However, the development of optimal sampling protocols is crucial for accurate and reproducible eDNA results (Deiner et al., 2015) that can be integrated globally (Chavez et al., 2021). Improvements in collection methods, DNA extractions, gene target choice, and bioinformatic databases have led to increasingly powerful eDNA studies in the marine environment showing good agreement with visual

and trawl surveys (Cilleros, 2019; Stoeckle et al., 2020; Thomsen et al., 2016; Yamamoto et al., 2017). Traditional surveys of fish or marine mammals can require weeks of ship time, while similar spatial scales of observation are achievable through eDNA within days (Acharya et al., 2019; Truelove et al., 2019; Watsa et al., 2020). eDNA metabarcoding analyses thus have the potential to greatly reduce the manual labor, resources, and time traditionally required for marine biodiversity surveys.

Despite their potential, the scale and pace of eDNA metabarcoding studies in the ocean have lagged behind those of freshwater environments (Beng & Corlett, 2020; Jerde et al., 2019). This discrepancy is largely due to the scale differences between the two environments; access to marine environments requires expensive oceanographic research vessels that sample over considerable temporal (months) and spatial (hundreds of square kilometers and multiple depths) scales (McClenaghan et al., 2020). Moreover, the manual labor required to filter water, preserve samples, and analyze them limits data collection, with significant expense, logistical constraints, and hundreds of hours of work required for a typical ship-based mission. The need for mobile, autonomous eDNA collection and processing systems is therefore pressing. The recent development of long-range autonomous underwater vehicles (LRAUVs) equipped with environmental sampling processors (ESPs) offers one means of meeting this requirement, to overcome many of the challenges associated with ship-based sampling (Scholin et al., 2017).

The Monterey Bay Aquarium Research Institute (MBARI) has successfully deployed LRAUVs fitted with ESPs that filter seawater *in situ* and preserve material collected for laboratory-based eDNA extraction and sequencing (Scholin et al., 2017; Yamahara et al., 2019). The current generation of the ESP (3G-ESP) used aboard the LRAUV employs a sample handling procedure that is notably distinct from what would typically be done manually in a shipboard setting. First, ESP-sampled water passes through a 1-mm mesh copper screen that reduces biofouling and prevents larger zooplankton from entering the intake tubing and potentially clogging the filtration system. Second, the ESP acquires a sample by "sipping" water over time (e.g., ~1 h) as the vehicle drifts in or propels through the water; this is in contrast to ship surveys, which generally collect water via Niskin bottle casts that rapidly capture a volume of water from a given depth, which is then processed onboard as a bulk sample. Finally, while both the 3G-ESP and manual methods in this study aim to filter 1 L of seawater through a 0.2-micron PVDF filter (Millipore), storage of the samples post-filtration differs. The filters on the ESP are incubated in RNAlater® (Thermo Fisher) for 20 min, are flushed with nitrogen gas, then remain at ambient temperature until returned to the laboratory after recovery, and finally stored at −80°C until processed. In contrast, filters collected aboard the ship are immediately frozen at −80°C or in liquid nitrogen after filtration and stored frozen until processed. Yamahara et al. (2019) showed that samples collected by the LRAUV-ESP yielded similar DNA concentrations as manually collected samples, suggesting DNA stability is not compromised by autonomous collection. Moreover, the LRAUV-ESP provided similar quantitative PCR (qPCR) results on the abundance of several keystone marine species, including the diatom *Pseudo-nitzschia*, krill, and anchovy.

Targeted gene assays such as qPCR assess the abundance of genes from specific organisms, but are limited in terms of assaying community diversity broadly. In contrast, metabarcoding methods allow for a much greater diversity of species to be detected from eDNA. Metabarcoding amplifies genes from a wide taxonomic range, and the resulting amplicons are sequenced at a depth that yields hundreds or thousands of amplicon sequence variants (ASVs). This approach has long been used to assess prokaryotic diversity, using the highly conserved 16S small subunit ribosomal RNA gene as a marker gene, and it offers similar potential for eukaryotic communities. However, metabarcoding results for higher organisms can vary widely depending on the target gene(s) (Berry et al., 2019; Djurhuus et al., 2018; Kelly et al., 2017; Stat et al., 2017; Zhang, Zhao, et al., 2020). For eukaryotic diversity in dilute aquatic environments, the mitochondrial cytochrome oxidase I (COI) gene is often preferred because of the relatively higher copy number compared with nuclear genes, and the mitochondrial 12S rRNA gene target has become popular for metazoans, particularly studies targeting fish, because of the higher taxonomic resolution afforded for Illumina short-read sequences (Rees et al., 2014). However, the nuclear 18S rRNA gene provides greater taxonomic breadth than mitochondrial genes (Pochon et al., 2013). As such, a combination of metabarcoding primer sets is considered an ideal strategy for a comprehensive survey of marine life (Kelly et al., 2017, Berry et al., 2019, Djurhuus et al., 2020, West et al., 2020). An important advantage of eDNA metabarcoding is the use of multiple markers that can be amplified from the same sample to generate a comprehensive survey of organismal diversity across trophic levels.

In this study, we compared community composition of seawater samples collected along coastal Northern California using shipboard eDNA collection and filtration methods with samples collected and filtered *in situ* by the LRAUV-ESP at approximately the same time and location (site and depth). We used a metabarcoding approach with four sets of marker genes (mitochondrial 12S rRNA targeting vertebrates, 16S rRNA targeting bacteria and archaea, nuclear 18S rRNA targeting eukaryotes, and mitochondrial COI targeting eukaryotes) to comprehensively assess qualitative and quantitative differences in detected taxa.

Ground-truthing studies are critical for evaluating the efficacy of autonomous systems. Our results provide valuable insight into the advantages and disadvantages of the two methods and highlight the substantial progress made in the field. We show that autonomous sampling by instruments such as the LRAUV-ESP compares favorably with commonly used manual eDNA sample collection methods and combining autonomous eDNA sampling technology with metabarcoding offers a promising future for expanding the spatial and temporal scales of environmental monitoring in both marine and freshwater settings. We further provide guidance on the potential applications for autonomous eDNA sample collection and suggest future improvements in autonomous eDNA sampling technology.

2 | METHODS

2.1 | Experimental design

In this study, we analyzed a set of samples collected aboard ship (i.e., by CTD Niskin bottle and shipboard filtration) on the R/V *Western Flyer* and autonomously by the LRAUV-ESP. The two sampling methods targeted the same depths, times, and locations. However, due to operational concerns about potential collisions between the ship CTD and the autonomous vehicle, sample collection averaged around 1 km apart (Table S1). In addition, the LRAUV-ESP collects a seawater sample slowly for up to an hour compared with nearly instantaneously by the ship CTD, further increasing the time and space differences in sample collection. Given these constraints, samples were taken at the closest time and space possible using these two contrasting methods. Distances between autonomous and shipboard samples were calculated using the rdist.earth function in the R "fields" library, which given two way points computes the great circle (geographic) distance between them (Table S1). Samples were collected in the spring of 2017 and the fall and spring of 2018 as part of a larger sampling effort (MBARI's Controlled, Agile, and Novel Observing Network (CANON) Initiative); the spring 2018 sample collection was also in collaboration with the NOAA Ship *Reuben Lasker* during the Rockfish Recruitment and Ecosystem Assessment Survey (RREAS). Cruise sampling sites were located along coastal Northern California, and dates spanned May 3–9 in 2017 and May 30–June 11 and September 7–11 in 2018 (Table S1, Figure 1).

In the spring of 2017, the R/V *Western Flyer* cruise sampled across fronts within northern Monterey Bay. Samples were coordinated between shipboard CTD sampling, shipboard ESP filtering (with a benchtop ESP), and LRAUV-ESP sampling. As this was one of the first deployments of the LRAUV-ESP platform, only a limited number of samples (n = 5) could be collected autonomously with that early version of the instrument prototype. For later sampling, engineering advancements allowed the LRAUV-borne 3G-ESP (Figure S1) to contain a rotating carousel that contained 60 reusable cartridges (reusable between deployments but not within a deployment) during a mission.

In the spring of 2018, the R/V *Western Flyer* repeatedly sampled along a latitudinal line north of Monterey Bay, CA, that crossed an upwelling front and that was also sampled by the NOAA RREAS cruise on the *Reuben Lasker*. In addition to the two research vessels, multiple autonomous assets including the LRAUV-ESP were part of the experiment. The LRAUV-ESP was stationed at a single location on the latitudinal line and repeatedly acquired shallow (30-m) and deep (200-m) samples to create a time series over the course of the cruise. The R/V *Western Flyer* collected samples along this latitudinal line, following the NOAA Ship *Reuben Lasker* and collecting samples at the same location and depths as the LRAUV-ESP.

In the fall of 2018, cruise efforts focused on the same latitude line north of Monterey Bay, CA. During this time, the LRAUV-ESP initiated a drifting sampling pattern to collect a Lagrangian time series instead of targeting two depths in a set location as before. The LRAUV-ESP targeted an isotherm (12°C) and drifted freely along that feature collecting discrete samples at 2-h intervals from the same water parcel (tracking a feature as in Zhang, Kieft, et al., 2020). The R/V *Western Flyer* regularly sampled alongside the LRAUV-ESP by acoustically tracking its position using an autonomous Wave Glider at the surface (Zhang, Kieft, et al., 2020). Samples were collected on the 12-°C isotherm using the CTD. Since shipboard CTD sampling depth had to be estimated (there was no communication possible with the drifting LRAUV to get exact depth while submerged), there was a greater difference in sampling depths between CTD and ESP samples in the fall than in the spring. Furthermore, in the fall the LRAUV-ESP was sampling at regular time intervals (every 2 h), but exact sample start times while submerged were only estimated. Fall shipboard CTD sampling times alongside the LRAUV-ESP were set based on

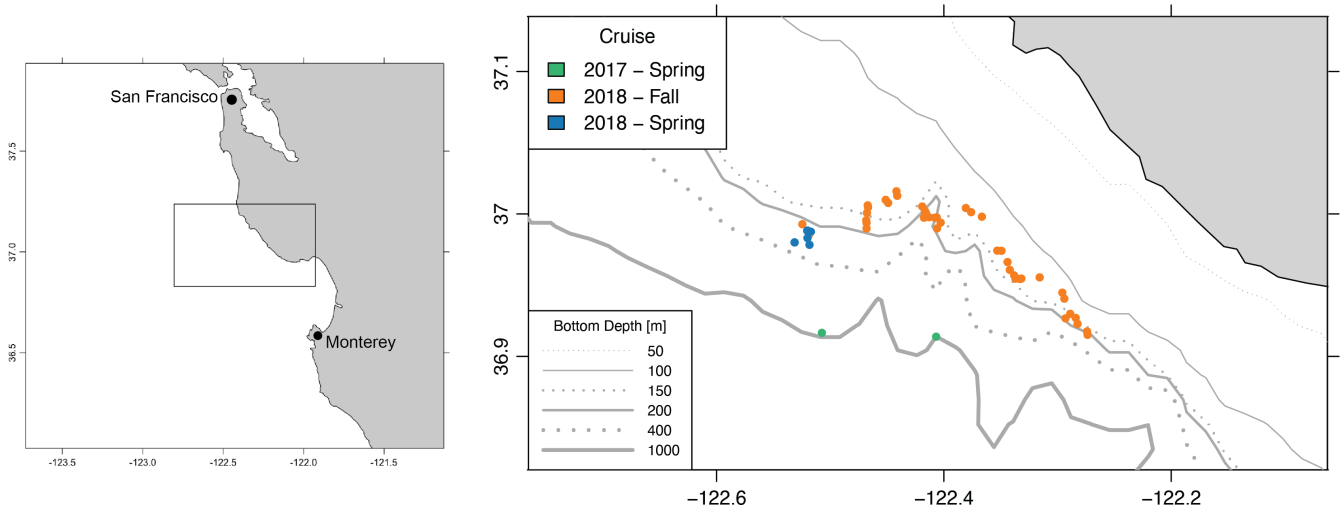

FIGURE 1 Map of sites off the coast of northern California sampled in this study. Sampling sites are represented by points colored by associated cruise (spring 2017, spring 2018, and fall 2018). CTD (shipboard) and LRAUV-ESP (autonomous) samples were collected at each site and at depths within the top 200 m of the water column

operational constraints and therefore occurred at varying times from ESP sample collection (Table S1). This is contrasted with spring sampling efforts where the LRAUV-ESP took samples at known depths and time points that could be coordinated with ship-based CTD sampling.

2.2 | Sample collection

Seawater samples from the ships were collected almost instantaneously at selected depths using 10-L Teflon-coated PVC Niskin bottles (142 mm diameter, 826-mm length) on a CTD rosette. Once back on deck, water from the Niskin bottles was diverted into sterile Whirl-Pak® bags and filtered through a peristaltic pump system onto 25-mm-diameter, 0.22-µm polyvinylidene difluoride (PVDF) filters (Millipore) in Swinnex (Millipore) cartridge housings. The target water volume was 1 L, but in the event of filter clogging, a lower total volume was collected and recorded. Filters were immediately placed in a labeled cryovial and preserved in liquid nitrogen or stored in a −80-°C freezer until processing.

The LRAUV-borne 3G-ESP has been described previously (Pargett et al., 2015), as has been shown to be comparable in terms of *in situ* eDNA sampling and preservation relative to manual sampling methods using targeted qPCR (Yamahara et al., 2019). The workflow used in this study is very similar and briefly summarized below. Water samples were collected and filtered from sites off coastal northern California in May 2017 and in May, June, and September 2018 (Figure 1, Table S1) using shipboard methods (Niskin bottle, shipboard peristaltic pump filtration, and flash freezing in liquid nitrogen; $n = 32$) and autonomous methods (3G-ESP filtration and preservation with RNAlater®; $n = 32$).

To achieve sample collection and archival for subsequent laboratory analysis of eDNA, pump heads and valves were used to pass seawater through the 1-mm-diameter fluidic path of a 3G-ESP cartridge. Cartridge parts of the 3G-ESP were decontaminated prior to deployment with 10% bleach, 10% hydrochloric acid, and/or UV irradiation, and cartridges were aseptically loaded with sterile, 0.22-µm PVDF membrane filters (MilliporeSigma) and with 0.1-µm filtered RNAlater® (Life Technologies) to preserve the material collected on the filter. A clean cartridge is used to collect and preserve a single sample. Pump heads and valves were used to pass 1 L of seawater through a 1-mm-diameter fluidic path through a cartridge for eDNA collection and preservation. Pumping 1 L of seawater through the filter membrane in the sampling cartridge can take up to 1 h with sampling completed if 1 L or 1 h is reached first. The material collected was preserved by displacing seawater from the filter with 1.6 ml of RNAlater® (Life Technologies, final concentration >95%), incubating for 10 min, and then purging that from the membrane using ambient air of the LRAUV-borne 3G-ESP (N_2 gas). Although the filter was not stored flooded with RNAlater®, it remains moistened by that solution. Upon recovery of a field-deployed LRAUV-ESP, filters were removed from cartridges, placed into 2-ml tubes, and stored at −80°C until processed. Nucleic acids recovered after DNA extraction (see below) were compatible with PCR-based metabarcoding sample preparation protocols. More specific details associated with the paired ship and ESP samples (e.g., negative controls, time and distance between paired samples, and isotherm sampling) are found in the supporting information section Supplemental Methods.

2.3 | Direct comparisons of shipboard and autonomous samples

To verify previous findings that found equivalency between the sample filtration methods used during shipboard and autonomous sampling (Yamahara et al., 2019), four samples collected during the spring of 2017 were filtered using either a shipboard peristaltic pump filtration system or the 3G-ESP situated onboard the ship (hereafter "benchtop 3G-ESP"). Processing the same water sample provided a direct comparison of sample processing methods. Each direct comparison sample consisted of 10 L of seawater that contained equal amounts of seawater (2 L) collected at the surface, 10, 20, 30, and 40 m depths. This sample was then divided into two samples of equal volume (5 L) with one partition filtered using the shipboard peristaltic pump and the other with the benchtop 3G-ESP.

2.4 | DNA extraction, library preparation, and bioinformatic processing

DNA from the full set of filters used in this paper was extracted using the DNeasy® Blood and Tissue Kit following the standard protocol with some modifications (Qiagen) (protocols.io: dx.doi.org/10.17504/protocols.io.xjufknw). DNA extraction concentrations were quantified using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Differences in yields between samples collected by manual and autonomous methods were evaluated by running Student's *t* test in R. The primer sequences, thermocycling parameters, purification of PCR products, equimolar pooling of PCR products, quantification of pool concentration, and library preparation methods for all of the metabarcoding primer sets used in this study are in the supporting information section Supplemental Methods and are also described in Chavez et al., 2021.

2.5 | Comparative analyses—alpha diversity comparisons

Raw read count tables for all four metabarcoding data sets were imported into R for alpha diversity analyses. Alpha diversity (Shannon's index) was calculated in Phyloseq version 1.34.0 (McMurdie & Holmes, 2013). Violin plots comparing alpha diversity of autonomous and shipboard samples grouped by season and depth were plotted in R version 4.0.2. Mean alpha diversity for manual and autonomous

samples was compared using paired Student's *t* test, based on the pairing of the manual and autonomous samples.

The strength of correlation between alpha diversity values of paired autonomous and ship-collected samples was determined by fitting a linear regression to autonomous sample alpha diversity (Figure S2), with the alpha diversity of the paired, ship-collected sample as the predictor variable. Strength of correlation was reported separately for each cruise as R^2, *F*-statistic, and *p*-values. Alpha diversity was calculated using the Shannon index, and the linear regression was fit in R using the formula lm(formula = ESP~CTD).

2.6 | Comparative analyses—beta diversity comparisons

Beta diversity analyses for each marker gene data set were tested for differences between (a) shipboard/autonomous samples, (b) seasons, and (c) depth groups (i.e., shallow: 10–45 m; deep: 200 m) with principal component analysis based on the Aitchison distance matrices using DEICODE (Martino et al., 2019) and visualized in R using ggplot2. A multifactorial PERMANOVA of beta diversity was run in R using the adonis2 function from the vegan package (Oksanen et al., 2018) using the formula adonis2(PCA_dist ~shallow_or_deep + sampling_cruise + CTD_or_ESP, data = pca_metadata, permutations = 999).

To assess the compositional similarity of paired samples, the Aitchison distances between paired samples were compared with the Aitchison distances between all possible pairs of samples. The distance between each unique pair of samples was plotted against whether the two samples were paired or not, as well as by the relationship between the two samples, in terms of whether samples were from the same cruise and/or depth (Figure S3). To test whether paired samples were significantly more similar than unpaired samples, we ran a permutation test on the pairwise distances to compare the mean pairwise distance between paired samples with the mean pairwise distance between randomly selected samples. To account for the fact that paired samples were inherently from the same cruise and depth, we compared paired sample distances with distances between pairs of samples only from the same cruise and depth. We conducted permutation tests for each cruise individually, as well as for all samples together.

3 | RESULTS

3.1 | Shipboard and autonomous sample comparisons

During spring and fall 2018 cruises, shipboard and autonomous samples were collected as "pairs" roughly matched in sampling location, depth, and time. The distances between paired samples ranged from 0.55 to 1.81 km in the spring (average distance 1.12 km) and from 0.16 to 1.56 km in the fall (average distance 0.54 km) (Table S1). There were also temporal differences in sample pairs, ranging from ~12 min to 3 h 39 min in the spring (average difference 1 h 13 min) and from ~1 min to 6 h 45 min in the fall (average difference 54 min) (Table S1). Associated environmental metadata for 2018 cruises can be found in the MBARI Spatial Temporal Oceanographic Query System (fall cruise: https://stoqs.mbari.org/stoqs_canon_september2018/query/, spring cruise: https://stoqs.mbari.org/stoqs_canon_may2018/query/).

Autonomous methods provided similar concentrations of extracted eDNA as manual methods (Table S1; $n = 32$, *t* test $p = 0.697$). However, the mean number of reads per sample was significantly higher ($p = 0.032$) in eDNA samples collected autonomously compared with manually collected samples across all four eDNA metabarcoding primer set, with fold differences ranging from 1.14 (12S reads) to 1.36 (18S reads). The four water samples that were subdivided for parallel sample processing by peristaltic pump versus benchtop ESP produced highly similar results for amplicon sequencing of the 16S and CO1 marker genes (PERMANOVA 16S $p = 0.770$, *F*-statistic 0.13; PERMANOVA COI $p = 0.794$, *F*-statistic 0.32; Figure 2), with CO1 results showing no consistent differences between filtration methods and 16S results showing a trend for relatively higher levels of Bacteroidota in samples processed by peristaltic pump.

3.2 | Alpha and beta diversity of *in situ* shipboard and autonomous samples

Alpha diversity (Shannon's index) was similar between the autonomous and shipboard sample groups for the 16S, 18S, and COI marker genes (Figure 3a–c; $p > 0.05$), while Shannon diversity was significantly different for the 12S marker gene, with shipboard samples having higher alpha diversity (Figure 3d; $p = 0.006$). Plots of alpha diversity by cruise and depth grouping indicate that the spring cruise shallow samples and the fall cruise drive the higher alpha diversity of the shipboard compared with the autonomous samples for 12S; however, sample sizes were too low for robust statistical analyses based on cruise/depth subgroups of the data. An analysis of the correlation between alpha diversity values for paired autonomous and shipboard samples (Figure S2, Table S3) indicated that for each marker and cruise pairing, three of eight were significantly correlated: 18S, spring ($p = 0.0001$, *F*-statistic = 45.21, $R^2 = 0.8496$); 18S, fall ($p = 0.005$, *F*-statistic = 10.13, $R^2 = 0.3361$); COI, spring ($p = 0.006$, *F*-statistic = 13.6, $R^2 = 0.6297$).

Community composition varied significantly among depths and between seasons, and these findings were consistent across all four primer sets for both shipboard and autonomously collected samples ($p < 0.002$; Figure 4, Table 1). Spring eDNA samples were collected from two distinct depth groups of either 30 m or 195–200 m (Table S1). The 195- to 200-m samples from the spring clustered in the upper right-hand portion of the PCA plots in each of the primer sets, while the 30-m samples clustered in the lower right-hand portion of the PCA plots (Figure 4a–d). This separation in multivariate PCA space suggests

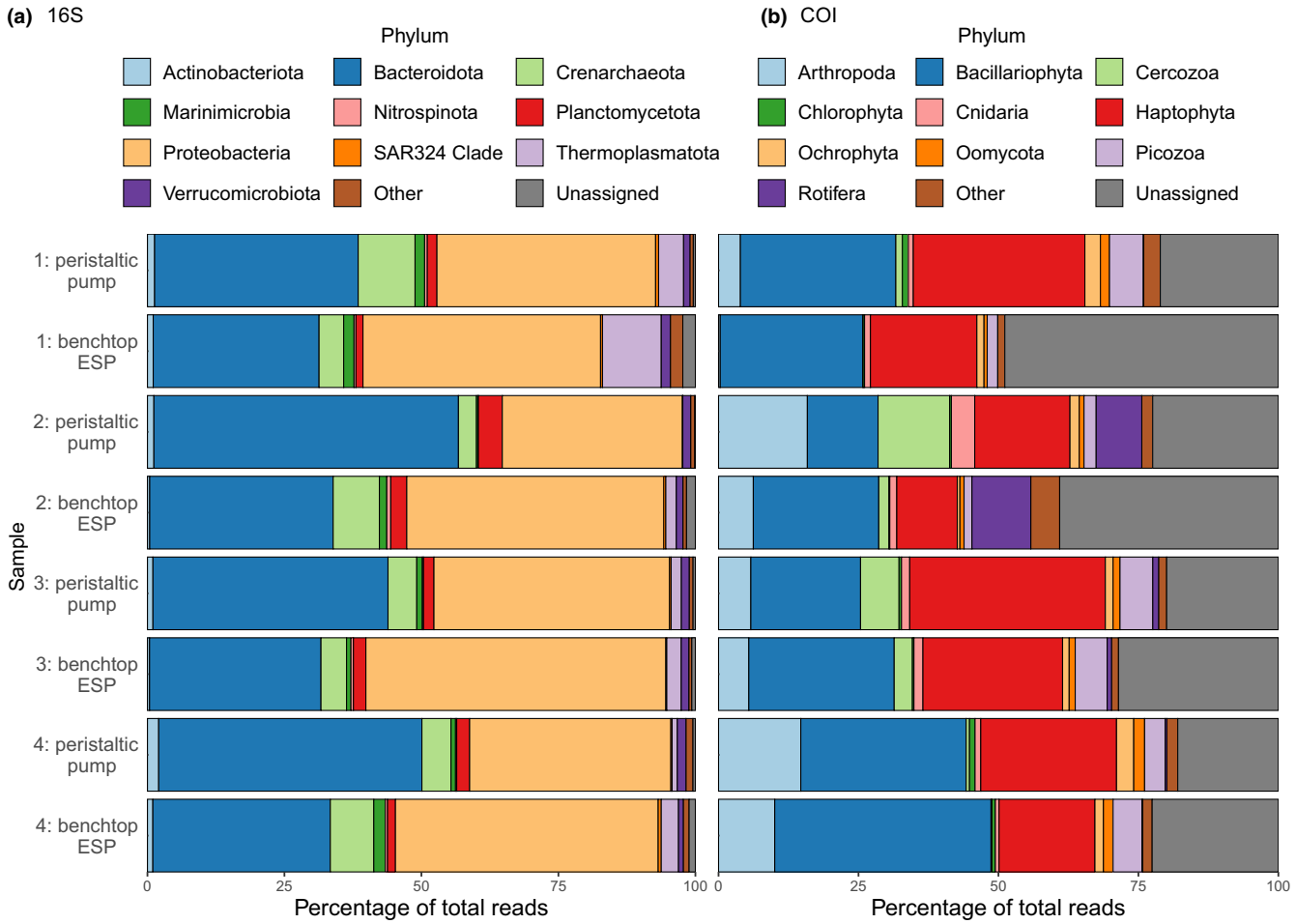

FIGURE 2 Community composition at the phylum level for four pairs of water samples filtered with a peristaltic pump and a benchtop ESP was highly similar between the two methods for both (a) microbial phyla detected by 16S rRNA metabarcoding and (b) eukaryotic phyla detected by COI metabarcoding results

that community composition differs between the 30-m and 195- to 200-m depth ranges, which is corroborated by significant differences found by the multifactor PERMANOVA ($p < 0.05$; Table 1) between these depth ranges in the 12S, 16S, 18S, and COI primer sets. The sampling depths in the fall were more variable and shallower than those of the spring, with depths ranging from 10 to 45 m (Table S1). The fall samples clustered together and were significantly different from spring samples ($p < 0.05$; Table 1) for all four marker gene data sets. This finding was consistent for both the autonomous and shipboard eDNA samples. A multifactor PERMANOVA found no significant differences between autonomous and shipboard sampling methods for the 16S and COI data sets ($p > 0.05$; Table 1), whereas 12S and 18S data sets were significantly different.

Beta diversity (Figure S3) was not significantly more similar between paired samples than between unpaired samples for any of the marker genes. The permutation test of the pairwise Aitchison distance values revealed that pairwise distances between paired samples were not significantly less than the distances between randomly selected unpaired samples that were also from the same cruise and depth for any marker (16S, $p = 0.237$; 18S, $p = 0.637$; COI, $p = 0.605$; and 12S, $p = 0.937$).

3.3 | Taxonomic composition variation by season and depth

The most commonly detected taxa in both autonomous and shipboard eDNA samples were similar across sampling methods and for all four primer sets (Figure 5). The most commonly detected phyla in the 16S rRNA primer set were consistently present in all samples, and the total number of reads was comparable between sampling methods (Figure 5). The 18S primer set also showed largely similar communities at the phylum level, with the exception of relatively higher levels of Arthropoda in the shipboard samples and relatively higher levels of Dinoflagellata in the autonomously collected samples (Figure 5). The most commonly detected phyla in the COI primer set were also consistently present between sampling methods with the highest proportions of reads in the phyla Arthropoda and Haptophyta. The results from the 12S primer set were more variable among samples overall; unlike in the other three primer sets, the results of this vertebrate-specific primer set are shown for the most commonly detected families instead of phyla, since all detected vertebrates belong to the same phylum. Both sampling methods detected the same families of vertebrates, with minor

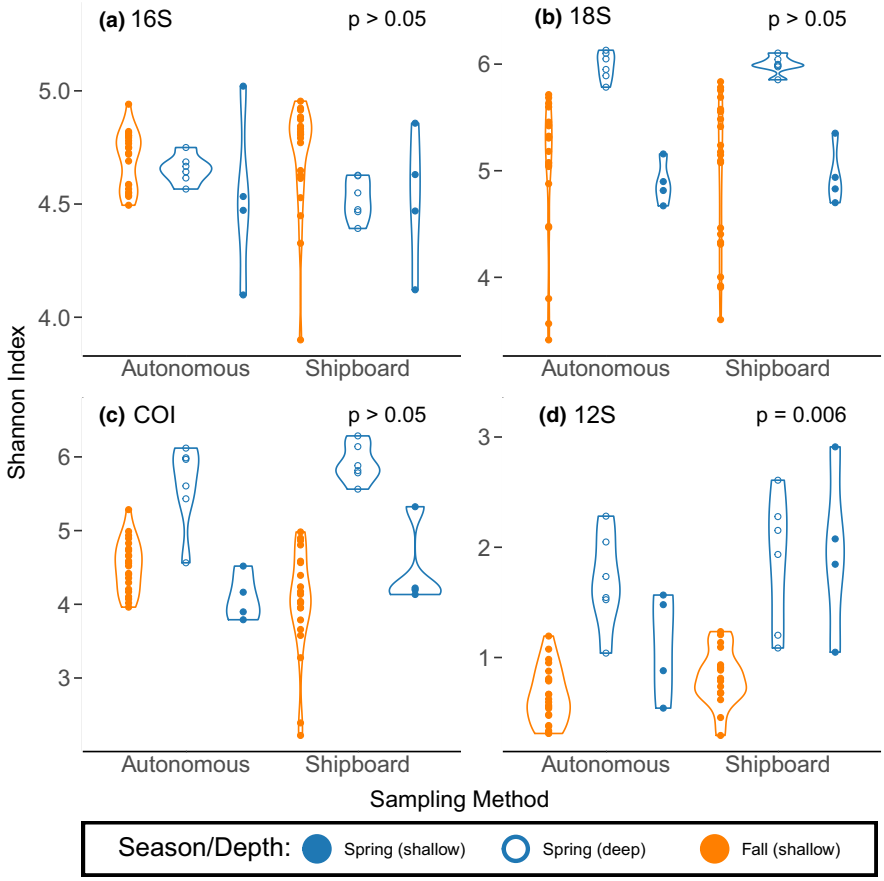

FIGURE 3 Comparison of alpha diversity (Shannon's index) for shipboard and autonomous samples according to results from four metabarcoding marker genes: (a) 16S rRNA, (b) 18S rRNA, (c) COI, and (d) 12S rRNA

proportional differences observed in the Engraulidae, Sebastidae, and Centrolophidae families. However, seven shipboard samples contained sequences for the family Chimaeridae, while none of the autonomous samples contained members of this taxonomic group.

3.4 | Amplicon sequence variants (ASVs) found in negative controls

A series of negative controls were sequenced along the entire sampling and processing pathway to detect contamination that may have occurred during shipboard, autonomous, or laboratory operations. For all the negative controls ($n = 48$; see methods for details), a total of 3057 ASVs were found by eDNA metabarcoding using the 16S, 18S, COI, and 12S primer sets. PCR blanks returned the lowest number of reads compared with all other control types, with the most reads assigned to ASVs classified as *Paracalanus* (18S; 1654), *Homo sapiens* (COI; 4067), and *Engraulis mordax* (12S; 337). For DNA extraction controls (16S: $n = 1$; 18S: $n = 7$; COI: $n = 7$; 12S: $n = 6$), the most abundant ASVs were assigned to the taxa *Parageobacillus toebii* (16S; 41 reads), Formicidae (18S; 5553 reads), Oomycota (COI; 123,807 reads), and *Homo sapiens* (12S; 108,914 reads).

As part of autonomous sampling, the 3G-ESP included pre- and post-deployment control cartridges processed onboard the LRAUV ($n = 8$), and the observed level of amplification was consistent across the four primer sets (16S, 18S, COI, and 12S). The taxonomy of ASVs with the most reads found in the pre-deployment controls for each of the four markers was *Aestuariicella* (9139 16S reads), Poaceae (3887 18S reads), Moerisiidae (143,999 CO1 reads), and *Homo sapiens* (3510 12S reads). The ASVs with the most reads in the post-deployment controls were classified as *Colwellia* (15,550 16S reads), *Chrysaora fuscescens* (40,684 18S reads), Moerisiidae (53,862 CO1 reads), and *Engraulis mordax* (35,150 12S reads).

Relatively few shipboard collection controls were collected ($n = 3$, from only the fall research cruise and sequenced with just the 18S and COI primer sets), with the most ASV reads assigned to Dinophyceae (6836 18S reads) and unassigned Eukaryota (56,463 CO1 reads). A detailed taxonomy table consisting of negative control type (shipboard, autonomous, DNA extraction, or PCR negative controls), number of ASVs in each control, number of reads for each ASV, and ASV taxonomy assignment using MEGAN6, and taxonomy barplots are available on GitHub (https://github.com/MBARI-BOG/ESP_CTD/tree/main/figures/supplemental).

Diversity statistics revealed significant differences in community composition (beta diversity) for the 3G-ESP onboard negative controls compared with environmental samples for three of the four markers (PERMANOVA; Figure S1). Similarly, alpha diversity (Shannon index) was significantly lower in the controls for the 16S, 18S, and CO1 markers (16S: PERMANOVA $p = 0.013$, F-statistic 4.46; t test $p = 6.08$ E−07, 18S: PERMANOVA $p = 0.002$, F-statistic 6.57; t test $p = 0.001$, COI: PERMANOVA $p = 0.002$, F-statistic 5.55; t test $p = 4.12$ E−07). The exception was the 12S primer set (PERMANOVA

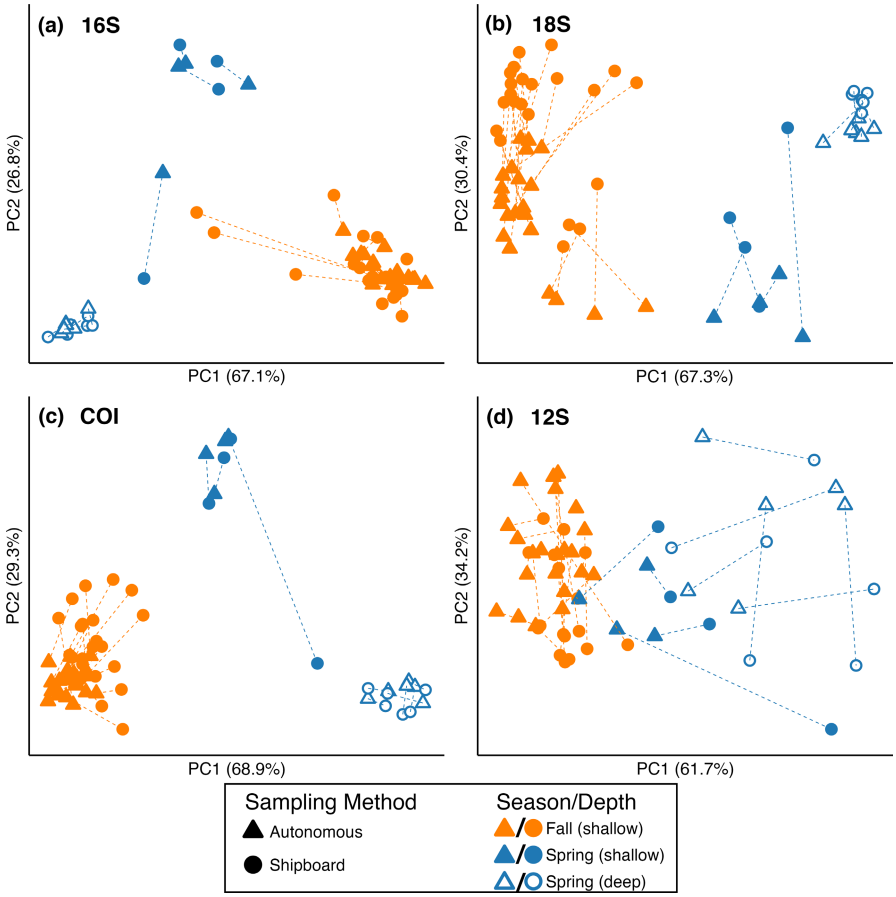

FIGURE 4 Principal component plot of autonomously collected (diamond) and shipboard peristaltic pump (circle) eDNA samples. Orange symbols indicate samples collected in the fall research cruise in 2018, and blue symbols indicate samples collected in the spring research cruise in and 2018. Samples in the fall cruise were collected at depths ranging from 10 to 45 m (shallow, solid orange symbols), and in the spring cruise, samples were collected at either 30 m (shallow, solid blue symbols) or 200 m (deep, hollow blue symbols). Panels show results from each of the four metabarcoding gene markers as follows: (a) 16S rRNA, (b) 18S rRNA, (c) COI, and (d) 12S rRNA

TABLE 1 *p*-values and *F*-statistics for the multifactor PERMANOVAs run on each marker gene data set

Data set	Variable tested	*N*	*p*-value	*F*-statistic
18S	Sampling method (ESP vs. CTD)	64	0.001	37.2
18S	Season (fall vs spring)	64	0.001	52.6
18S	Depth groups (0–25, 27–50, 70–200)	64	0.001	99.3
COI	Sampling method (ESP vs. CTD)	64	0.129	2.2
COI	Season (fall vs. spring)	64	0.001	170.1
COI	Depth groups (0–25, 27–50, 70–200)	64	0.001	195.2
12S	Sampling method (ESP vs. CTD)	60	0.001	10.7
12S	Season (fall vs. spring)	60	0.001	9.6
12S	Depth groups (0–25, 27–50, 70–200)	60	0.001	36.3
16S	Sampling method (ESP vs. CTD)	62	0.472	0.7
16S	Season (fall vs. spring)	62	0.001	157.4
16S	Depth groups (0–25, 27–50, 70–200)	62	0.001	173.5

$p = 0.423$, *F*-statistic 0.87; *t* test $p = 0.738$) (Student's *t* test; Table S2). Overall, less reads were seen in the controls although a few marine taxa, such as the Pacific sea nettle (*Chrysaora fuscescens*), the copepod *Arcatia*, and the Californian anchovy (*Engraulis mordax*), had a higher number of reads in the 3G-ESP negative controls compared with the environmental samples. The low number of samples ($n = 3$) and targets for the shipboard controls limited the statistical power of diversity analyses; however, the trend of lower alpha diversity present in the shipboard controls compared with environmental samples was consistent for the 18S and COI primer sets (Table S2).

4 | DISCUSSION

4.1 | Efficacy of autonomous eDNA sampling

Autonomous methods can greatly increase the spatial and temporal scales of eDNA sampling in marine and freshwater ecosystems. This increased resolution is required to first determine the mean baseline of aquatic biodiversity and how it may vary over time due to environmental changes. The development of instruments capable of capturing and preserving eDNA *in situ* has accelerated over the last decade, and

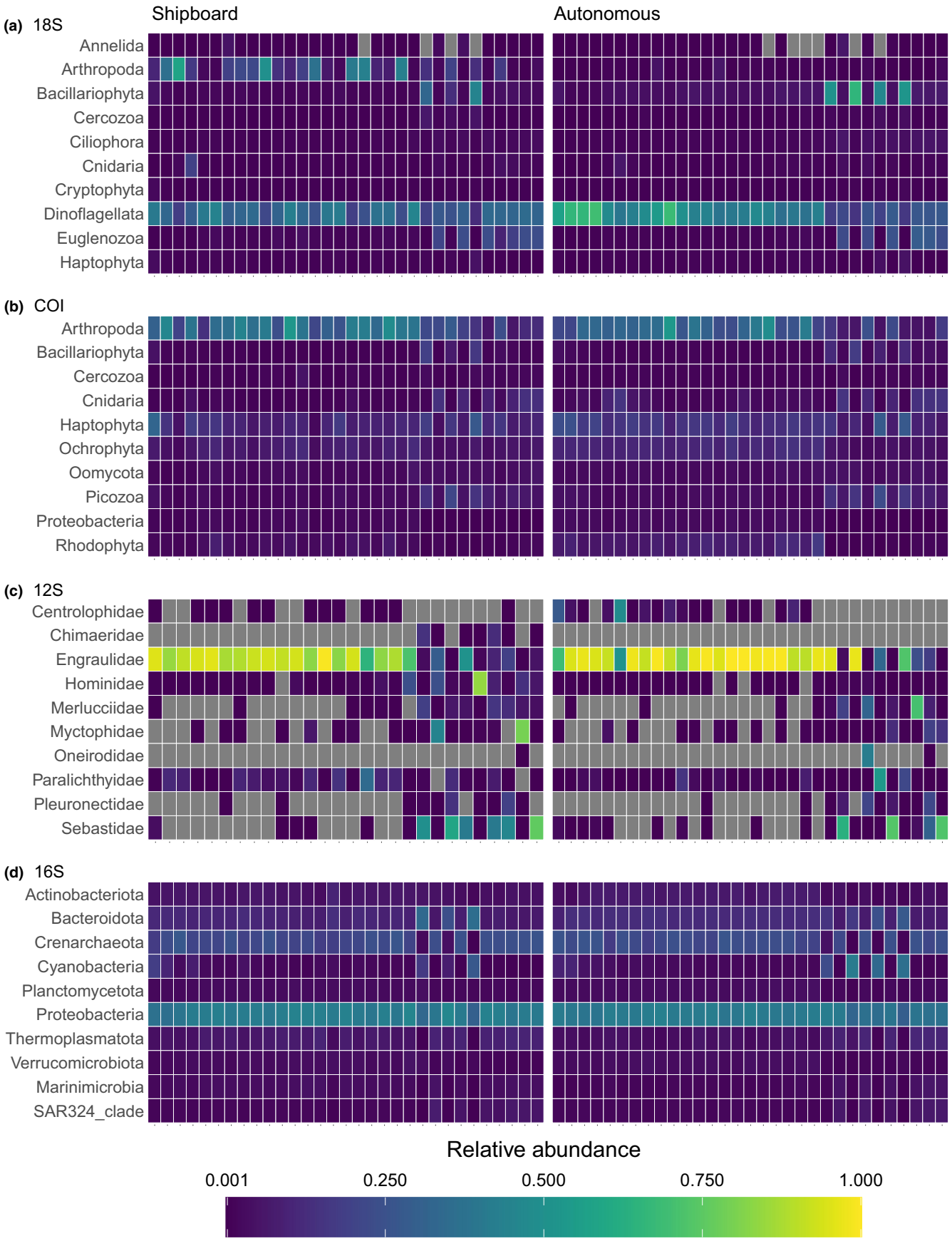
Shipboard
Autonomous
(a) 18S
Annelida
Arthropoda
Bacillariophyta
Cercozoa
Ciliophora
Cnidaria
Cryptophyta
Dinoflagellata
Euglenozoa
Haptophyta
(b) COI
Arthropoda
Bacillariophyta
Cercozoa
Cnidaria
Haptophyta
Ochrophyta
Oomycota
Picozoa
Proteobacteria
Rhodophyta
(c) 12S
Centrolophidae
Chimaeridae
Engraulidae
Hominidae
Merlucciidae
Myctophidae
Oneirodidae
Paralichthyidae
Pleuronectidae
Sebastidae
(d) 16S
Actinobacteriota
Bacteroidota
Crenarchaeota
Cyanobacteria
Planctomycetota
Proteobacteria
Thermoplasmatota
Verrucomicrobiota
Marinimicrobia
SAR324_clade
Relative abundance
0.001
0.250
0.500
0.750
1.000

FIGURE 5 Heatmap of the relative abundance of the most commonly observed taxa between CTD rosette samples (shipboard) and ESP samples (autonomous). Sample boxes are ordered according to sample pairing from left to right for shipboard (left-handed heatmap) and autonomous (right-handed heatmap) methods. The gray boxes in the heatmap correspond to a relative abundance of zero. Observed phyla are represented in panels a–c, corresponding to the following metabarcoding markers as follows: (a) 18S rRNA, (b) COI, (c) 12S rRNA, and (d) 16S rRNA. The most commonly observed families are shown for the 12S rRNA marker in (c) since all vertebrate species detected by 12S belong to the same phylum

multiple systems capable of extended deployments now exist (Hansen et al., 2020; Lindsay, 2021; Sepulveda et al., 2020). More recently, the 3G-ESP has successfully been integrated with the LRAUV, allowing for targeted autonomous sample collection to occur *in situ* over the course of days to weeks and at depths up to 300 m (Zhang et al., 2019). The 3G-ESP has been previously validated as a tool for efficient eDNA collection based on targeted qPCR assays (Yamahara et al., 2017). Here, we found that the LRAUV-ESP is comparable to shipboard sampling for marine biomonitoring surveys using eDNA metabarcoding.

Our study demonstrates that eDNA collected and preserved *in situ* by the 3G-ESP produced similar metabarcoding results to ship-based methods when total eDNA yield, alpha diversity (Figure 3), and beta diversity (Figure 4) were compared. Moreover, the 3G-ESP samples yielded significantly more sequence data than eDNA samples filtered with the peristaltic pump despite similar DNA inputs for library preparation. The overall quality of autonomously collected eDNA from the 3G-ESP may explain these results. The 3G-ESP reduces the rate of water filtration (range 0.5–0.2 ml/s) to maintain a pressure difference (12–26 psi) across the filter and thus does not likely reach the pressures seen using peristaltic pump filtration (Thomas et al., 2018). This may have resulted in higher molecular weight eDNA by avoiding flow rates that can negatively affect cell retention, particularly for phytoplankton that are prone to lyse under rigorous filtration (Goldman & Dennett, 1985). The 3G-ESP samples were also preserved with RNAlater under an N_2 atmosphere, which may provide higher DNA quality than freezing without a preservative as was done for the manually collected samples. Higher molecular weight template DNA can result in more sequencing reads as the PCR products will contain a higher proportion of amplified target gene relative to unamplified template or nonspecific amplification products (Quail et al., 2008; White et al., 2009). Thus, despite combining PCR products in equimolar concentrations for the sequencing library, sequence data from samples with higher quality eDNA may also contain more target amplicons. Further investigation of the relationships among peristaltic filtration rate, resulting DNA molecular weight, and sequencing read output may provide more quantitative results and valuable guidance for marine eDNA sample collection.

The sequencing results of eDNA samples collected and preserved by the 3G-ESP revealed the same trends in taxonomic composition associated with depth and seasonality as the shipboard methods. These findings suggest that the Shannon alpha diversity and beta diversity were driven by the biological characteristics of the *in situ* samples rather than the sampling method. These findings were consistent across four commonly used eDNA metabarcoding primer sets spanning a wide range of prokaryotic and eukaryotic taxa. Alpha diversity was comparable between sample groups across all metabarcoding markers (Figure 3), suggesting that the two methods recover similar species richness, while taxonomic composition results (Figure 5) support the idea that no major taxa are consistently excluded by either method, with the lone exception of organisms of the family Chimaeridae, which were detected only by the shipboard method. Beta diversity analyses also showed samples cluster largely by season and depth rather than by the sampling method (Figure 4). The 12S data sets were more variable within each sample group and yielded fewer ASVs than the other marker genes, as reflected by the lower alpha diversity values (Figure 3). This difference may be due to lower levels of the 12S target gene in environmental samples compared with the 16S rRNA, 18S rRNA, and COI markers and, consequently, greater incidence of amplification bias, trends that have been observed previously for this marker (Gold et al., 2021; Kelly et al., 2019; Port et al., 2016). Further, some of the observed variability may be due to the relatively high number of PCR amplification cycles (41 initial + 15 indexing = 56 total) performed for this marker gene. Extremely low levels of vertebrate eDNA in some marine samples can necessitate this level of amplification; however, PCR biases can become much more pronounced and lead to higher variability among samples as cycles increase (Nichols et al., 2018). Thus, a small number of samples enriched with certain taxa could have more easily skewed the comparison between data sets. We observed minor differences in relative abundance for three vertebrate families (Engraulidae, Sebastidae, and Centrolophidae), but the major difference was in the family Chimaeridae, which appeared in 7 shipboard collected samples and none of the 3G-ESP samples (Figure 5). Additionally, fine-scale spatiotemporal variability in fish distributions in the Monterey Bay (Andruszkiewicz et al., 2017) may explain the differences between the shipboard and 3G-ESP samples for the 12S vertebrate primer set, since the comparative samples were collected ~1 km away and ~1 h apart from each other.

The 18S rRNA gene data set also showed more separation by method in beta diversity plots than those of other markers (Figure 4). A higher relative abundance of arthropods was observed in the shipboard sample group, while the reverse trend existed for dinoflagellates (Figure 5). The phylum Arthropoda includes many common zooplankton species, including copepods and krill, which could easily have been captured by the Niskin bottles but excluded from the 3G-ESP given its mode of sample acquisition (steady "sipping" from a small intake covered with a mesh screen). In contrast, two dinoflagellate species in the genus *Protoperidinium* had consistently higher relative abundance in the 3G-ESP samples compared with shipboard CTD rosette samples (>20% compared with <2% summed across samples within each group). *Protoperidinium* is a heterotrophic, armored genus with relatively large (60–300 μm length) cell sizes; it is thus unclear why the 3G-ESP might have a sampling bias for members of this genus. Their prey consists largely of diatoms (Jeong et al., 2004;

Menden-Deuer et al., 2005), and the diatom phylum Bacillariophyta was also higher in the autonomous samples than the shipboard samples (Figure 5), suggesting a possible predator–prey association detected with eDNA. Overall, community structure was very similar at the phylum (16S, 18S, COI) and family (12S) levels between the two sampling methods (Figures 4 and 5). Thus, community-level trends were consistent across sample groups, further validating the robustness of autonomous sampling in replicating shipboard sampling.

The full series of negative controls indicated that there were sources of contamination throughout sample collection and processing, with human DNA found in negative PCR controls indicating issues during laboratory phases of operation. With regard to the 3G-ESP, additional onboard flushing steps have since been implemented into subsequent 3G-ESP LRAUV deployment designs. Potential carryover between environmental samples did not appear to affect the overall patterns of diversity found here for 16S, 18S, and COI data, which were primarily driven by depth and seasonality (Figure S1a–c). The exception to this trend was the 12S primer set, which appeared more susceptible to broader effects of contamination due to the far fewer number of taxa detected than the other three primer sets (Figure S1d). These results highlight the importance of using a suite of negative controls to diagnose and mitigate issues that may occur throughout the course of eDNA monitoring surveys.

4.2 | Advantages and disadvantages of autonomous eDNA sampling

A critical goal of marine eDNA research, particularly with respect to long-term survey and management applications, is the development of a consistent and standardized protocol capable of producing high-quality, reproducible results. This goal is not easily achieved in marine surveys that rely on shipboard operations, variable skill sets of individual scientists, and often, the complex management of scientific priorities among an interdisciplinary team aboard oceanographic vessels that have only a limited amount of time at sea at particular locations. We provide some of the first evidence that autonomous *in situ* eDNA sample collection instruments, such as the 3G-ESP, can overcome many of these challenges with respect to collection of material for metabarcoding analyses, and provide a sampling methodology that is equivalent to and in some instances preferable to shipboard sampling methods. For example, during filtration the 3G-ESP monitors pressure in real time to attempt to prevent loss of cell material during eDNA sample collection. The 3G-ESP sampling procedure can be adjusted to meet specific requirements for target organisms, making this autonomous method more replicable than shipboard benchtop filtration (i.e., operator experience).

Targeted sampling of eDNA from dynamically changing ocean conditions is another advantage that autonomous methods have over ship-based eDNA sampling. The LRAUV-borne 3G-ESP, for example, is engineered to be a much more agile sampling platform capable of responding to and pairing eDNA samples to ecologically important ocean features such as fast-moving water currents, isotherms, and upwelling events, as well as using real-time sensor data onboard the AUV to directly inform sampling (Zhang, Kieft, et al., 2020; Zhang et al., 2019). Improving our understanding of how well these new types of autonomous sampling techniques capture the dynamics of biological communities over space and time, relative to what limited shipboard collection methods offer due to expense and logistical constraints, will help guide the adoption of this technology into future sampling biomonitoring studies.

While autonomous sampling can provide several advantages over manual bottle sampling, it has some disadvantages as well. For example, the 3G-ESP is not capable of rapidly collecting several liters of a discrete water parcel like the Niskin bottles commonly used onboard research vessels. The much larger intake diameter of the Niskin water sampling device compared with that of the 3G-ESP (~150× larger) allows the Niskin to collect larger invertebrates such as copepods and larger pieces of marine snow than the 3G-ESP. Additionally, the LRAUV-borne 3G-ESP can currently only be deployed to depths <300 m, thus limiting its ability to sample eDNA from deep water environments.

5 | CONCLUSIONS

Overall, this comparative study provides evidence that autonomous eDNA sample collection and preservation methods provided by the 3G-ESP LRAUV compare favorably with commonly used shipboard methods. Some marker genes, in particular the 12S and 18S markers, exhibited fine-scale differences between methods in alpha and beta diversity; however, these differences were minor relative to the diversity patterns attributable to the ecological drivers of depth and seasonality. Marine biological assemblages are apparently captured equally well between autonomous and shipboard eDNA sample collection methods as ecological patterns were consistently observed with both methods across the four commonly used eDNA metabarcoding marker genes used in this study. The study further highlights advantages that the autonomous environmental samplers provide in terms of reproducibility of results and the potential to expand eDNA sampling in space and time without the strict requirement or expense of accessing ship time. Such an expansion of scale is urgently needed to help inform aquatic conservation and management practices by increasing our ability to detect community changes in sensitive habitats (McClenaghan et al., 2020, McDevitt et al., 2019), identify the presence of rare or endangered species (Pfleger et al., 2016; Strickland & Roberts, 2019), and track the spread of invasive species or pathogens (Klymus et al., 2017; Robson et al., 2016). Eventually, we foresee the autonomous sampling capabilities being extended to include near-real-time *in situ* analyses (e.g., sequencing) at sea in a scalable and distributed context, providing critical information to observe and manage life in the sea and the ecosystem services provided.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors designed and planned the study and provided feedback that helped develop the manuscript. NKT, NVP, MM, and KJP drafted the manuscript, analyzed data, and interpreted results. KDG oversaw the 16S metabarcoding aspects of the work. CMP and KMY prepared the autonomous environmental sample processors for deployment, assisted with autonomous sample collection, and interpreted results. YZ, BYR, BK, and BH prepared the autonomous underwater vehicles for deployment and managed field operations. KJP and FPC designed and coordinated the joint mission with the NOAA ship *Reuben Lasker*.

DATA AVAILABILITY STATEMENT

Illumina MiSeq FASTQ files are available for download on NCBI (BioProject ID PRJNA726280). Sample metadata, final ASV tables, and all R-Scripts used for data analysis and generating the figures and tables presented in the manuscript are available on GitHub (https://github.com/MBARI-BOG/ESP_CTD).

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SUPPORTING INFORMATION

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