A Pilot Environmental DNA Study for Demonstrating Autonomous Sampling and Optimizing Sample Volumes

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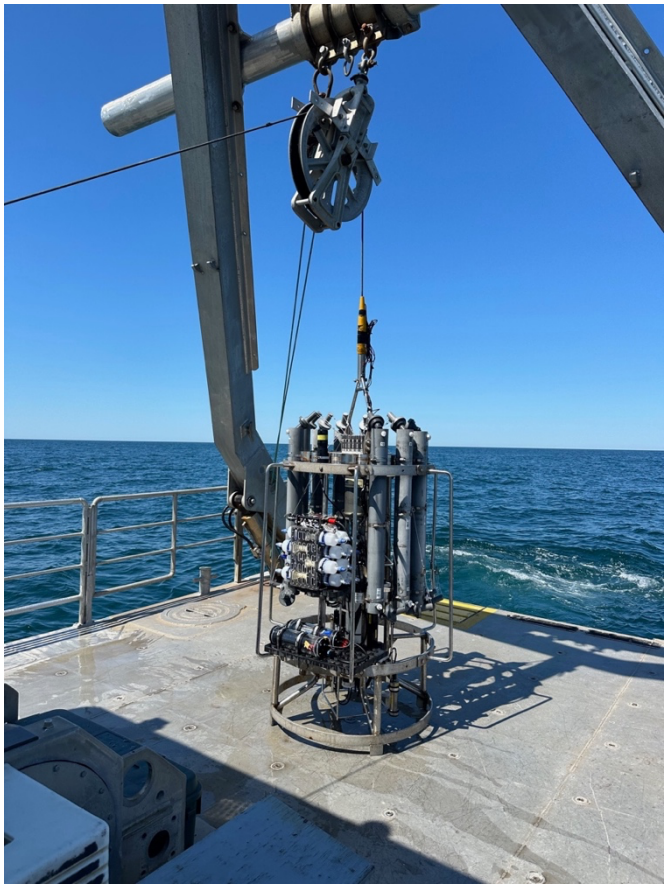

Table of Contents

1. Executive Summary

Environmental DNA metabarcoding analyses are an efficient and scalable approach for biodiversity monitoring, and optimizing sampling parameters is important for effective implementation of a monitoring program. Sampling factors, including collection methods, filter choices, and sample volumes can potentially influence the detection of taxa and ecosystem metrics and should be evaluated. Autonomous sample collection with in situ filtration has many practical advantages, including reducing processing time and increased control over sampling parameters such as the volume of seawater filtered. Larger sample volumes, greater than those that can be recovered in conventional Niskin bottle water sampling, may result in the detection of more taxa. Therefore, the objectives of this pilot study were to: 1) determine optimal sample volume for the detection of animal taxa by comparing metabarcoding results from eDNA samples originating from a range of sample volumes; 2) assess variability in community composition between sample replicates as a function of volume; 3) assess the impact of seasonal variability in our eDNA metabarcoding assessments; and 4) provide further validation on autonomous eDNA sampling. This study employed the autonomous Oceanic-WHOI eDNA sampler, which has a relatively high flow rate (~2 liters/minute) and allows for real-time control of sample volume. Sampling was conducted at a site located south of the island of Nantucket (Massachusetts, USA) during April and July 2024 and entailed collecting triplicate samples encompassing a range of volumes at 10 meters and 30 meters depth. A CTD rosette possessing 12 3.3-liter Niskin bottles (for conventional small-volume 0.5, 1, and 2 liter samples on Sterivex filters) and the integrated Oceanic-WHOI multisampler (for the 2-20 liter samples on large-area Waterra filters) was used to collect water and filtered samples. Animal diversity was assessed across season, depth, and sample volume from 18S and 12S metabarcode datasets. Results from both metabarcode markers showed seasonal and depth differences with season being the largest driver of variability, and overall indicated that there is an optimal sample volume in terms of taxon detection, consistency of the results, and sampling effort. However, the two markers varied in terms of how taxon detection was impacted by sample volume. These differences may be due to filter type and surface area, the taxonomic resolution of the target taxa, and the distribution of the target taxa eDNA. Future research will be necessary to disentangle the impact of these factors on metabarcoding results. Overall, the results of this study demonstrate the utility of autonomous sampling in terms of practicality and efficiency, and in the ability to control sampling parameters. Study results also indicate the need for regional pilot studies to optimize sampling parameters in the context of specific environments, seasons, and monitoring goals.

2. Introduction

2.1 Background

Environmental DNA metabarcoding analysis is a powerful new approach for animal biodiversity observing, and optimizing sampling parameters and obtaining baseline data will enable effective implementation of a monitoring program (Hestetun et al., 2023). While eDNA approaches are relatively new, research on the development and application of eDNA is increasing exponentially (Patin and Goodwin, 2023; Yang et al., 2024). As a scalable and efficient approach, eDNA-based monitoring is poised to become a practical solution for ocean observing needs (Chavez et al., 2021).

Many factors need to be considered when interpreting animal eDNA metabarcoding datasets. Environmental processes, such as the rates at which eDNA sheds and decays, physical factors such as eDNA dispersal and transport, and laboratory factors such as molecular protocol parameters, are known to influence eDNA analyses (**Figure 1**). An important, but often overlooked consideration is eDNA sample volume, which impacts the detection of animal taxa (Govindarajan et al., 2022).

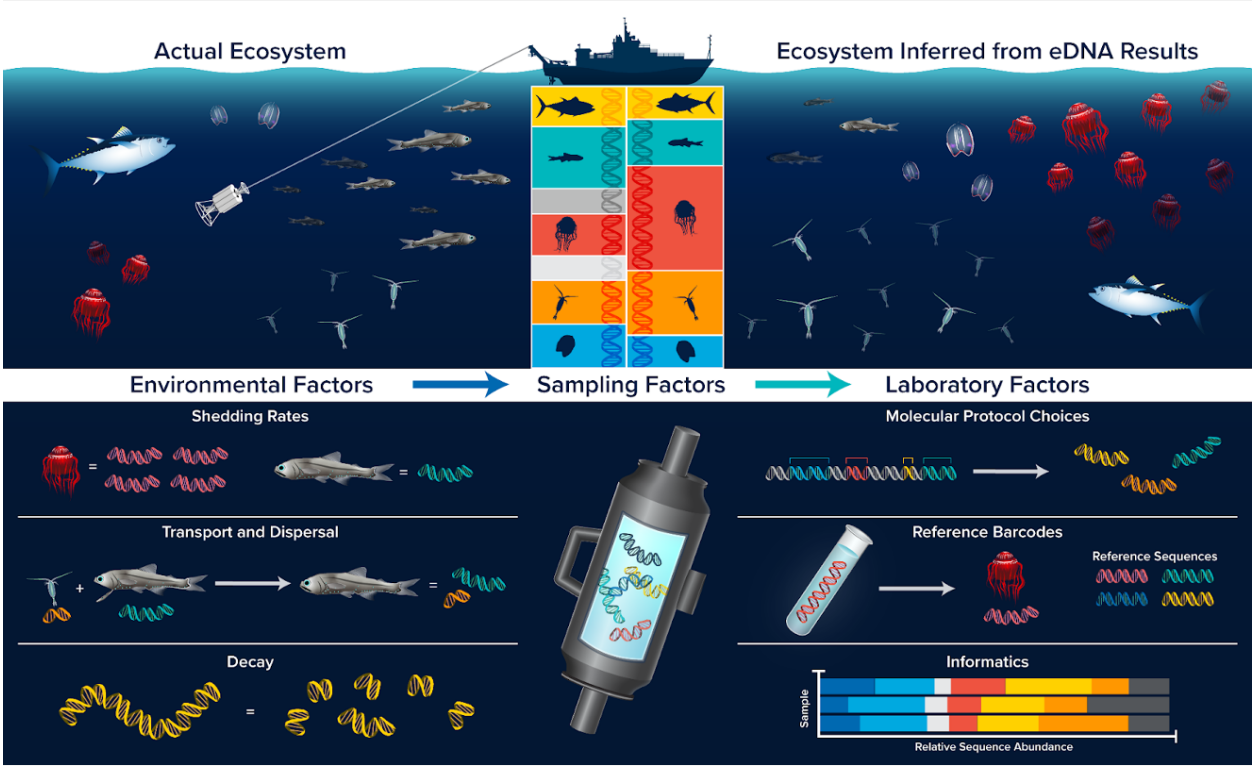

Figure 1. An interpretive framework for eDNA analysis. *Top panel:* The ecosystem as inferred by eDNA analysis differs from the actual ecosystem. *Bottom panel:* Environmental, sampling, and laboratory factors all contribute to biases in eDNA datasets. It is necessary to understand how these factors impact eDNA results to improve the accuracy of eDNA assessments. Figure from Govindarajan et al., 2023.

Environmental DNA is collected from seawater samples by filtering the water and extracting the DNA from the filters. In conventional oceanic sampling, the seawater is collected in Niskin bottles from a CTD rosette, and then filtered once the bottles are retrieved. Sample volumes are limited by the Niskin bottle size, number of available bottles, and other demands on the collected water (e.g., nutrient analyses). Typical eDNA sample volumes are 1-5 liters, but optimal volumes for detecting biodiversity are rarely assessed even though eDNA concentrations are known to vary greatly with water column depth (Govindarajan et al., 2022).

To address deep pelagic sampling needs, Govindarajan et al. (2022) developed a large volume autonomous eDNA sampler with in situ filtration. The sampler was integrated onto the midwater AUV *Mesobot* and large volume (40-60 liter) samples were taken at different depths in the water column. These samples revealed more taxa than co-collected small volume (2 liter) samples obtained from CTD-mounted Niskin bottles.

A newer version of the Govindarajan et al. (2022) sampler has been successfully deployed on *Mesobot* as well as other ocean observing platforms (Govindarajan et al., 2023). The sampler, referred to as the "Oceanic-WHOI" sampler, can collect up to 16 samples per deployment and filters water through large area filters at approximately 2 liters/minute. Each filter is connected to an individual pump, which can be controlled in real time from a shipboard computer.

Animal abundances, environmental DNA concentrations, and corresponding optimal eDNA sampling volumes, are expected to vary with depth, region, and season - reflecting shifts in biomass (the source of eDNA) and physical factors such as ocean currents and temperature (affecting eDNA dispersal and decay). Because sample volume impacts the likelihood of species detection (Govindarajan et al., 2022), it should be carefully considered when initiating a monitoring program.

2.2 Goals

The objectives of this pilot study are to: 1) determine optimal sample volume for the detection of animal taxa by comparing metabarcoding results from eDNA samples originating from a range of sample volumes; 2) assess variability in community composition between sample replicates as a function of volume; 3) assess the impact of seasonal variability in our eDNA metabarcoding assessments; and 4) provide further validation on autonomous eDNA sampling. The proposed study will take place south of the island of Nantucket (Massachusetts, USA). The study includes sampling missions at two times of the year (spring and summer) when biological production and biodiversity are expected to differ; and includes sample collection along vertical transects with depths ranging from the surface to near-bottom. The study also includes metabarcoding analysis of the 18S V9 marker (targeting a broad range of invertebrate groups) and the 12S marker (targeting fish species).

3. Methods

3.1 Fieldwork

Fieldwork consisted of one day of field sampling in the spring (April 17, 2024) and one day in the summer (July 2, 2024). The field site was located 60 miles east of Montauk and 20 miles south of Nantucket MA, USA at coordinates provided by Equinor (41-5.0'N / 70-39.14'W; **Figure 2**). The study's aim was further validation of eDNA technology as a research and development

activity financed by Equinor, and not linked to any requirements for the lease or consenting issues. The choice of location for sampling also had to allow for accessibility for WHOI staff and vessel, being based in MA, USA. The field site was accessed by WHOI's vessel, the R/V Tioga. The water depth at the sampling site was ~35-50 m and our sampling targeted two depths on both dates (10 m and 30 m). To address our goal of assessing sample volume, we collected triplicate samples from volumes ranging from 0.5 to 20 liters. Specifically, for each date and depth, we collected sample sets based on 0.5, 1, and 2 liter sample volumes using Niskin bottle water collections, and 2, 6, 12, 16, and 20 liter sample volumes using the Oceanic-WHOI multisampler. Sample volumes were controlled by monitoring the flow from a laptop computer on the ship. In the July 10 m deployment, flow slowed substantially as filtration progressed in the 16-liter samples indicating that the filters had become clogged (**Appendix A**). Therefore, to avoid damaging the sampler pumps, we did not attempt to collect 20-liter samples and instead collected a set of 9-liter samples (**Appendix B**).

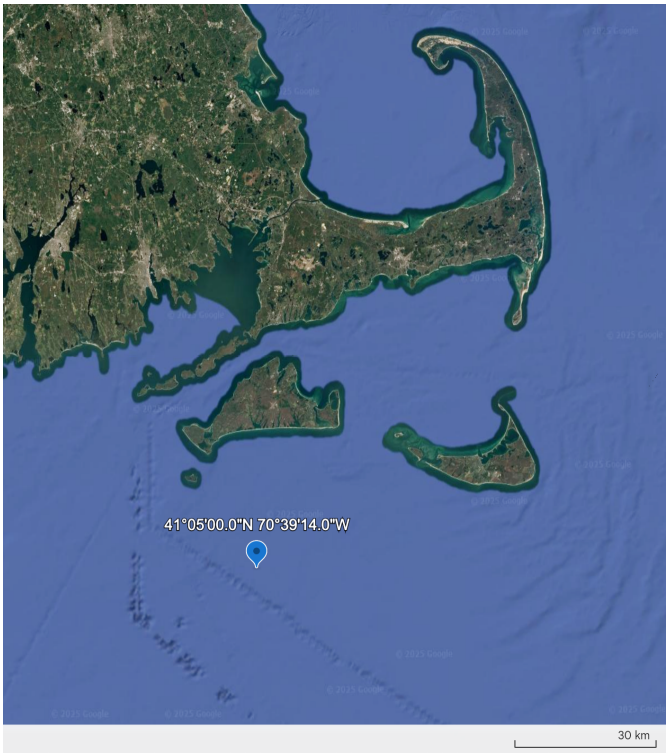

Figure 2. Study site, located south of the island of Martha's Vineyard, MA.

A CTD rosette possessing 12 3.3-liter Niskin bottles (for the 0.5, 1, and 2 liter samples) and the integrated Oceanic-WHOI multisampler (for the 2-20 liter samples) was used to collect water and filtered samples (**FIgure 3**). For the Niskin bottle water samples, water was sampled on the upcasts. Immediately upon retrieval of the CTD rosette, the Niskin bottle water was transferred to sterile containers and placed in coolers with dry ice or Techni-Ice icepacks, for filtering upon return to the lab on shore. The laboratory filtering process entailed pumping the water samples through 0.2 µm PES Sterivex filters, which were then preserved in a -80°C freezer. Sterile, molecular grade water sample was also filtered on each date to serve as a negative control. For the large-volume samples, the Oceanic-WHOI multisampler, controlled by a laptop

from the ship, filtered water in situ through sterile Waterra 0.2 µm PES capsule filters. Additional Waterra filters, which did not have water pumped through them, were placed on the sampler to serve as field blanks (negative controls). Once retrieved, the Waterra capsules were stored on ice and transferred to a -80°C freezer upon return to WHOI. Data from the CTD were plotted to characterize the water column on each sampling date (**Appendix C**).

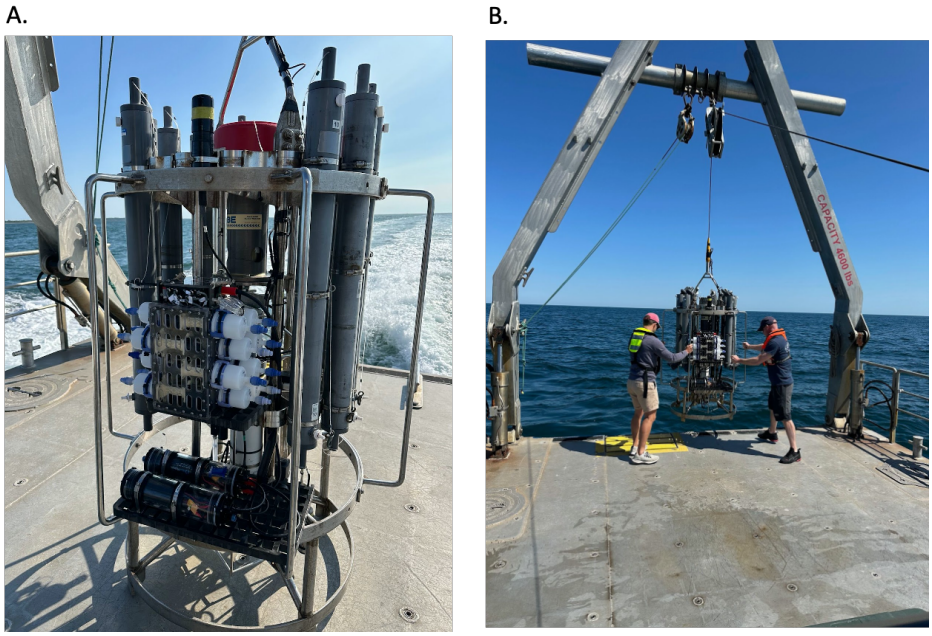

Figure 3. A) The Oceanic-WHOI sampler with attached Waterra capsule filters mounted on the CTD rosette. **B)** Deploying the CTD rosette with the sampler on the R/V Tioga in July.

3.2 DNA Extractions

Genomic DNA was extracted from the Sterivex and Waterra filters using DNeasy Blood and Tissue Kits (Qiagen) following established protocols (Govindarajan et al., 2021, 2022). For the Waterra filters, the extraction method followed the protocol described for large-area capsule filters (Pall MiniKleenpaks; Govindarajan et al., 2022). The Waterra capsules were opened using a pipe cutter and the filters were removed using sterile scalpels and forceps. The outer pre-filter and inner filter were each sectioned into 6 slices, extracted individually using the DNEasy Blood and Tissue Kit, and pooled. Thus, the eDNA aliquots included DNA from both the filter and pre-filter, to capture a wider range of biodiversity (Govindarajan et al., 2022). Extraction blanks were processed with the sample extractions to identify potential contamination occurring during this step. DNA extractions were quantified using a Qubit fluorometer.

3.3 Controls

Rigorous controls, all of which are routinely applied in Govindarajan's laboratory, were taken to prevent and identify contamination during all stages of the study. In the field operations, these included incorporation of negative controls (field blanks) in both the Niskin and autonomous sampling which were processed with the samples all the way through sequencing, the use of pre-

sterilized capsule filters (Sterivex and Waterra filters), and bleach sterilization of sampling components (e.g., peristaltic pump tubing, Waterra filter housings). In the laboratory, processing was conducted in an eDNA-dedicated space. Before each use, all surfaces were decontaminated with 10% bleach, followed by wipes with MilliQ water and ethanol to remove the bleach. Waterra filter handling (removal of the filters from capsules and slicing) was conducted in a PCR hood with UV-sterilization. All pipettes were regularly decontaminated by UV sterilization with a nUVaClean UV Pipette Sterilizer. Only sterile pipette tips with filters were used. In all stages, project personnel wore gloves and changed them frequently, as needed. Negative controls (blanks) were run during each lab step (extraction blanks, and PCR blanks). Sequences detected in the negative control data were subtracted from the samples during the data analysis.

3.4 PCRs, library preparation and amplicon sequencing

The 18S V9 protocol utilizes primers that target a broad range of eukaryotes including Metazoa (animals) (Amaral-Zettler et al., 2009). The first PCR step was conducted at WHOI as described in detail in Govindarajan et al. (2022). Samples were then sent to the University of Illinois, Chicago for the second PCR step and paired-end Illumina MiSeq sequencing. While the 18S V9 marker detects many invertebrates, it is not well-suited for fish (Govindarajan et al., 2021; 2023b), so the 12S barcode marker was also sequenced. The 12S protocol incorporates the MiFish-U primers (Miya et al., 2015) which target fishes, but can also amplify other vertebrates including marine mammals (Govindarajan et al., 2023b). Aliquots of genomic DNA and negative controls were sent to Applied Genomics for library preparation and sequencing according to their protocols, which followed a standard 2-PCR procedure with a size-selection step to remove non-target amplicons. Briefly, the amplification process employed AmpliTaq Gold 360 Master Mix (Applied Biosystems) and the 12S Mifish primers. Each sample was amplified in triplicate and subsequently pooled to increase ASV diversity. Pooled products were purified using Cytiva Sera-Mag Select magnetic beads and quality-checked via capillary electrophoresis on a QIAGEN QIAxcel Connect instrument with a DNA Screening kit. As part of the rigorous quality control procedures, Applied Genomics included four amplification negative controls taken from the PCR amplification step (“PCR NEG”), and two indexing negative controls (“IDX NEG”) during the indexing PCR step. Negative control samples were included in all downstream processes. To prepare amplicon sequencing libraries, a Nextera XT Index Kit v2 (Illumina) was used, following the manufacturer’s guidelines. Library purification was carried out using Cytiva Sera-Mag Select magnetic beads, and quality was checked using capillary electrophoresis. Library concentrations were quantified, adjusted, pooled, denatured with NaOH, and supplemented with PhiX control following Illumina's protocols. The eDNA amplicon library was sequenced on an Illumina MiSeq sequencer using the MiSeq Reagent Kit v3 (2x300bp chemistry).

3.5 Bioinformatics

Data received from the sequencing facilities was analyzed at WHOI using standard bioinformatics approaches in the Quantitative Insights Into Microbial Ecology 2 (QIIME 2) software platform (Boylen et al., 2019) as described in Govindarajan et al., 2021; 2022; 2023b). Both datasets were processed similarly unless otherwise noted. Sequence quality plots were examined, forward primer sequences at the 5′ end and reverse complements of reverse primers at the 3’ end were trimmed using the Cutadapt QIIME 2 plugin (Martin, 2011). Sequences were

quality filtered, truncated, denoised, and merged to generate amplicon sequence variants (ASVs) using DADA2 (Callahan et al., 2016) within QIIME 2. Sequences found in the negative control samples were subtracted, as were potentially erroneous ASVs that had a summed frequency of less than 10 across the dataset.

For the 18S dataset, which yields information on broad categories (e.g., to the level of Order) of a wide range of invertebrates taxonomic assignment of the ASVs was based on a classifier that was trained on the Silva v.132 99% small subunit rRNA database (Quast et al., 2013). The dataset was filtered using the term “Metazoa” to create a metazoan (animal)-only dataset (Yang et al., in prep.). Unclassified ASVs were Blasted against GenBank, and sequences with a grade of at least 95% were classified to Order. Unclassified, arachnid, and insect sequences were removed from the dataset.

For the 12S dataset - which provides species and genus-level resolution, taxonomic assignment was based on Blast searches of the NCBI GenBank database. Species-level matches were based on at least 98% identity with the reference sequence over at least 90% of the sequence length (Govindarajan et al., 2023b). If multiple species belonging to the same genus fit that criterion, the ASV was identified only to genus. Several non-fish vertebrates were detected in the 12S dataset, some of which were likely contaminants (e.g., human) and others that were outside of the project scope (e.g., bird, freshwater fish) and these were removed.

For both datasets, rarefaction curves (# of unique ASVs as a function of sequencing depth) were generated in QIIME 2 and re-plotted in R (R Core Team, 2021). Stacked bar plots of the 20 most abundant taxa were generated for both markers using ggplot in R. To assess potential impacts of sample volume on community composition, the dataset was normalized in QIIME 2 by randomly subsampling to the lowest sequencing depth and Jaccard similarities. Non-metric multidimensional scaling (NMDS) plots were generated with vegan v2.3_5 (Oksanen et al., 2016) in R. Significant patterns relative to volume, depth, and season were assessed with PERMANOVA tests using the adonis function in R and pairwise comparisons with pairwiseAdonis (Martin, 2020). For the pairwise comparisons, sample volumes were merged into small (0.5-2 liter), medium (6-12 liter), and large (16-20 liter) categories to increase statistical power. Significant differences were found with respect to sampling date (season) and depth, so the impacts of sample volume were assessed with the dataset as a whole and in separate date/depth strata.

4. Results

4.1 18S dataset

The 18S dataset consisted of 12,451,758 raw reads and 11,219,655 reads after the denoising and decontamination steps. The final curated metazoan-only dataset consisted of 7,067,701 reads distributed amongst 119 ASVs. Rarefaction curves were generated for each date/depth combination (April 10m, April 30m, July 10m, July 30m) (**Figure 4**). These plots show an initial increase in the number of ASVs detected followed by a leveling off, indicating that our sequencing depth (i.e., the number of sequences per sample) was sufficient to recover the diversity (represented by the number of ASVs) present in our samples. These plots also showed that diversity was greater in the larger-volume samples even at lower sequencing depths. However, for sample volumes >6 liters, there was no clear relationship between sample volume and diversity (i.e., samples with volumes <20 liters had more ASVs than 20-liter samples).

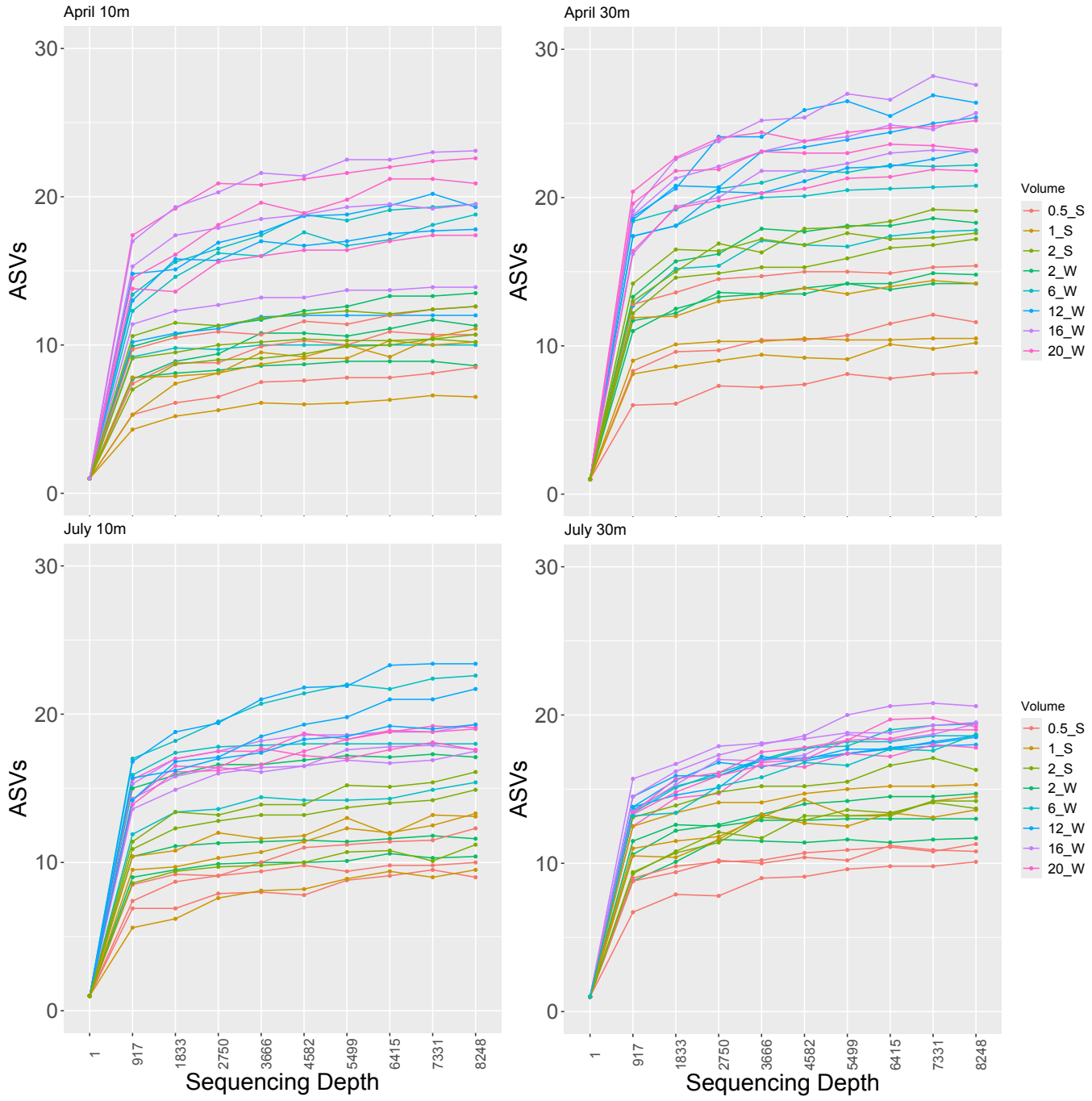

Figure 4. Number of ASVs as a function of sampling depth for the 18S dataset. Plots are terminated at the lowest sequencing depth (8,248).

The composition of the samples varied with sampling date (season) and depth in terms of the relative abundance of the most common orders of invertebrates (**Figure 5**). Sequence reads from calanoid copepods dominated in all datasets, and cyclopoid copepods were the next most

abundant group overall. Aphragmophora (chaetognaths, or predatory invertebrates also known as arrow worms) sequences were commonly observed and had the highest relative abundance in the April 30m dataset. Leptothecata (hydromedusae - a category including some jellyfish) were observed in the July 10m dataset. A variety of other invertebrates were found at lesser relative abundances, particularly in April, which had the greatest diversity of ASVs. Some of these (e.g., Sessilia, or barnacles) likely indicate pulses of larvae of benthic taxa in the water column.

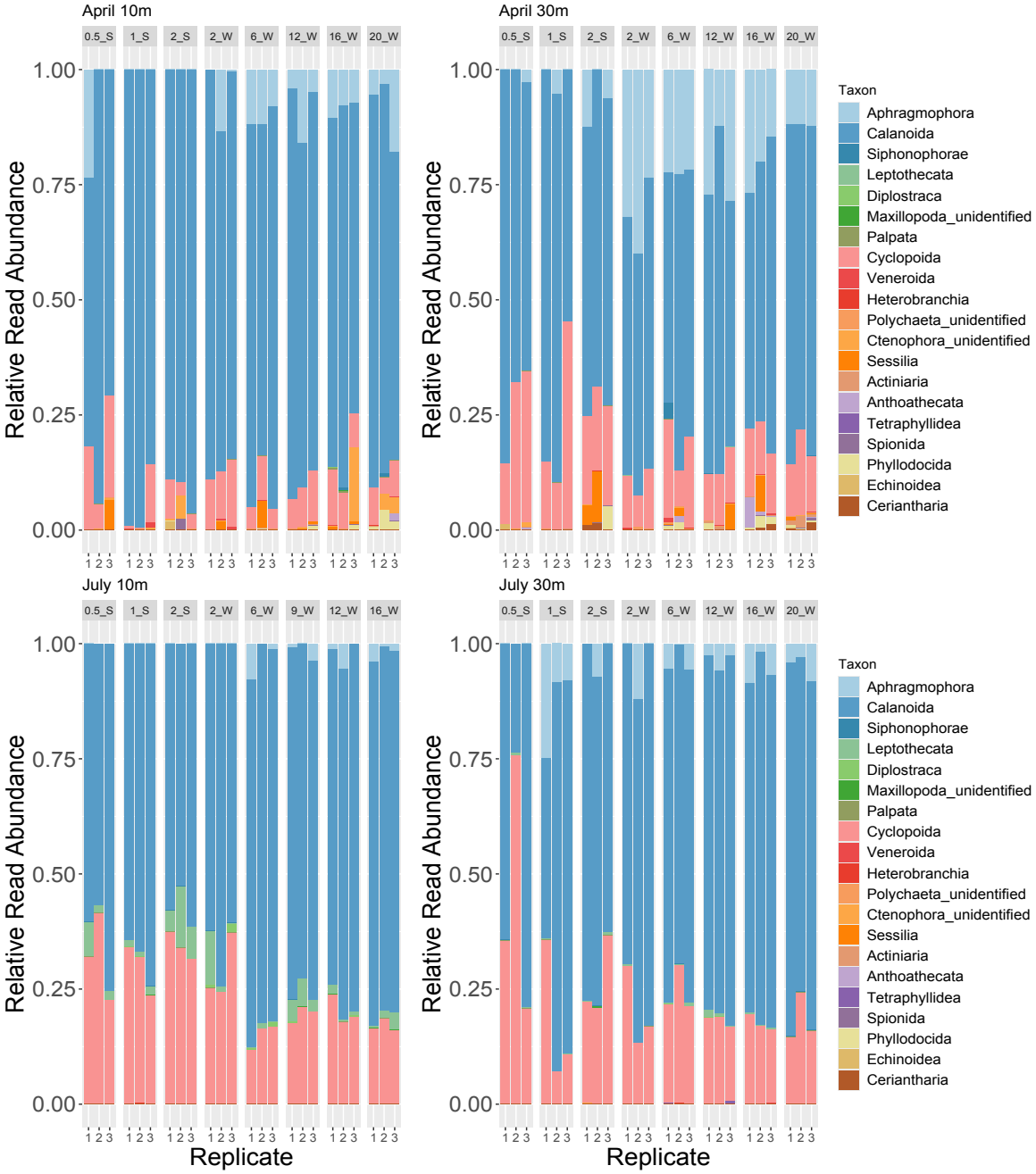

Figure 5. Relative read abundances from the 18S dataset. Taxa represent Order-level classification.

Multivariate analyses indicated impacts of sample volume, observed as differential clustering and greater spread of small, medium, and large volume samples in the NMDS plots (**Figure 6A,** R^2 = 0.16833, p = 0.001). Season and depth also had significant impacts (Season: R^2 = 0.21343, p = 0.001; Depth: R^2 = 0.06258, p = 0.001; **Figure 6B**). Therefore, to reduce the impact of season and depth, the dataset was partitioned and sample volume was assessed separately in each date/depth category (**Figure 7**). In all four cases, small volume samples differed significantly from medium and large volume samples, and exhibited greater variability between replicates (**Table 1**). The large volume samples were generally clustered more tightly than the medium volume samples, but the effect was less pronounced than in comparison to the small volume samples. The 2-liter Waterra samples clustered at times with the 6-liter Waterra samples (**Figure 7B**) but otherwise appeared more similar to the 2-liter Sterivex and smaller volume samples.

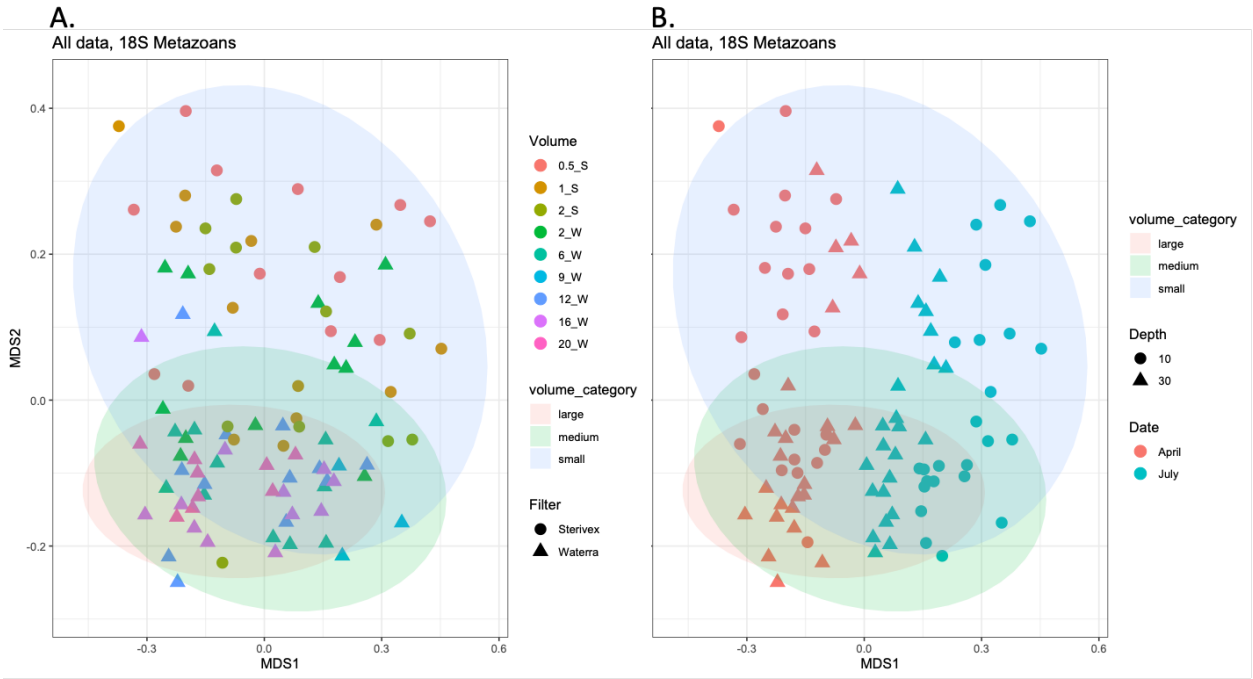

Figure 6. Non-metric multidimensional scaling (NMDS) plots of the 18S dataset. **A)** Samples coded by volume (color) and filter type (shape). **B)** Samples coded by sampling date (color) and water depth (shape). In both plots, sample volumes were classified as “small” (0.5-2 L), “medium” (6-12 L), and “large” (16-20L). Ellipses represent 95% confidence intervals.

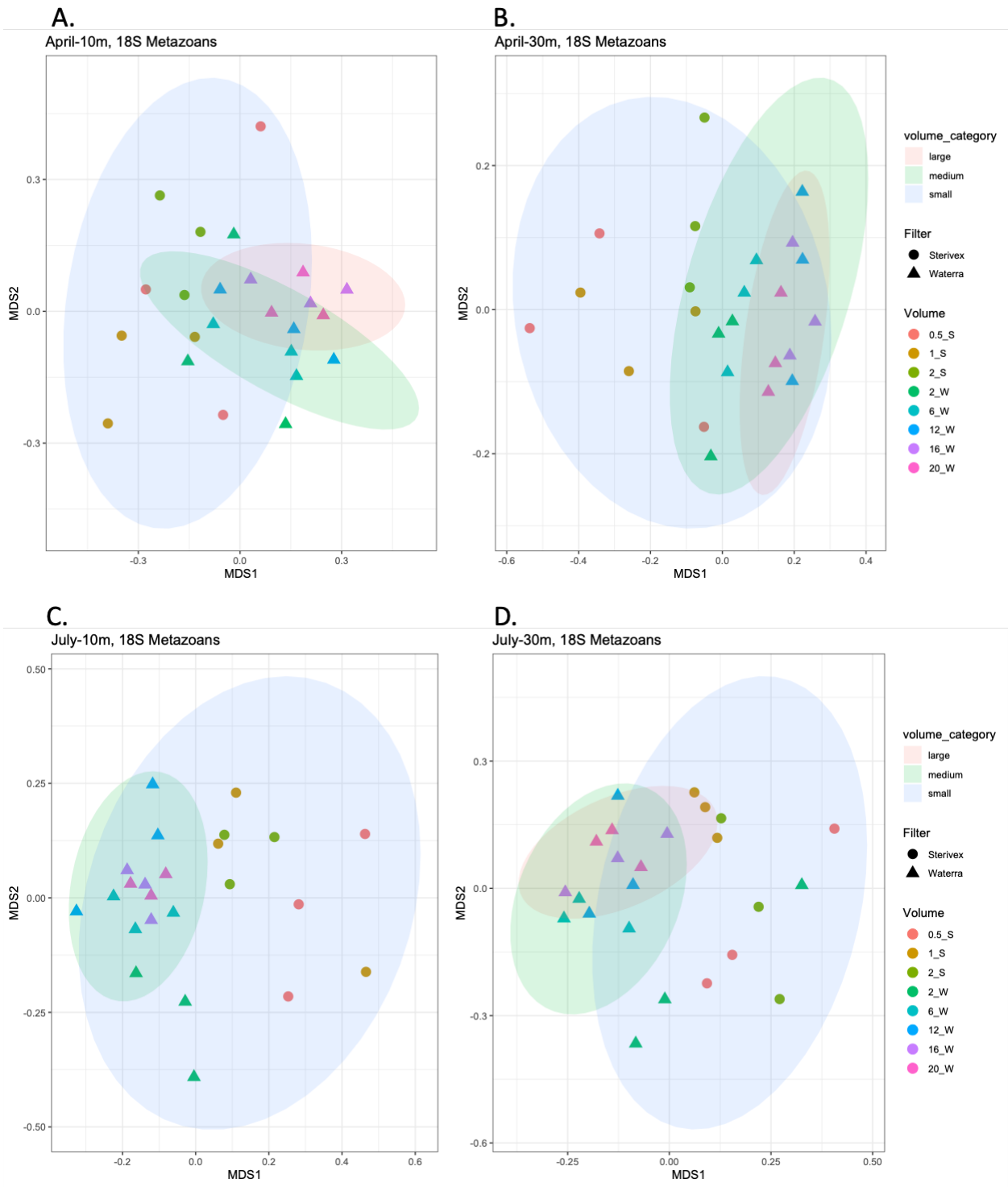

Figure 7. Non-metric multidimensional scaling (NMDS) plots of the 18S dataset. **A)** April - 10m; **B)** April - 30m; **C)** July - 10m; **D)** July - 30m. Sample volumes were classified as “small” (0.5-2 L), “medium” (6-12 L), and “large” (16-20L). Because filter clogging prevented us from obtaining 20-liter samples in the July - 10m case, there were insufficient points to generate an ellipse for large volume samples. Ellipses represent 95% confidence intervals.

Table 1. PERMANOVA results (R^2 and p values) and post-hoc pairwise comparisons (p values) for the 18S dataset.

Season/Depth	*R^2*	*p*	small vs. medium	small vs. large	medium vs. large
April 10m	0.40866	0.013	0.003	0.003	0.299
April 30m	0.47938	0.001	0.009	0.009	0.222
July 10m	0.51618	0.001	0.003	0.0045	0.035
July 30m	0.48293	0.001	0.0015	0.0015	0.039

4.2 12S dataset

The 12S dataset consisted of 10,120,963 raw reads and 6,291,322 after the denoising and decontamination steps and removal of vertebrate taxa beyond the scope of this study (e.g., freshwater fish, birds). Rarefaction curves were generated for each date/depth combination (April 10m, April 30m, July 10m, July 30m) (**Figure 8**). As we found with the 18S data, the sequencing depth was sufficient to capture the ASV diversity in the samples. However, the relationship between the number of ASVs recovered and sample volume was less clear. Small-volume Sterivex samples recovered the greatest number of ASVs in all cases, and the large-volume samples generally contained an intermediate number of ASVs. The small-volume Waterra samples tended to have the least numbers of ASVs.

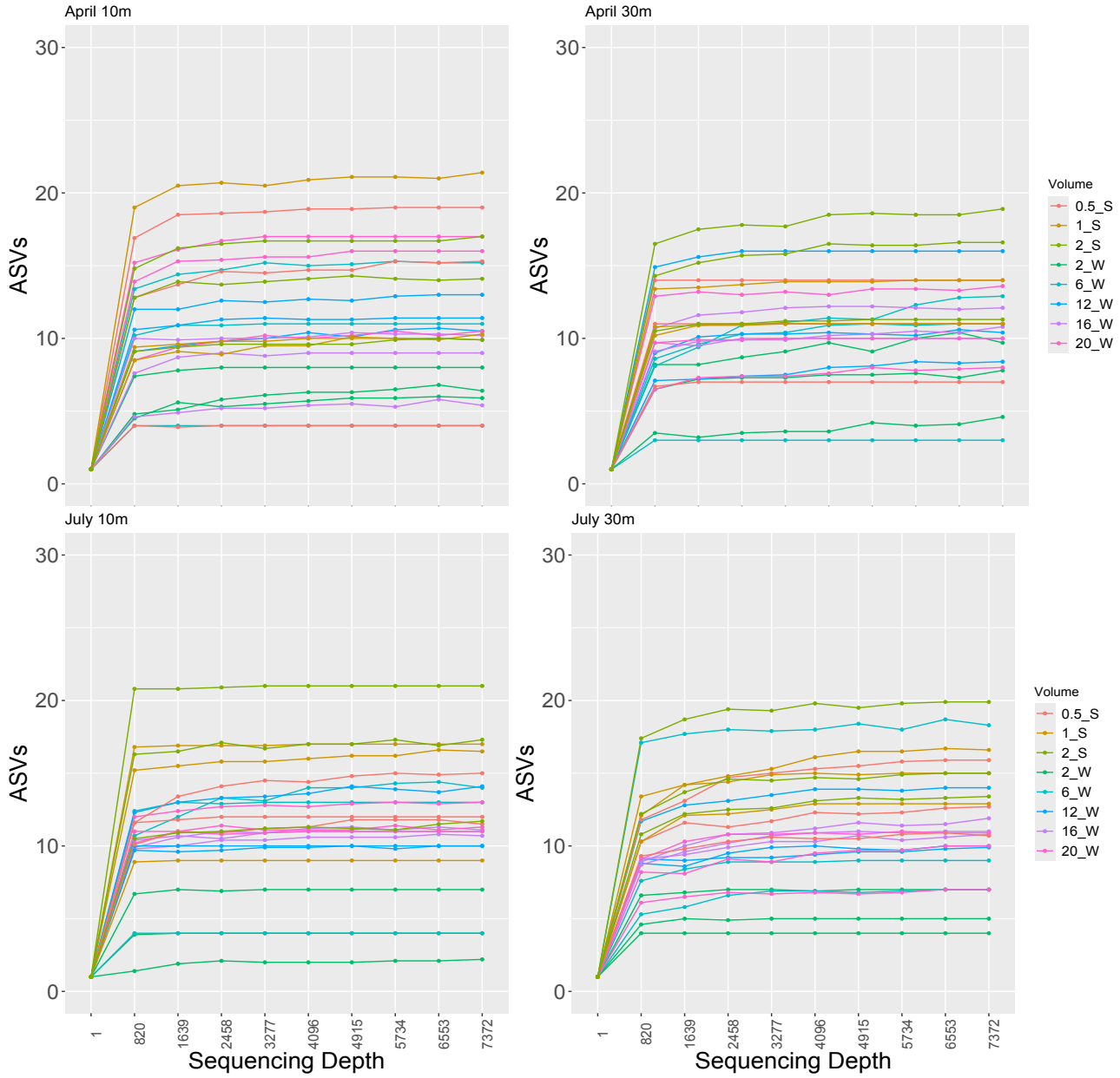

Figure 8. Number of unique ASVs as a function of sequencing depth for the 12S dataset, **A)** April - 10m; **B)** April - 30m; **C)** July - 10m; **D)** July - 30m.

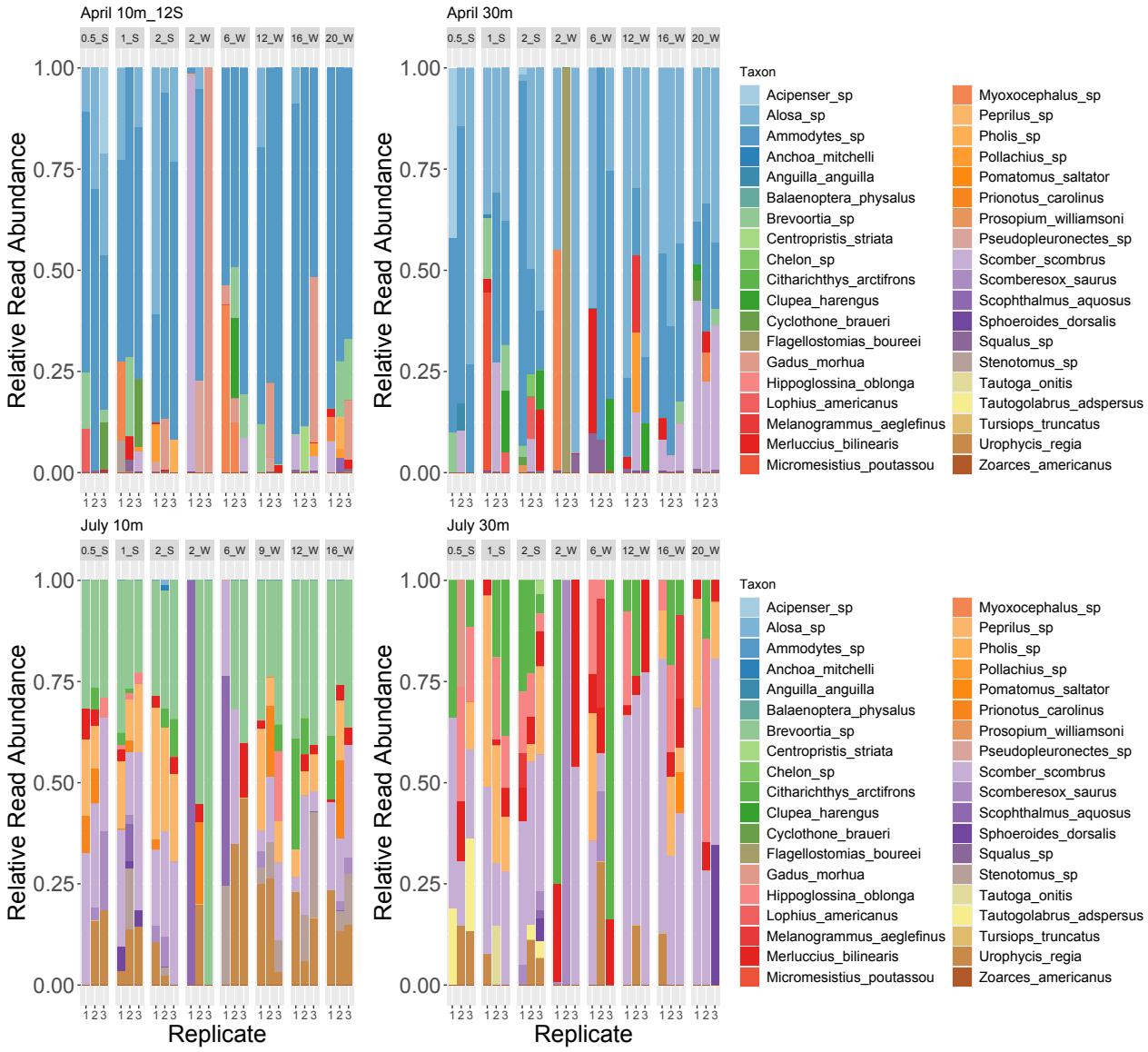

Figure 9. Relative read abundances of vertebrates (marine fish and mammals) identified from the 12S dataset.

The composition of the samples varied with sampling date and depth in terms of the relative abundance of the most common taxonomic groups (**Figure 9; Figure 10**). However, as a whole, sample volume category (small, medium, and large) did not impact composition (**Figure 10**). When the date/depth datasets were examined separately, sample volume category was important in some cases (e.g., April 30m; **Figure 11; Table 2**). Overall, the small-volume Waterra samples were the most and consistently variable on the NMDS plots, as indicated by the larger area of the blue ellipses (**Figure 11**). While the PERMANOVA results indicated significant

differences related to sample volume category, the post-hoc comparisons between specific volume categories (**Table 2**) were less consistent than for the 18S data (**Table 1**).

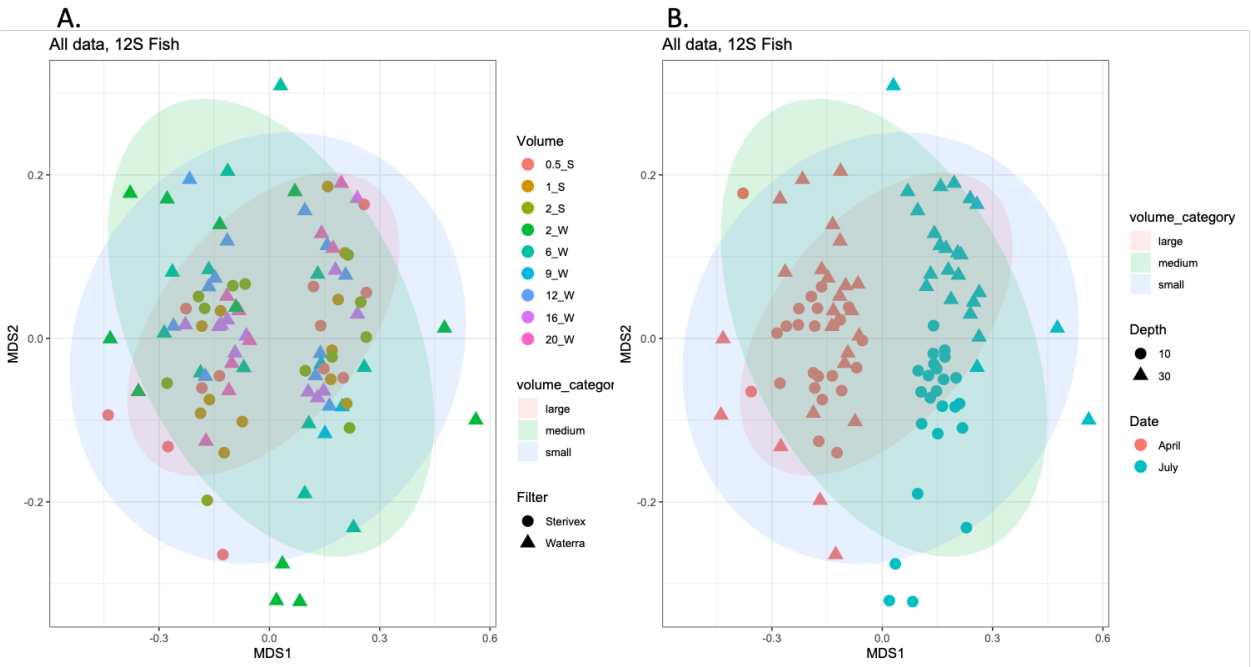

Figure 10. Non-metric multidimensional scaling (NMDS) plots of the 12S vertebrate dataset. **A)** Samples coded by volume (color) and filter type (shape). **B)** Samples coded by sampling date (color) and water depth (shape). As with the 18S data, sample were classified as "small" (0.5-2 L), "medium" (6-12 L), and "large" (16-20 L). Ellipses represent 95% confidence intervals.

Table 2. PERMANOVA results (R^2 and *p* values) and post-hoc pairwise comparisons (*p* values) for the 12S dataset.

Season/Depth	*R^2*	*p*	small vs. medium	small vs. large	medium vs. large
April 10 m	0.33472	0.012	0.0675	0.057	0.717
April 30 m	0.33685	0.03	0.232	0.009	0.015
July 10 m	0.35331	0.001	0.4995	0.4995	0.801
July 30 m	0.32736	0.063	0.473	0.473	0.473

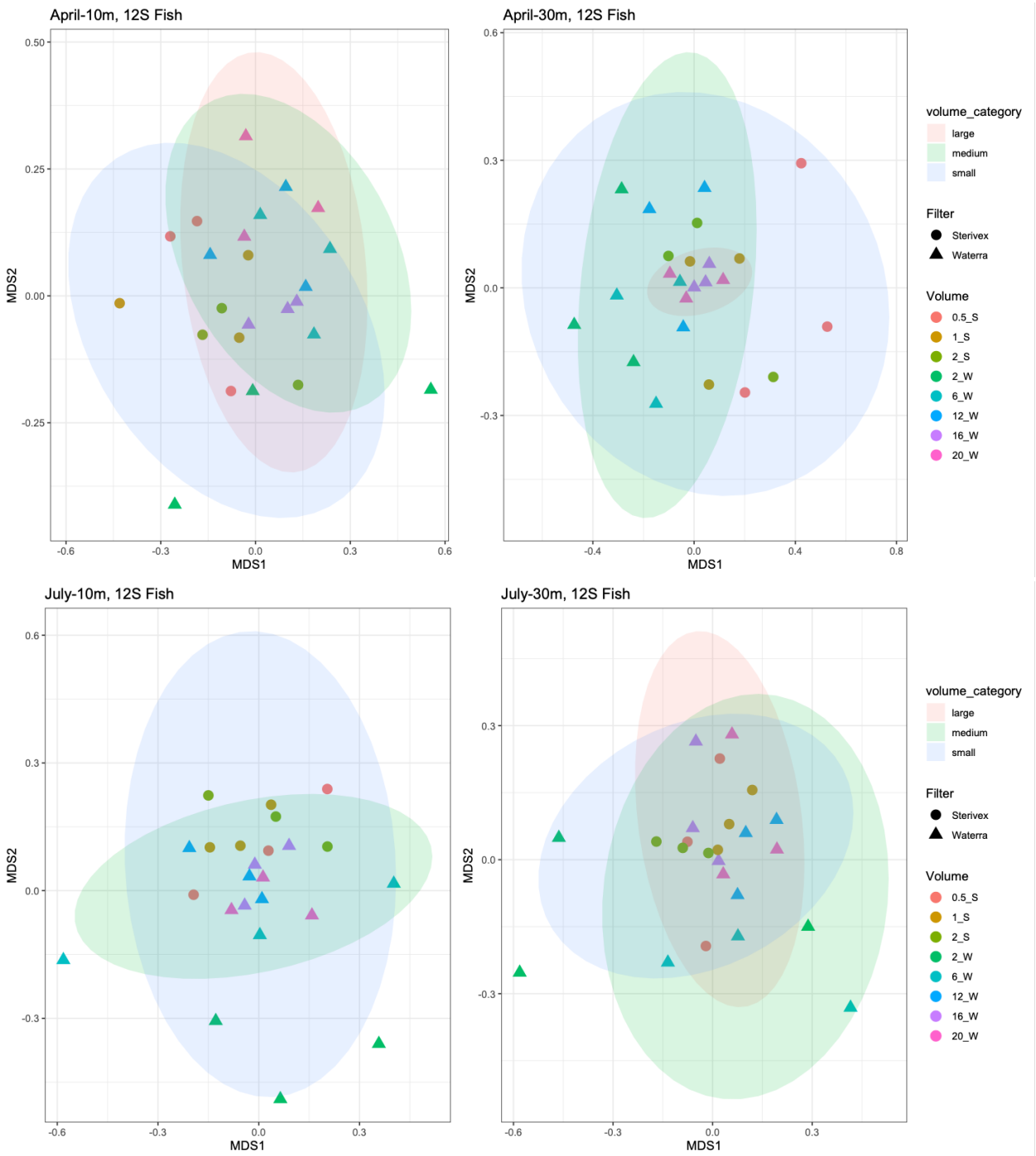

Figure 11. Non-metric multidimensional scaling (NMDS) plots of the 12S vertebrate dataset. A) April - 10m; B) April - 30m; C) July - 10m; D) July - 30m. Sample volumes were classified as “small” (0.5-2 L), “medium” (6-12 L), and “large” (16-20 L). Because filter clogging prevented us from obtaining 20 liter samples in the July - 10m case, there were insufficient points to generate an ellipse for large volume samples. Ellipses represent 95% confidence intervals.

5. Conclusions, recommendations, and next steps

We successfully demonstrated the utility of autonomous eDNA sampling through the application of the Oceanic-WHOI large-volume multisampler to collect eDNA samples at a study location south of Nantucket, Massachusetts, USA. The sampler was mounted on to a CTD rosette, which enabled collection of samples at different depths. The sampler filtered seawater in situ, so samples could be immediately preserved upon retrieval. Filtration was controlled via a laptop on board the vessel, and sample volume and flow rate could be monitored in real time.

To further facilitate the optimization of eDNA - based ecosystem monitoring parameters, we examined animal community composition recovered over a range of sample volumes, depths, and seasons. We analyzed data from two markers – 18S V9, which detected invertebrate taxa, which were analyzed at the level of order; and 12S, which detected fish, which were analyzed at the level of species. Results from both markers showed seasonal and depth differences, and overall indicated that there is an optimal sample volume in terms of taxon detection, consistency of the results, and sampling effort. However, the two markers varied in terms of how taxon detection was impacted by sample volume. These differences may be due to filter type, the range of target taxa, and the taxonomic resolution of the target taxa. To summarize:

- Sample volume is an important parameter that may impact both the number of taxa detected and community composition metrics. Low sample volumes (2 liters or less) that are typically used in eDNA studies may result in fewer taxa discovered, as seen our 18S results, and significantly greater variability in community assessments, as seen in results from both markers. The 18S data indicated that larger sample volumes may lead to the detection of more taxa. However, these results also suggest that there are diminishing returns for very large sample volumes. Both the 18S and 12S markers yielded more consistent community metrics with larger sample volumes. An efficient approach would be to determine an optimal sample volume that balances dataset benefits (greater taxon detection and consistency) with sampling effort.

- Filter type may have influenced our results. For some taxa that may be less common or more heterogeneously distributed (i.e., fish), greater species richness observed in small-volume samples on Sterivex filters compared to large-volume samples on Waterra filters may reflect differences in the filter surface area. For Waterra filters, the sample is distributed over a much larger surface area (300 cm^2) than for the Sterivex filters (10 cm^2). While the Sterivex sample volumes were small, the eDNA on the filter may have been more concentrated, potentially resulting in higher detection of taxa in the 12S dataset. Future research could test the impact of filtering larger volumes over smaller filters to concentrate the DNA, especially if the aim is to detect target taxa that may be less abundant. Another approach that could be considered is to increase the number of PCR replicates, as this has also been shown to increase the number of taxa that are detected (Ficetola et al., 2015; Ruppert et al., 2019). This could be especially true for large-volume samples that yield larger volumes of DNA extracts as in this study, as a greater proportion of the extract volume would be amplified. Potential differences between Waterra and Sterivex filters independent of filter area could be assessed in future work by

subsectioning the Waterra filters to equalize filter area with Sterivex, and running comparisons over a range of sample volumes.

- Target taxa differed between the two datasets, and these differences may impact data interpretation. The 12S marker targets a specific group (vertebrates) and provides species level taxonomic resolution. The 18S marker targets a broad range of animal phyla but provides less (order-level) taxonomic resolution. As seen in the relative read abundance plots, the order-level analyses in this study for 18S likely smoothed over variation inherent in species-level analyses. Additionally, it is possible that fish eDNA, originating from a relatively small number of numerically less abundant species, is less common and more heterogeneously distributed than invertebrate eDNA in the environment, leading to more variable eDNA results. Thus, optimal sampling parameters may differ if the monitoring objectives focus on target species versus general community characteristics.

- Season was the largest driver of community composition, as seen in the relative read abundance and NMDS plots for both markers. This may be due to differences in what the markers are detecting and their relative abundances in the environment. The findings in this study reflect a temperate, relatively shallow, coastal study site. Locations that are at different latitudes, deeper, farther offshore, or differ in other ways may have different optimal sampling parameters. Assessing optimal sample volume is recommended in representative study sites in monitoring areas. Offshore and deeper study locations will likely require greater sample volumes due to lower biomass and eDNA concentrations (Govindarajan et al., 2022).

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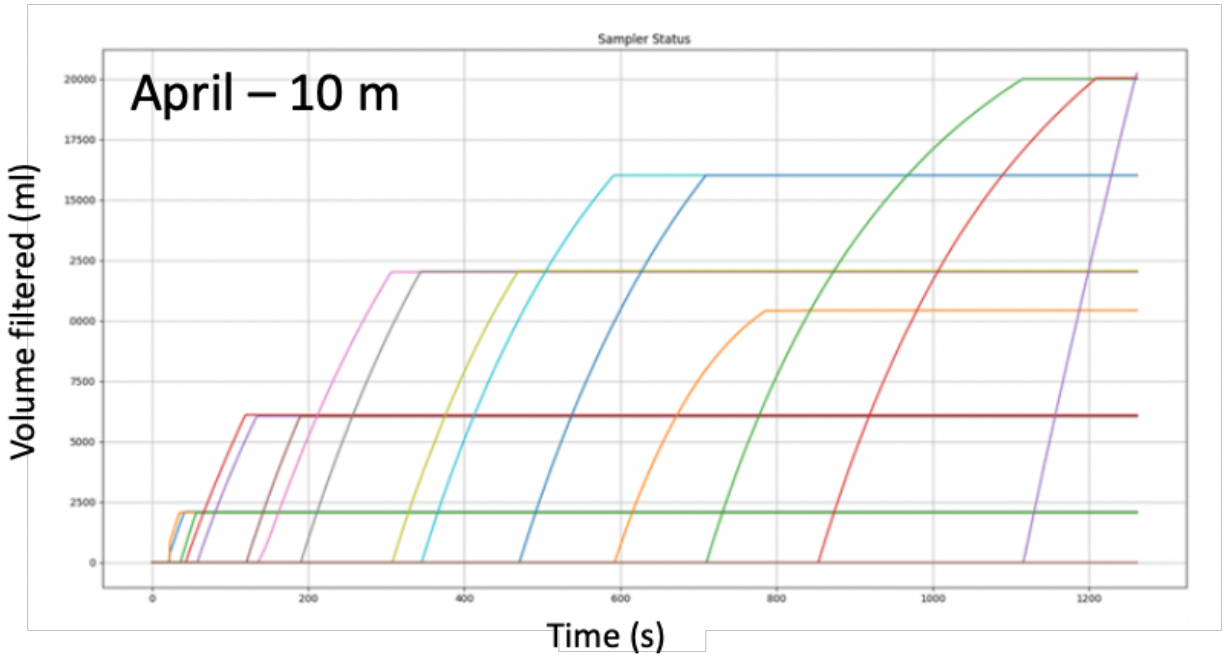
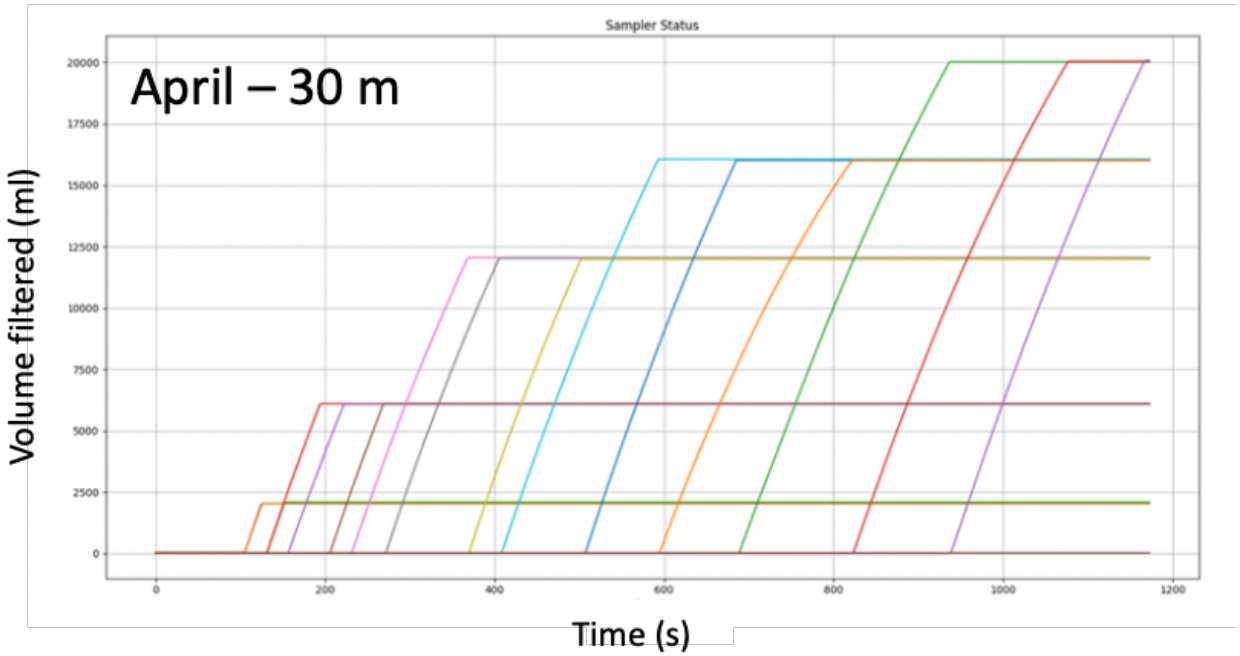
7.1 Appendix A. Sampler flow rates. Images are screenshots displaying the water volume filtered over the course of each deployment. Colors represent individual pumps.

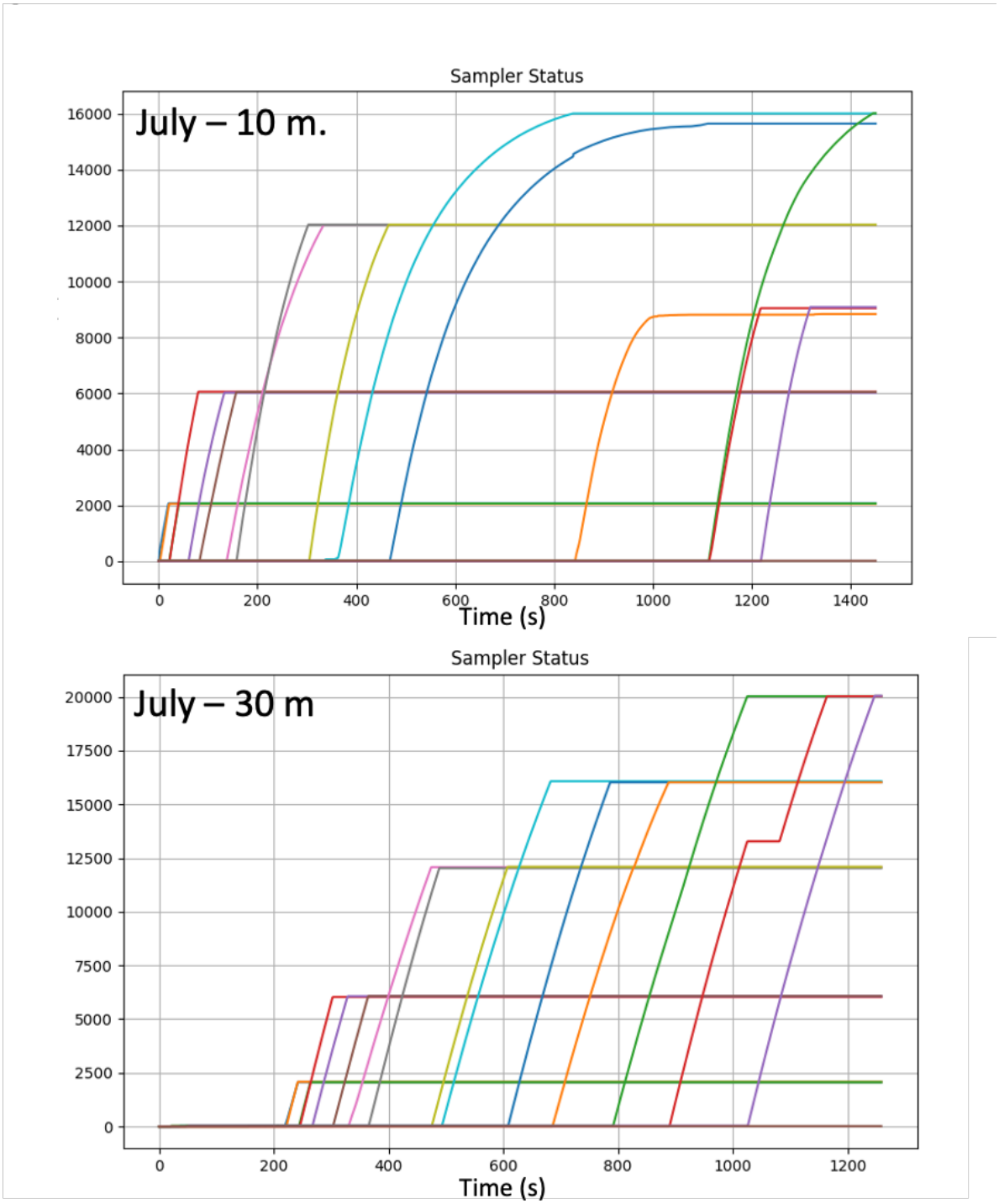
April Deployments





Appendix A, continued

July Deployments - For the 10 m deployment, filtration slowed during the 16-liter collections (teal blue, dark blue, and green lines), indicating that the filters were clogged. To prevent pumps from being damaged, 20-liter samples were not collected. Three 9-liter samples (orange, red, and purple lines) were collected instead.



7.2 Appendix B. Table of samples collected and associated metadata.

Depth (m)	Filter	Replicates	Volume (April)	Volume (July)
10	Sterivex	3	0.5	0.5
10	Sterivex	3	1	1
10	Sterivex	3	2	2
10	Waterra	3	2	2
10	Waterra	3	6	6
10	Waterra	3	12	9
10	Waterra	3	16	12
10	Waterra	3	20	16
30	Sterivex	3	0.5	0.5
30	Sterivex	3	1	1
30	Sterivex	3	2	2
30	Waterra	3	2	2
30	Waterra	3	6	6
30	Waterra	3	12	12
30	Waterra	3	16	16
30	Waterra	3	20	20

7.3 Appendix C. Temperature, salinity, oxygen, and fluorescence profiles from **A)** April; and **B)** July.