To freeze or to scoop? Dealing with the turbid waters of California's coastal lagoons

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Abstract

Coastal lagoons are an important habitat for endemic and threatened species in California that have suffered impacts from urbanization and increased drought. Environmental DNA has been promoted as a way to aid in the monitoring of biological communities, but much remains to be understood on the biases introduced by different protocols meant to overcome challenges presented by unique systems under study. Turbid water is one methodologic challenge to eDNA recovery in these systems as it quickly clogs filters, preventing timely processing of samples. We investigated biases in community composition produced by two solutions to overcome slow filtration due to turbidity: freezing of water prior to filtration (for storage purposes and long-term processing), and use of sediment (as opposed to water samples). Bias assessments of community composition in downstream eDNA analysis was conducted for two sets of primers, 12S (fish) and 16S (bacteria and archaea). Our results show that freezing water prior to filtration had no effects on community composition for either primer, even when using a filter of larger pore size (3 μm), and therefore it is a viable approach in this system for comparison of water borne fish, bacteria and archaea. However, the 16S primer showed significantly different community

composition in sediments compared to water samples, although still recovering eDNA of organisms from the water column. Sediment sample replicates were heterogeneous, and therefore increasing the number of replicates would be recommended for similar habitats.

Introduction

Coastal lagoons in California are the numerically dominant form of coastal wetland (Jacobs et al., 2011; Stein et al., 2014) and are important in many other Mediterranean climates and subtropical environments. These lagoons are characterized by seasonal and episodic breaching (opening of the lagoon to the sea, usually by stream flow) and closure (isolation of the lagoon by a high sandbar), which provide a suite of ecological services: from groundwater infiltration to support of unique biodiversity (Ballard et al., n.d.). This system serves as important habitat and nursery for endemic and endangered fishes and amphibians, such as the steelhead (*Oncorhynchus mykiss*), red-legged frog (*Rana aurora draytonii*), and the tidewater goby (*Eucyclogobius newberryi*) (Earl et al., 2010; Shaffer et al., 2004; Swift et al., 1993, 2016). Thus, California lagoons are spatially and temporally variable systems with unique biodiversity and biodiversity assessment challenges.

Coastal lagoons have been drastically reduced in numbers along the California coastline, driven mostly by the impact of coastal land use for transport structures, agriculture, and development. These are further exacerbated by ongoing changes in the hydrological cycles due to climate change (SCWRP, 2018). While these sites are critical for endangered species conservation, they are also subject to frequent invasion and their response to environmental variation is poorly documented. However, monitoring of this habitat can be limited by a variety of issues, ranging from limited human power and access to challenges driven by the natural complexity and dynamism of these lagoons.

The use of environmental DNA (eDNA) has been advocated as an alternative for monitoring communities and target species (Thomsen & Willerslev, 2015), and can overcome and complement certain field limitations from traditional methods (e.g. seining, trapping). On-site collection can be relatively fast, and therefore allow field workers to cover more ground. It can also recover the DNA signal of species that are rare, cryptic and/or hard to capture by traditional methods, and being non-intrusive, it offers an alternative when working with endangered species for which permits are necessary (Deiner et al., 2017; Dejean et al., 2012; Sard et al., 2019). In addition, metabarcoding approaches allow the investigation of multiple species from a single collection (Taberlet, Coissac, et al., 2012).

Nevertheless, it is important to recognize that this approach also brings its own limitations and biases (van der Loos & Nijland, 2021). In some circumstances, eDNA sampling can be more expensive than traditional, more established methods (Smart et al., 2016). Since there are no voucher specimens from collections, contamination is a major issue that needs to be addressed early on, following best practices in the field (Goldberg et al., 2016). The lack of voucher specimens also leads to an overdependence on the use of barcodes and genetic databases for taxonomic identification, which introduces another set of biases, from misidentification to lack of species representation (Taberlet, Coissac, et al., 2012). Other challenges arise from the non-universality of sampling methods and downstream processing, with the probability of detection varying depending on the species and their density, as well as the type of environment, which affects rates of DNA degradation (Deiner et al., 2015; Rees et al., 2014; Williams et al., 2017).

Coastal lagoons can vary in their environmental qualities quite drastically. One major challenge is the high and variable turbidity of the water. High turbidity usually occurs when

lagoons are closed to the ocean by a sandbar and driven by organic and inorganic matter. In this case, filtering water on-site becomes a problem. Filtration is a widespread method for handling water samples (Laramie et al., 2015; Tsuji et al., 2019). Set volumes of water are run through a small filter to concentrate DNA before extractions. However, high concentration of fine sediment or organic matter in water quickly obstructs filters, making the filtration process time-consuming (although it could actually aid recovery by binding DNA to suspended particles: Kumar et al., 2022; Liang & Keeley, 2013; Torti et al., 2015).

To overcome this issue, some stakeholders have relied on a tiered filtration step (prefiltration) to reduce particles and avoid clogging filters (Tsuji et al., 2019), but this approach increases costs, labor and opportunities for potential contamination (Li et al., 2018; Majaneva et al., 2018; Robson et al., 2016). The use of filters of bigger pore sizes, up to 20 µm, has been previously tested and in cases of turbid waters is generally preferred, but requires filtering larger volumes of water to capture the same amount of DNA recovered in smaller pore size filters (Robson et al., 2016; Turner, Barnes, et al., 2014).

Freezing water for storage purposes prior to filtration can mitigate the issue of slow filtration in the field and allow it to be done in batches in the laboratory at a later time, but this type of sample storage might introduce bias on DNA capture and community composition (Kwambana et al., 2011; Sekar et al., 2009). Cells can disrupt and extrude their DNA in the environment, an issue that has been demonstrated in certain cases (e.g. Suomalainen et al., 2006), which would then make it easier for it to pass through the filter pores. In the case of turbid waters, increasing the pore size of filters to speed the filtration process could worsen this problem by letting DNA in solution flow through the pores more easily.

When dealing with turbid waters, some stakeholders have opted to use the centrifugation approach (e.g. Williams et al., 2017). Extracellular DNA (i.e. DNA not contained within a cell wall) can be bound to particles (Torti et al., 2015) and consequently be captured and detected more easily following centrifugation of particles into pellets. However, the amount of water used is limited by centrifuge size, usually around 15-30 mL per replicate (Doi et al., 2017; Ficetola et al., 2008), which might limit recovery of diluted DNA (Deiner et al., 2015).

Processing sediment samples may be preferable to processing highly turbid water samples. However, it is important to understand how DNA recovery from these different media compare to one another. Turner et al. (2015) and Perkins et al. (2014) have shown that sediment can have a higher concentration of fish eDNA and some bacteria, respectively. This could be related to the organic-particle binding and sinking properties, and a longer DNA persistence in sediment compared to water samples. However, as is the case with water samples, there is no consensus on the rate of degradation of eDNA in soil and sediment (Dell'Anno & Corinaldesi, 2004; Levy-Booth et al., 2007; Torti et al., 2015), and this will depend on multiple local biotic and abiotic factors. In addition, biological communities will naturally differ between water column and sediments, even though we expect some level of overlap due to both DNA sinking and suspension.

Previous work have been done comparing different approaches to processing eDNA, such as filtration and storage methods (Hinlo et al., 2017; Takahara et al., 2015), including some work on turbid waters (Kumar et al., 2022; Robson et al., 2016; Williams et al., 2017), and comparisons between water and sediment eDNA recovery (Sales et al., 2019; Turner et al., 2015). But results have been contradictory, or limited to looking at just DNA concentration, or at a single targeted species.

The goal of the present study is to compare how freezing water prior to filtration and using water versus sediment samples induce and/or exacerbate biases in taxa detection for a set of universal primers targeting different biological communities–12S (fish) and 16S (bacteria and archaea)—in coastal lagoons. By understanding the biases introduced when processing environmental samples, we will be able to inform decisions regarding experimental design for monitoring such a dynamic and challenging habitat, which has invaluable importance for the maintenance of ecosystem services for both wild and urban populations. We expect these results will be of interest relative to eDNA sampling in other aquatic systems as well.

Material and Methods

Site - Topanga Lagoon

To determine the variability of species detection for each protocol, water and sediment samples were collected from a south-facing coastal lagoon in southern California, located in Malibu, a stretch of coast that runs from Santa Monica to Point Mugu. This lagoon is part of the Topanga State Park and is currently undergoing plannings for restoration. It is the only lagoon on this stretch of coast that still harbors a stable population of tidewater goby (*E. newberryi*), a federally endangered species, and is relatively less impacted than other lagoons in the same region. The endangered southern steelhead trout (*O. mykiss*) is also found in this system during anadromy when the lagoon is breached. Due to the presence of these species, Topanga lagoon has been periodically surveyed by the Jacobs' lab members and collaborators such as researchers at the Resource Conservation District of The Santa Monica Mountains (RCDSMM), and therefore its macrobiota is regularly studied, especially the fish fauna. The lagoon was sampled on September 6th, 2018, at the end of the Summer season, and as is typical of this time of the year, the weather was dry with no record of precipitation since June (WeatherSpark.com, n.d.). The lagoon was

closed to the ocean by a sandbar and the water was murky (Fig. 1), which in the author's experience, such turbidity slowed filtration and easily clogged 0.45 µm cellulose nitrate filters.

Protocols and samples

A sterilized water jug was used to collect a single water sample in the lagoon, at a mid-point between the mouth margin and the road bridge (Fig. 1). The sample was then placed on ice and brought to the laboratory (~1 hr car ride). This method of "grab-and-hold" has proven to be similarly effective as on-site filtration in a previous study (Pilliod et al., 2013). Once in the laboratory, the total volume was divided in three batches for each treatment: (i) centrifugation followed by filtration of supernatant (5 replicates of 50 mL falcon tube) (Doi et al., 2017); (ii) pre-freezing followed by double filtration (5 replicates of 500 mL Nalgene bottles) (Turner, Miller, et al., 2014); and (iii) no freezing followed by double filtration on the same day of collection (5 replicates of 500 mL Nalgene bottles) (Turner, Miller, et al., 2014).

For the pre-freezing protocol, water bottles were frozen at -20 °C for 3 days before thawing for filtration. Double filtration for both pre-freezing and no-freezing treatments was done through cellulose nitrate filters, firstly on a 3 µm pore size filter, then followed by a 0.45 µm pore size using an adapted vacuum pump in the pre-PCR room of the laboratory (Fig. S1). The centrifugation protocol also included a second stage filtration of the supernatant using a 0.45 µm pore size filter. Here, we will focus only on the results from the first filtration step of the water filtration protocol. More details on that are explained further in the supplemental material.

Surficial sediment was collected in triplicates at the same location where water was sampled (5 replicates of triplicate 2 mL cryotubes, 15 tubes total), following instructions as defined by the CALeDNA program (https://ucedna.com/methods-for-researchers). These were also kept on ice during field work and stored in a -80°C freezer upon arrival at the laboratory

until DNA extractions. Results from sediment samples were compared against both filtration protocols: (1) pre-freezing followed by filtration; (2) no freezing followed by filtration.

DNA Extraction

DNA from sediments and filters were extracted following the PowerSoil extraction protocol. Filters were chopped into thin strips before being added to the bead tubes, and sediment triplicates were pooled in small batches to reach 0.25-0.3 g before processing. We used the soil extraction kit on the filters as well to reduce potential PCR inhibition caused by the water turbidity (Kumar et al., 2022), but also to limit the number of variables in the research design by adding another extraction protocol.

Contamination best practices

Care was taken to avoid contamination both in the field and the lab. Before collection, bottles and water jug were cleaned and bleached and then handled with clean gloves on site. Extractions and PCR were done in a separate pre-PCR room. Utensils and bench top were cleaned with 10% bleach, followed by 70% ethanol. Forceps and scissors for handling filters were seared and cleaned with bleach and ethanol after dealing with each sample. PCR reagents were prepared in a clean, PCR-free, positive pressure hood. Sediment samples were collected with new 2 mL cryotubes and following field protocol as recommended by the CALeDNA program. Blanks were made for the field collection, laboratory filtration and PCR (5 blanks in total) and included in the library for sequencing.

Sequencing

Library preparation followed CALeDNA protocols (https://ucedna.com/methods-for-researchers). Metabarcode libraries were generated for bacteria and archaea (16S rRNA), fish (12S rRNA) and metazoans (CO1). Sequences for each primer can be found at Table 1. All

libraries consisted of triplicate PCR reactions. PCR products were visualized using gel electrophoresis, and for each barcode, PCR triplicates were pooled by sample. After bead cleaning, all markers were pooled by sample and tagged for sequencing (single indexing). Libraries were pooled and run on a MiSeq SBS Sequencing v3 in a pair-end 2x300 bp format [Technology Center for Genomics & Bioinformatics (TCGB), UCLA] with a target sequencing depth of 25,000 reads/sample/metabarcode. Two sequencing runs were conducted, but the CO1 primer was still below the sequencing depth threshold and therefore its results will not be discussed here (see Figs. S2-3). For each run, our library was pooled with different samples from different collaborators to maximize efficiency of the sequencing run.

Bioinformatics and data pre-processing

Sequence data was bioinformatically processed in Hoffman2, the High Performance Computing cluster at UC Los Angeles, using the Anacapa Toolkit (Curd, Gomer, et al., 2018) with default settings. Briefly, reads are demultiplexed and trimmed for adapters (cutadapt, Martin, 2013) and low-quality reads (FastX Toolkit, *FASTX-Toolkit*, n.d.). Dada2 (Callahan et al., 2016) is used to denoise, dereplicate, merge and remove chimeras, and the resulting clean Amplicon Sequence Variants (ASVs) have their taxonomy assigned using Bowtie2 (Langmead & Salzberg, 2012), matched to a custom reference library (CRUX, Curd, Kandlikar, et al., 2018). Confidence levels are determined by the BLCA algorithm (Gao et al., 2017) to generate a table of best taxonomic hits, from super-kingdom to species level. The pipeline was designed to process not only paired, but also unmerged and unpaired reads.

Taxonomic tables with a bootstrap confidence cutoff score of 0.6 were used for downstream analyses. Except when noted, all bioinformatic analyses mentioned beyond this point were performed using R v.3.6.2 (R Core Team, 2018) in RStudio v.1.2.1335 (RStudio

Team, 2020). Decontamination was done separately for each primer set and each run (since the dataset was pooled with different combinations of samples for sequencing). We used the package metabaR (Zinger et al., 2020) to lower tag-jumping and remove contaminants through detection of ASVs whose relative abundance is highest in negative controls. We also ran a modification of the gruinard pipeline (https://github.com/zjgold/gruinard_decon), including only steps 4 (site occupancy modeling) and 5 (dissimilarity between replicates), since previous steps were redundant with the metabaR decontamination steps. Lastly, taxa classified as "Not_found", "Unclassified", "*Canis lupus*", "*Bos taurus*", and "*Homo sapiens*" were removed from the final tables before being merged and used in downstream analyses.

Diversity analysis

We used the laboratory's own sampling record and the Global Biodiversity Information Facility database (Gbif.Org, 2022) to manually check the 12S primer final taxonomic table. The number of species captured by each treatment was visualized using Venn Diagrams (package VennDiagram, Chen, 2018). Species rarefaction curves were made for each metabarcode to inspect the level of species saturation for each protocol replicate. The slope of each curve was calculated using the rareslope function in the vegan package (Oksanen et al., 2019), and the confidence interval for each protocol was calculated using pairwiseCI (Schaarschmidt & Gerhard, 2019) with confidence level at 95%. Rarefaction curves were plotted using the ggrare function from the ranacapa package (using step = 5).

Differential abundance

The raw dataset was analyzed using DESeq2 to look at differential abundance between protocols (Love et al., 2014). The default testing framework was used (test = "Wald", fitType = "parametric"), which includes the Benjamini-Hochberg multiple inference correction. The

sfType option was defined as poscounts since this estimator is able to handle zeros. The log2 fold change of each pairwise comparison for which there were significant differences in abundances was plotted.

Beta diversity

For the beta diversity analysis, samples were standardized by using either the eDNA index (Kelly et al., 2019) or by rarefying them as a way to equalize sequencing effort and minimize stochasticity and bias. For the eDNA index, we followed the Wisconsin double standardization method in the vegan package. The custom_rarefaction function in the R package ranacapa (Kandlikar, 2020) was used to rarefy the dataset with 10 replicates.

For the 12S primer, samples were rarefied to 20 000 reads. Three sediment samples were excluded due to very low read numbers (<100). For the 16S, samples were rarefied to 15 000 and one sediment sample that had ~5000 reads was excluded. The number of reads per taxa for each protocol replicate was plotted using the phyloseq package (McMurdie & Holmes, 2013), for both the raw and rarefied dataset.

The rarefied dataset followed a Constrained Analysis of Principal Coordinates (CAP) using the capscale function in vegan and Bray-Curtis distance. This ordination method, which can be used with non-Euclidean dissimilarity indices, explains the ordination of assemblage composition based on species abundances. The difference in community composition for each treatment was then analyzed using a PERMANOVA and Bray-Curtis dissimilarity, followed by a pairwise PERMANOVA comparison (all with the vegan package). P-values were adjusted using the FDR (False Discovery Rate) approach.

Results

Sequencing

The first run generated a total of 6 407 371 reads: 3 817 216 reads for the 12S primer, 2 393 627 for 16S, and 196 528 for CO1. In the second run there were a total of 9 088 496 reads: 6 685 673 reads for the 12S metabarcode, 1 904 283 reads for 16S and 498 540 for the CO1. For the 12S and 16S primers, we were able to reach our threshold of 25 000 reads/sample in most cases, while that was not the case for all except one sample of the CO1 primer. Because of this limitation on the number of reads/sample, the CO1 metabarcode will not be discussed further in the main paper (but check the supplemental material for more details).

Bioinformatics and data pre-processing

The number of reads per sample after decontamination and combining both runs is illustrated in Figure S3. We manually checked the final taxonomic tables of each separate run for the 12S primer to look for signs of contamination and evaluate how well the bioinformatic decontamination steps worked (metabaR and gruinard). The taxonomic tables for the 12S primer have substantially less species than the 16S, and the local fish fauna is relatively well known, making the process more tractable.

For the run that was pooled with samples from Palmyra Atoll, the output still retained some tropical reef and pelagic fish and elasmobranch species that are not found in coastal lagoons in California. We can expect that tag-jumping contamination is also present in the other sequencing runs and primers as well. Interestingly, eight out of 28 of those tropical species (ca. 28%) were found exclusively on the sediment samples and not the water samples (e.g. *Acanthurus achilles, Scarus altipinnis, Lutjanus russellii*).

Barplots for both the raw and rarefied dataset (Figs. S3-4, respectively) show that sediment replicates had greater variability amongst themselves, both in number of reads and community composition, compared to the replicates of either water protocols. Water replicates were more consistent within and between protocols, and had an overall higher number of reads than the sediment samples.

Diversity

After the decontamination steps (metabaR and gruinard) and removing specific, uninformative ASVs (as listed above), the total number of species assigned to 12S was 39, distributed in 20 orders and 22 families. Of these 39 species, only four had been previously recorded for the site (Table S1). For 16S, the total number of taxa assigned to species was 2 625, distributed in 45 phyla and 335 families.

We have also noticed some dubious taxonomic assignments. For example, for the 12S primer, we had one hit for *Fundulus diaphanus*, which is a species of killifish native to the northeast of North America. However, the californian species *F. parvipinnis* has been previously documented in Topanga by lab members sampling at the site. Similarly, there were two hits for *Phoxinus phoxinus*, which has a European distribution with a closely related North American counterpart, *P. eos*, although this species has not been identified in collections from Topanga lagoon. Another dubious identification occurred for two species of *Odontesthes*, *O. incisa* and *O. smitti*, which were among the most abundant hits in our dataset but are native to the southwest Atlantic. These two species, however, are relatives of topsmelt (*Atherinops affinis*), commonly found in coastal lagoons and estuaries in California (Table S1).

The Venn Diagram (Fig. 2) shows that even though sediment samples had lower numbers of reads overall (Figs. S2-3), they had the highest number of species recovered (12S primer:

N=27, 19 unique; 16S primer: N=1 929, 1 178 unique). The species overlap between protocols for the 12S was only 1.2% (n=1), and for the 16S primer it was 3.5% (n=402).

Species rarefaction curves also show that sediment samples are further from reaching saturation compared to water samples, both for 12S and 16S primers (Fig. 3), although there was more variation between the replicates for the 12S sediment samples. For 12S primer, there is a significant difference in the slope of the species curves between the sediment and no freezing protocols (Fig. 4), while for 16S, all pairwise comparisons between protocols showed significant differences.

Differential Abundance

For the 12S primer, there was no significant difference between species abundance for any of the protocols' pairwise comparisons. For the 16S primer, there was no significant difference in comparison between the water protocols (pre- and no freezing). However, there were significant differences in the pairwise comparisons of water samples and sediment samples (Fig. 5, Tables S2-3). The top five differentially abundant species in the water protocols were representatives of the families Aphanizomenonaceae, Comamonadaceae and Flavobacteriaceae (in both pre- and no freezing); plus Hemiselmidaceae and Geminigeraceae (pre-freezing protocol only). These comprise groups of cyanobacteria (Aphanizomenonaceae) and algae (Hemiselmidaceae and Geminigeraceae), as well as environmental bacteria (Comamonadaceae and Flavobacteriaceae).

The most differentially abundant species found in the sediment were representatives of the families Catenulaceae, Fragilariaceae and an archaea assigned to the Thaumarchaeota phylum (both pre- and no freezing); plus Woeseiaceae and Elphidiidae (no freezing protocol only); and Anaerolineaceae and Desulfobacteraceae (pre-freezing protocol only). These comprise groups of diatoms (Catenulaceae and Fragilariaceae), environmental bacteria (Woeseiaceae,

318 Anaerolineaceae and Desulfobacteraceae) and archaea (Thaumarchaeota), and foraminiferans
319 (Elphidiidae).

320 ### Beta diversity

321 When using the eDNA index, the CAP analysis for the 12S primer showed that many of the
322 species driving the differences in assemblage composition were the tropical species that are
323 coming from the tag-jumping contamination (Fig. S6). For example, we see overrepresentation
324 in the sediment samples of *Stegastes nigricans* and *Caranx melampygus*; and in the no freezing
325 water samples, *Sphyraena barracuda*. Nevertheless, we also see some other species that are
326 known to be found in the lagoon, such as the *Eucyclogobius newberryi*, being mostly
327 overrepresented in the water samples compared to the sediments; and *Gila orcutii*,
328 overrepresented in the no freezing protocol. Two species of dubious taxonomic assignment are
329 also overrepresented in the sediment: *Phoxinus phoxinus* (as discussed in the previous
330 'Diversity' section); and *Acanthogobius flavimanus*, which is a species of goby native to Asia,
331 but that has been recorded previously in California estuaries (Nico et al., 2022). The
332 PERMANOVA results were not significant ($p = 0.067$).

333 For the rarefied dataset, the CAP analysis was not able to recover any differences in
334 assemblage composition for the 12S primer for any of the protocols (Fig. S5). One sediment
335 replicate is driving most of the difference (CAP1=86%) with the overrepresentation of many
336 tropical species, likely tag-jump contaminants. The PERMANOVA results were at the threshold
337 of significance ($p = 0.05$), but the pairwise test was not significant for any protocol comparison
338 (Table 2). The lack of significant differences between water and sediment samples could have
339 been driven by the loss of three sediment replicates when rarefying the dataset.

For the rarefied 16S primer dataset, the different protocols showed significant differences in assemblage composition. The first axis explains most of the total variation (CAP1=86%), with the tidewater goby being the most underrepresented in the sediment compared to the water samples, especially in the no freezing protocol (Fig. 6). Sediment samples were also slightly overrepresented by a few other species compared to water samples. One of them was identified as Candidatus *Nitrosopelagicus brevis*, which is a species of ammonia-oxidizing archaea (Thaumarchaeota) found mainly in the epi- and upper mesopelagic environments of the open oceans (Santoro et al., 2015). There are also two species of *Monomorphina*, (*M. pyrum* and *M. pseudonordstedti*) that belong to the Euglenaceae family, a group of eukaryotic flagellates found in freshwater environments. Lastly, there is *Elphidium williamsoni*, a foraminifera belonging to the family Elphidiidae found in tidal flats of the North Sea. CAP2 is representing the remaining variation (14%) found between the water protocols, with the most distinguishing species being the *Guillardia theta*, a species of flagellate algae belonging to the family Geminigeraceae, overrepresented in the pre-freezing protocol. The PERMANOVA result was significant for the 16S primer ($p = 0.001$), as well as for all the pairwise comparisons (Table 2).

The species represented in the rarefied dataset differ from the ones found when using the eDNA index for the 16S primer. Most of the community assemblage difference (CAP1=85%) is driven by differences between water and sediment samples, with six species being underrepresented in the latter: *Burkholderiales bacterium* TP637, *Curvibacter* sp. UKPF8, beta proteobacterium Mzo1, *Diaphorobacter ruginosibacter, Stella humosa* and *Verminephrobacter aporrectodeae*. All of them, with the exception of the last one, *V. aporrectodeae*, were also found as significantly different in the DeSeq2 analysis. The PERMANOVA result was also significant in this case ($p = 0.001$), as well as for all the pairwise comparisons (Table 3).

Discussion

Standardized protocols to process eDNA are under development (e.g. Bohmann et al., 2021), but to implement these efficiently it is necessary to compare biases in taxa detection associated with different protocols. Here, we have explored the detection biases in community composition introduced by freezing water samples prior to filtration (for storage purposes), and the use of sediment samples as an alternative to sampling turbid waters. We find that pre-freezing water does not affect the recovery of community composition either for the 12S and 16S primers, compared to the no freezing protocol. This is the case even when filters of larger pore size (3 μm) are used. Sediment samples recovered eDNA from organisms that inhabit the water column, however, due to high variability among replicates in read abundance, we suggest increasing the number of biological replicates in the field.

Tag-jumping contamination

Contamination concerns are usually centered around pre-sequencing, during the field and wet laboratory work. These are of fundamental importance and care should be taken by sterilizing equipment and using negative controls. However, previous literature shows that the sequencing phase can be another source of contamination, generating up to 10% of contaminated reads by tag-jumping (Larsson et al., 2018; Schnell et al., 2015), which can skew analyses of taxa abundance and composition towards the rare taxa. There are ways to help minimize this issue by making use of dual indexing (Kircher et al., 2012)—although see Caroe and Bohmann (2020) for a library approach without dual indexing—, and amplification positive controls. The latter can be used to track the rate and level of contamination after sequencing to guide read cutoffs on samples (Deiner et al., 2017; Port et al., 2016).

Bioinformatics and data pre-processing

We relied on a bioinformatic approach developed by the metabaR package, adapted from Esling et al. (2015), to reduce the issue of contamination from tag-jumping, since it does not rely solely on the use of positive controls (which we lacked in this analysis) to make the estimated cutoff thresholds. However, after manually checking the fish dataset (12S primer), the final taxonomic tables still contained reads assigned to taxa that are not found in coastal lagoons in California (Table S1). Some of it might be contamination from tag-jumping, although we cannot rule out the possibility that for a few of these species the eDNA could have come from local aquaria, as some are known in the pet trade (e.g. *Acanthurus achilles*). We also cannot disregard the limitations of the reference database, especially related to the absence of estuarine and lagoonal taxa that may lead to dubious assignments to non-local related species. Due to inability to completely remove potential tag-jump contaminants from the dataset, we can expect a bias towards the rare taxa that will inflate diversity metrics in our samples for all primer sets.

Sediment samples generally showed higher variability among replicates compared to water samples for both primer sets, both in number of reads and community composition (Fig. S3-4). The greater consistency of water replicates is an artifact of the single source for the water samples (the large jug), while sediment replicates were done by individually sampling the bottom of the lagoon. Although replicates were done a few centimeters apart, the bottom of the lagoon appears to have small-scale heterogeneity. The spatial variation of soil and sediment samples is recognized in the literature (Perkins et al., 2014; Taberlet, Prud'Homme, et al., 2012), and can be caused by sediment composition but also by the flow dynamic and distribution of eDNA in the water column. While this variability has been shown to occur for water samples as well in lentic environments (Harper et al., 2019 and references therein), the heterogeneity of water replicates in this system still requires further investigation.

Sediment samples also had an overall lower number of reads compared to water samples for both primer sets (Fig. S3). The lower number of reads seems to go against the expectations that eDNA can be more concentrated in sediments (Dell'Anno & Corinaldesi, 2004; Harper et al., 2019; Turner et al., 2015). This could be due to a few issues, some of which may interact. First, it could be related to a faster degradation and/or turn-over rates of eDNA in the sediment, which are determined by the soil and eDNA characteristics, as well as enzymatic and microbial activities (Levy-Booth et al., 2007; Pietramellara et al., 2009; Torti et al., 2015). The overall lower abundance of eDNA in the sediments could also be driven by increased inhibition (Buxton et al., 2017; Pawlowski et al., 2022). Even though we used a specific soil extraction kit for both sediment and filtered water samples, the purification steps in the protocol could still not have been enough to reduce inhibition in the sediment as well as for the water samples. Lastly, this could have been driven by the much lower volume of sediment used: 0.25-0.3 g versus 500 mL for water samples.

There is also the fact that this type of environment is affected by scouring (purging of sediment to the ocean) during high precipitation events and increased flow of freshwater. However, since the sediment collection was done out of the rainy season and the lagoon was closed by a sandbar with no signs of scouring, we are confident that this was not a factor that could have caused the decreased ability to recover eDNA from the sediments. Therefore, we expect that this difference in read abundance between sediment and water samples would be more related to the other factors mentioned above, such as eDNA degradation and turn-over rates, inhibition, and different process volumes. Considering both the high variability and the lower sequencing throughput of the sediment replicates, we advise using a modified sampling

protocol, e.g. the one developed by Taberlet, Prud'Homme, et al. (2012) that includes increasing the number of replicates and mixing larger volumes before processing.

Diversity

Considering that contamination through tag-jumping could be inflating the numbers of rare species in the dataset, the steepness and lack of a plateau for many of the species rarefaction curves could be artificial. This is especially evident for the 12S primer, since we were able to manually investigate the taxonomy tables (Figs. 2-3). However, this lack of a plateau is an expected outcome from environmental samples (Alberdi et al., 2018), and has been shown to occur more acutely in a coastal lagoon in California when compared to other environments in California (Shirazi et al., 2021)—albeit the authors were looking specifically at plants and fungi. The high number of species recovered from the sediment for the 16S primer (Fig. 2) is likely driven by the recovery of a rich and complex sediment biota that is not paralleled in the water column.

The low taxonomic assignment to the species level for some of the dubious fish species found in our dataset, e.g *Phoxinus phoxinus*, *Odontesthes* spp. and *Sebastes pachycephalus*, also highlight the need to expand barcoding efforts to the local estuarine taxa to improve reference databases. On the other hand, *Fundulus diaphanus*, the northeastern killifish, did receive a few high taxonomic scores at the species level, which merit further consideration for biomonitoring of coastal lagoons in the region.

Pre-freezing water prior to filtration had an effect on the species curves of the 16S primer dataset, but not on the 12S. This could be explained by how differently eDNA molecules are found in the environment for these two different groups of organisms, and how freezing and thawing water would impact them. In the case of the fish fauna, the DNA that is shed from the

organisms would be either found within cells, or adsorbed to colloids (Liang & Keeley, 2013; Torti et al., 2015; Turner, Barnes, et al., 2014). Even if cell walls were to disintegrate from the freezing and thawing process, they could still release intact mitochondria (which range from 1-8 μm in length) that could still be captured by our 3 μm pore size filters. On the other hand, bacteria and archaea, which are prokaryotic and often single celled organisms, would have their DNA released directly to the medium and pass through the larger pore size filters (>0.2 μm). Nevertheless, this freezing effect on cell walls has been shown to not always occur and likely be species-dependent (Sekar et al., 2009; Suomalainen et al., 2006).

Differential abundance

Pre-freezing water did not introduce any significant bias in species abundance compared to the "grab-and-hold", no freezing protocol, for any of the primer sets, even when using larger pore size filters (3 μm). Our results differ from other reports, where it was shown that freezing had differential effects on detection and relative abundance of different prokaryotic taxa (Kwambana et al., 2011; Sekar et al., 2009; Suomalainen et al., 2006). This could have been due to several reasons. First, the lack of effect pre-freezing had on community composition could be related to water properties of coastal lagoons that would have promoted the retention of DNA in the cellulose filters used in this analysis. Liang and Keeley (2013) have shown that presence and size of colloids, and the strength of ionic components, have an effect on increasing the binding affinities of DNA to the filters, especially the mixed cellulose esters filters (MCE). Another important aspect to consider is that the 'nominal' size of cellulose filters does not necessarily correspond to their 'effective' size. MCE filters do not have a uniform pore size like polycarbonate and nylon filters; rather, they are characterized by a 'tortuous flow path' from which particles are trapped more easily (Turner, Barnes, et al., 2014). This property of cellulose

filters likely worked to our advantage, but also causes cellulose filters to be more susceptible to clogging than others.

Due to eDNA precipitation and resuspension, we expect to capture some community overlap between water and surficial sediment samples, however abundances should be different following the origin and fate of the eDNA in the environment and the processes acting on it throughout (Torti et al., 2015). Not surprisingly, with the DeSeq2 analysis, we see more algae (Hemiselmidaceae and Geminigeraceae) and cyanobacteria (Aphanizomenonaceae) in the water samples, and statistically higher representation of presumptively benthic diatoms (Catenulaceae and Fragilariaceae) and foraminiferans (Elphidiidae) in the sediment. In addition, the types of environmental bacteria most abundant in the sediments were typical of soil and sediments elsewhere. Of particular note are those from anoxic environments (e.g. Anaerolineaceae and Desulfobacteraceae) as lagoon sediments are often dark and sulfide-rich.

The family Flavobacteriaceae was overrepresented in the water samples relative to the sediment, both in the pre- and no freezing protocols. In this family, there are important pathogens of fish and humans that belong to the genus *Flavobacterium*. (Suomalainen et al., 2006) found that *F. columnare* was more susceptible to having its cell walls disrupted to freezing due to high amounts of DNAases, lyases and proteases, likely connected to its pathogenicity, which then led to lower rates of DNA recovery. The species found in our dataset was *F. johnsoniae,* a species not known to be pathogenic–albeit with low species taxonomic score. Given that there was no difference in abundance for this species in our pre- and no freezing protocols, different from the results for the pathogenic species, *F. columnare,* this might relate to a true non-pathogenic species. However, considering that the endangered northern tidewater goby often achieves high

499 abundance in this lagoon, more detailed assessment of the *Flavobacterium* species inhabiting this
500 site would be of interest.

501 The other species assignment that draws our attention is the archea Candidatus
502 *Nitrosopelagicus brevis* (Thaumarchaeota), which is significantly more abundant in sediment
503 than water samples. As mentioned earlier, this is a pelagic species, normally found in the open
504 ocean worldwide. Although coastal lagoons are subject to marine input, the relatively high
505 concentration in sediment is unexpected and merits inquiry, especially considering that the
506 confidence in its taxonomic assignment was low across reads. Likely, this represents a new
507 environmental archaea that is abundant in coastal lagoon sediments.

508 Beta diversity

509 McMurdie and Holmes (2014) recommends against rarefying datasets due to the risk of
510 removing true, rare ASVs. However, in our case, where we were unable to completely remove
511 tag-jumping contaminants, this pre-process could help alleviate some of the noise caused by
512 contaminants. Nevertheless, the CAP and PERMANOVA results on both the rarefied and
513 standardized (eDNA index) dataset mostly corroborate some of our previous findings with the
514 DeSeq2 analysis ('Differential abundance' section), showing significant differences in
515 assemblage composition for the 16S primer, but not the 12S primer.

516 For the rarefied 16S primer dataset, all the species that were over- and underrepresented
517 by CAP and PERMANOVA analyses were the same as those found by DeSeq2, such as
518 *Guillardia theta* (Geminigeraceae), which was overrepresented in the pre-freezing protocol
519 compared to the no freezing protocol. In addition, the species of foraminifera, *Elphidium*
520 *williamsoni* (Elphidiidae) and the archea Candidatus *Nitrosopelagicus brevis* (Thaumarchaeota)
521 were found to be overrepresented in sediment samples compared to water samples for both

freezing protocols. The CAP results on the 16S primer dataset standardized using the eDNA index (Fig. S6) showed different species as underrepresented in the sediment compared to water samples but those also showed up as significantly differentially represented in the DeSeq2 analysis, with the exception of one, *Verminephrobacter aporrectodeae*.

Interestingly, the CAP analysis was also able to capture the underrepresentation of tidewater gobies (*E. newberryi*) in sediment samples on the 16S primer when compared to the no freezing protocol (Fig. 6B). This reinforces the idea discussed earlier ('Bioinformatics and data pre-processing' section) that fish eDNA, at least in this environment, is less concentrated in the sediment than in the water column, which contradicts other findings from the literature (Perkins et al., 2014; Turner et al., 2015). But it is worth noting that this underrepresentation of fish eDNA in the sediment was found not significant for the 12S primer, though, and there could be some bias related to how these two genes behave and degrade differently in the environment for the fish fauna.

Lessons Learned

Here is a list of recommendations and best practices for eDNA sampling and analysis in coastal environments that we have learned throughout this work and believe will be useful for others working in similar environments with turbid water and highly heterogeneous sediment/soil:

1. Filtered water samples had an overall higher number of reads compared to sediment for both primer sets. Therefore, we recommend the use of this protocol as it will increase chances of species detection;
2. If using sediment samples, we recommend increasing the number of replicates and mixing larger volumes before processing for DNA extractions (as in Taberlet, Prud'Homme, et al., 2012);

3. Pre-freezing water samples prior to filtration are an effective long-term storage solution and, at least for 3 μm pore size filters, it did not introduce bias in community composition compared to no freezing;
4. The use of dual-indexing and positive controls during library preparation will help minimize and address cross-contamination from tag-jumping, as is now widely recognized in many best-practice protocols (e.g. Deiner et al., 2017; Goldberg et al., 2016);
5. Although rarefying the dataset is not recommended (McMurdie & Holmes, 2014), we recognize that it can aid in reducing the noise of contaminants from your dataset, as long as they are rare. Otherwise, the use of eDNA index (Kelly et al., 2019) can be an alternative to standardize your dataset.

Conclusions

In this work, we assessed environmental DNA protocols for use in coastal lagoons, a highly dynamic habitat at the intersection of terrestrial, freshwater and marine environments. Pre-freezing water combined with the use of larger pore size filters (at least up to 3 μm) is a viable alternative for storage and processing of turbid water samples and, at least in the case of coastal lagoons, can work for the investigation of both fish (12S, MiFish) and bacteria and archaea (16S) communities. However, the use of sediment samples as an alternative to processing water samples should be done with caution, and at minimum the number of biological replicates should be increased to more than the five used in this work. Also, while sediment samples were able to recover eDNA from organisms commonly found in the water column, such as the tidewater goby, this was achieved during a period of relatively long lagoon closure, when there was no recent scouring of sediments to the ocean.

While we expect these guidelines to be helpful in the development of strategies to use eDNA as a monitoring resource in similar environments, protocol testing is still strongly advised whenever possible, especially when working in a new system. Much work is necessary to understand the full potential eDNA brings for the conservation and restoration of endangered species and habitats.

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Tables

Table 1: Detailed information of the primers used.

Primer	Targets	Forward Primer	Reverse Primer	Reference
12S	Fish	GTCGGTAAAACTCGTGCCAGC	CATAGTGGGGTATCTAATCCCAGTTTG	Miya *et al.* 2015
16S	Bacteria and archaea	GTGYCAGCMGCCGCGGTAA	GGACTACNVGGGTWTCTAAT	Caporaso *et al.*, 2012 (F: 515F and R: 806R)
CO1	Animals	GGWACWGGWTGAACWGTWTAYCCYCC	TANACYTCnGGRTGNCCRAARAAYCA	Leray *et al.* 2013

Table 2: Pairwise PERMANOVA (rarefied dataset) between all three protocols: pre- and no freezing water prior to filtration and sediment samples. P.adjusted is the adjusted p-value after FDR correction.

Primer	Comparison	F.Model	R^2	p.value	p.adjusted
12S	No freezing vs Pre-freezing	2.07252	0.20576	0.151	0.297
	No freezing vs Sediment	3.56051	0.41592	0.297	0.297
	Pre-freezing vs Sediment	2.25713	0.31102	0.224	0.297
16S	No freezing vs Pre-freezing	10.3356	0.56369	0.008	0.012
	No freezing vs Sediment	12.1022	0.63355	0.012	0.012
	Pre-freezing vs Sediment	12.5474	0.6419	0.008	0.012

Table 3: Pairwise PERMANOVA (eDNA index dataset) between all three protocols: pre- and no freezing water prior to filtration and sediment samples. P.adjusted is the adjusted p-value after FDR correction.

Primer	Comparison	F.Model	R^2	p.value	p.adjusted
16S	No freezing vs Pre-freezing	1.479053	0.156034	0.007	0.016
	No freezing vs Sediment	5.965368	0.427154	0.011	0.016
	Pre-freezing vs Sediment	6.514592	0.448831	0.016	0.016

Figure legends

Figure 1: Photo of Topanga lagoon taken on August 22nd, 2018, a few weeks after collection. There was no record of precipitation for the previous three months and the lagoon was closed to the ocean by a sandbar. There was also no sign of recent waves topping over the sandbar and reaching the lagoon.

Figure 2: Venn diagrams of A) 12S and B) 16S primers showing the number of species found at and between each protocol. Sediment samples showed the highest number of unique species for both primers, although for the 12S dataset, about 28% are the result of contamination from tag-jumping.

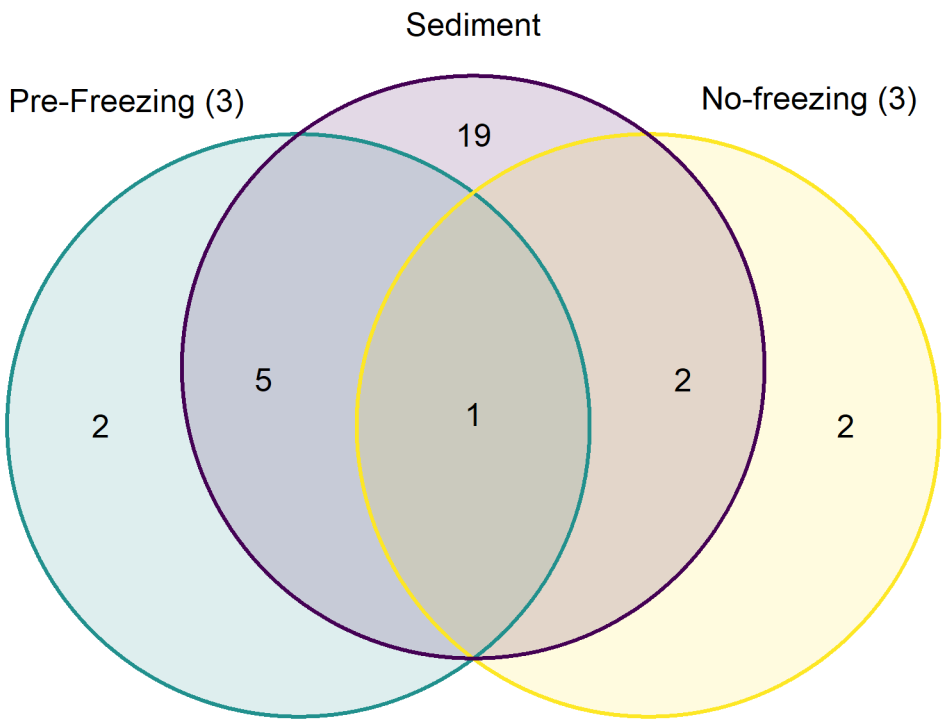
Figure 3: Species rarefaction curves based on sequencing effort for each protocol. A) 12S primer; B) 16S primer. With the exception of the water samples for the 12S primer, none of the curves have reached a plateau, although we expect the high diversity seen for the 12S sediment samples be due to contamination from tag-jumping.

Figure 4: Confidence interval (CI) for slopes of rarefaction curves (Fig. 3) for each pairwise comparison of the different protocols. Only the comparison between pre- versus no freezing water samples, and pre-freezing versus sediment samples for the 12S primer (A) have come out non significant. The remaining comparisons showed significant differences between rarefaction slopes.

Figure 5: Plots of log2fold change of families of bacteria and archaea (16S primer) for the pairwise comparison between A) no freezing versus sediment; and B) pre-freezing versus sediment. Circles are colored by phylum. Species present above zero are overrepresented in the pre- or no freezing protocol, and species below the zero threshold are overrepresented in the sediments.

Figure 6: Constrained Analysis of Principal Coordinates (CAP) of A) 12S and B) 16S primer rarefied datasets. Circles are colored by protocol.

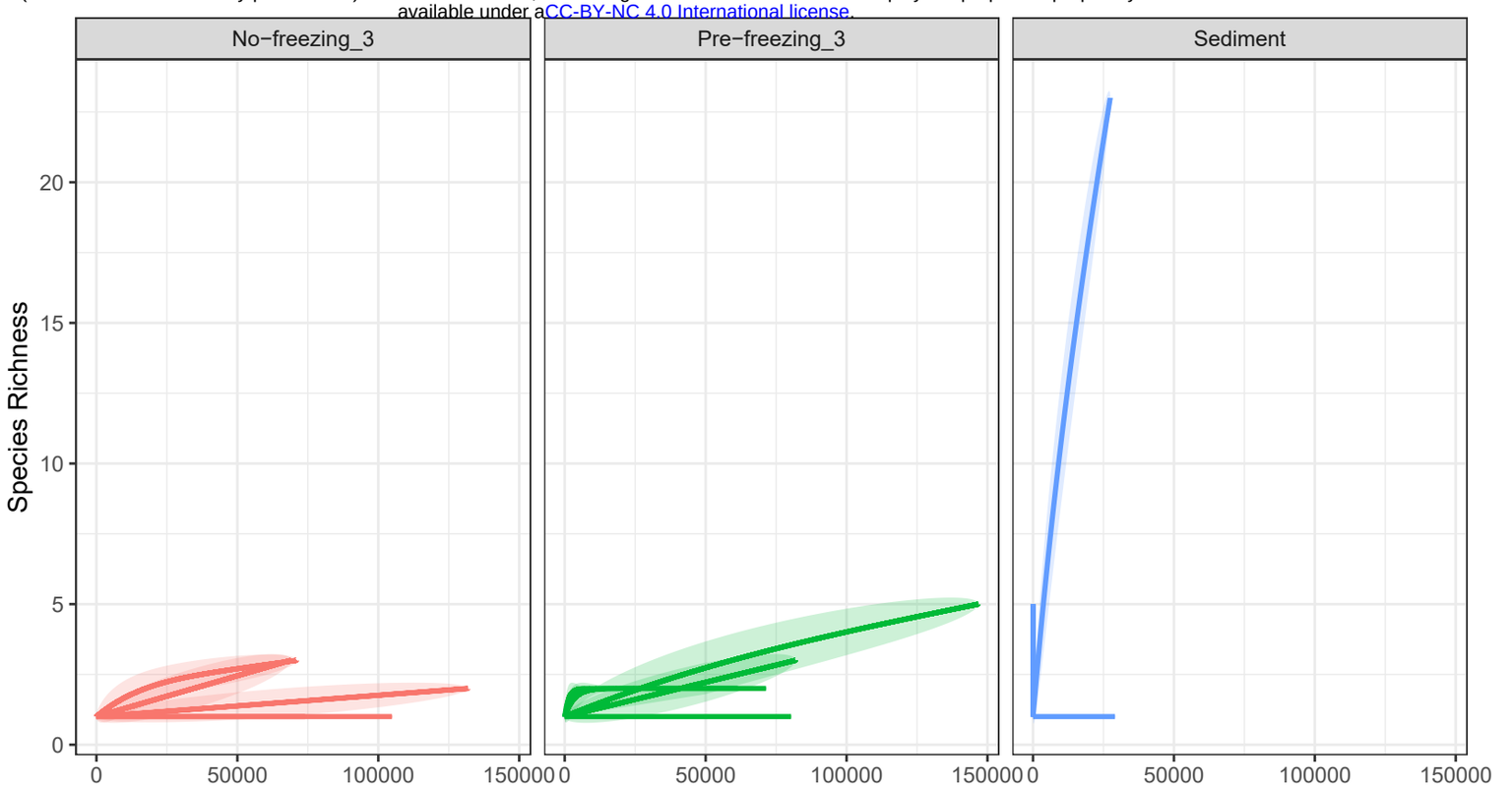
A) 12S


Sediment
Pre-Freezing (3)
No-freezing (3)
19
2
5
1
2
2

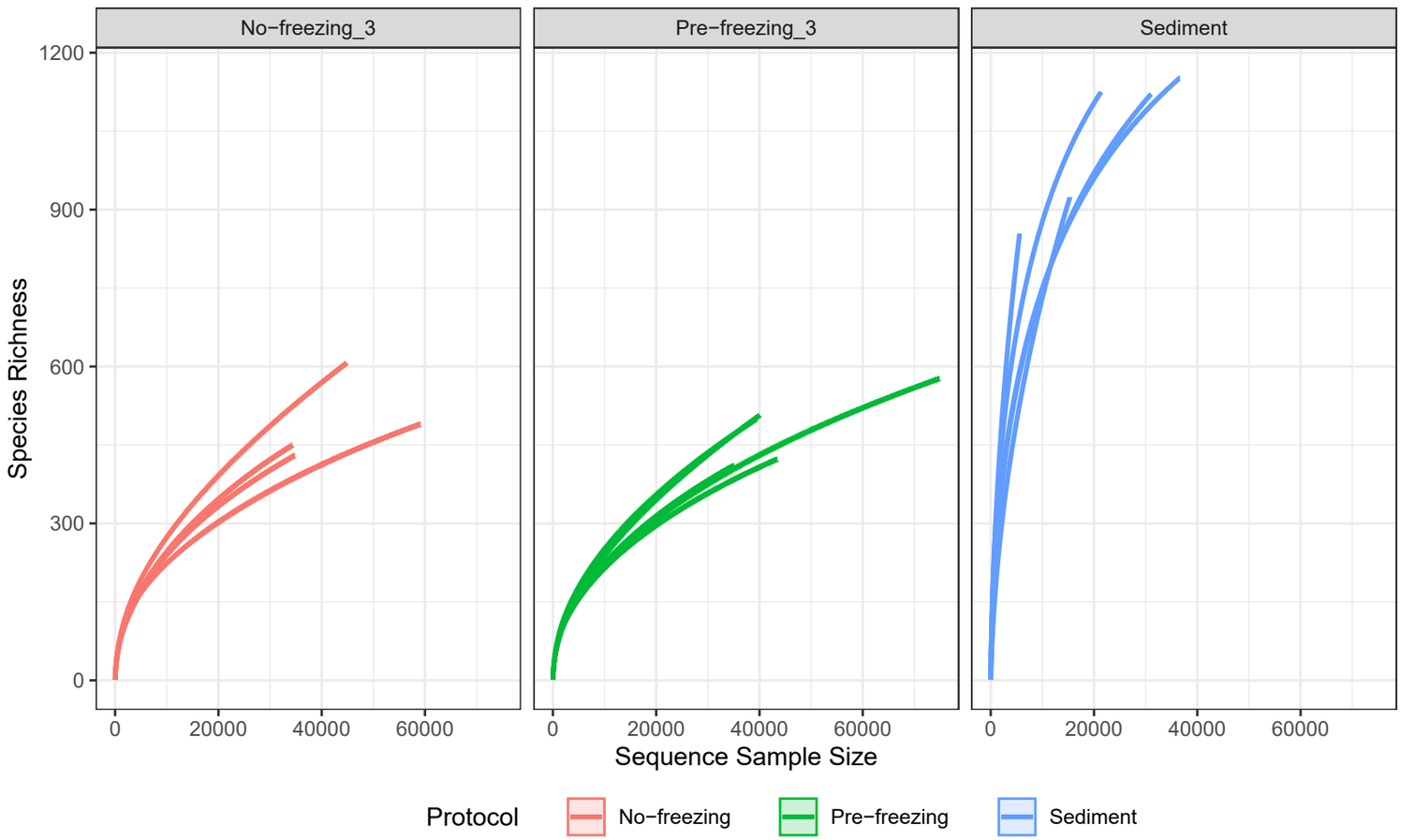
B) 16S


Sediment
Pre-Freezing (3)
No-freezing (3)
1178
182
181
402
177
286
185

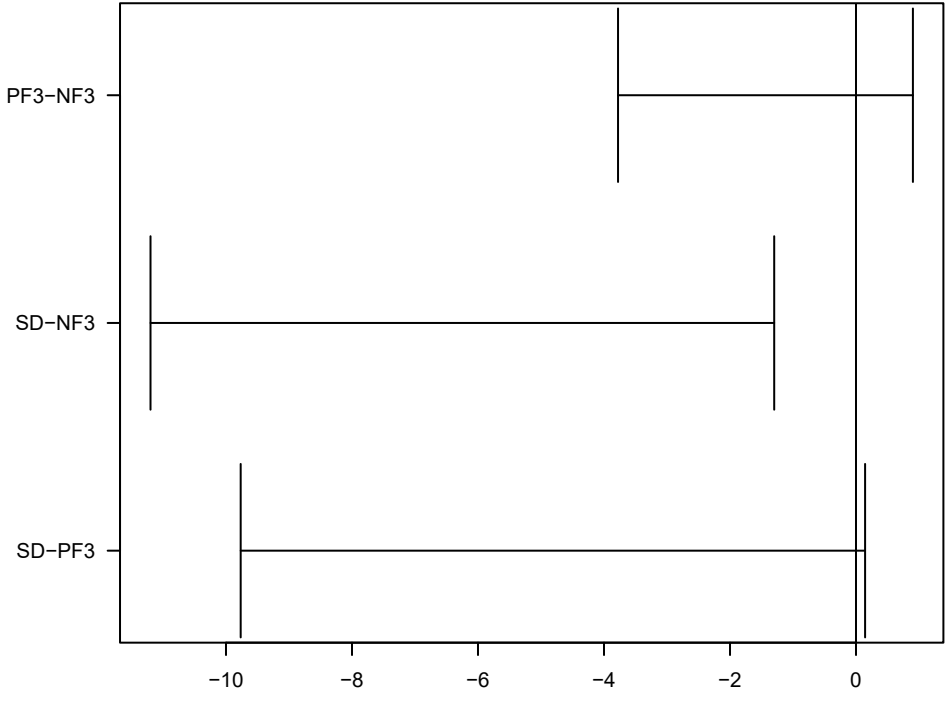
A) 12S



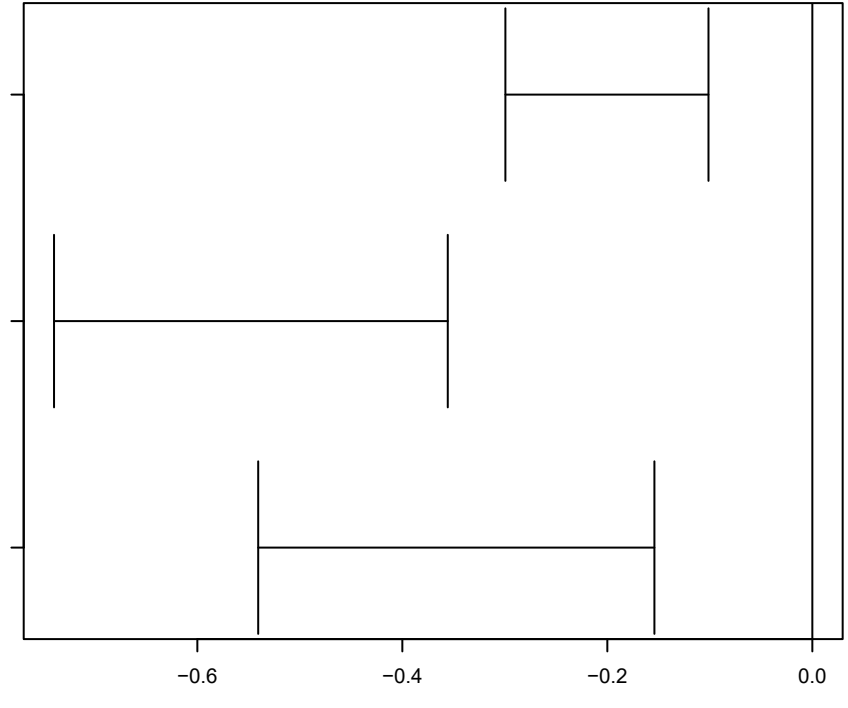
B) 16S

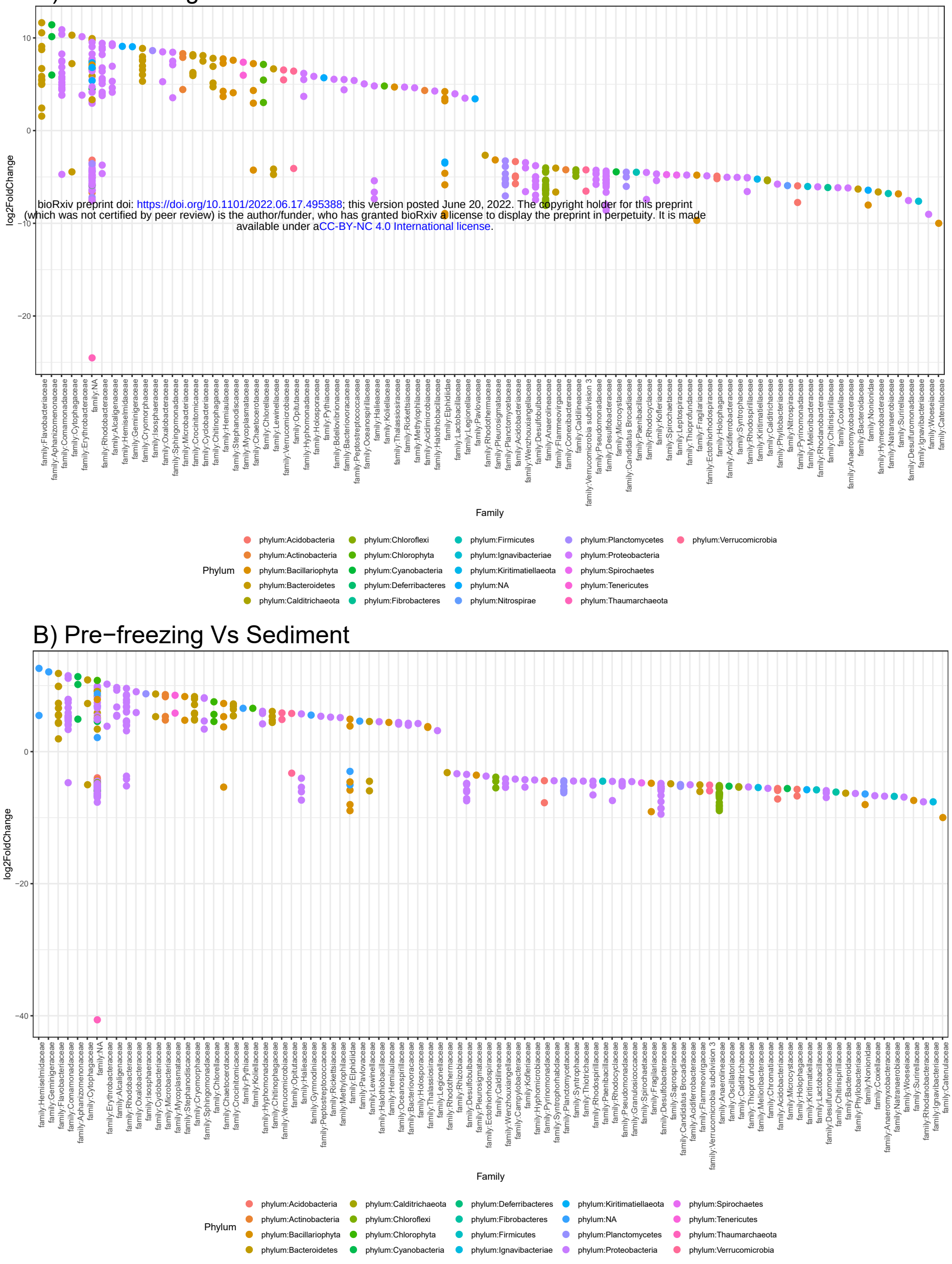


A) 12S

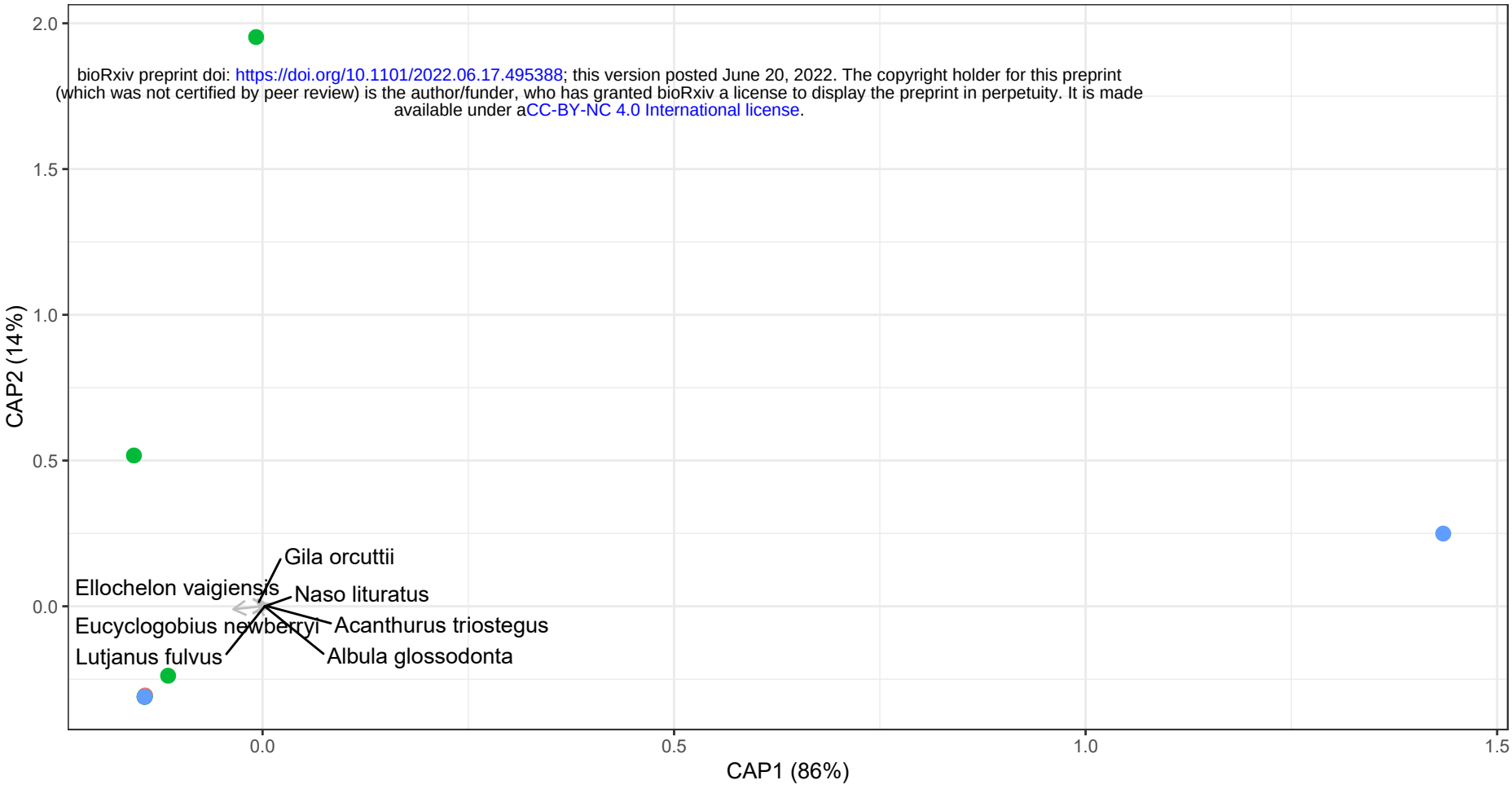


B) 16S





A) 12S



B) 16S