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ORIGINAL ARTICLE

Short-term temporal variation of coastal marine eDNA

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Abstract

Temporal variation in eDNA signals is increasingly explored for understanding community ecology in aquatic habitats. Seasonal changes have been addressed using eDNA sampling, but very little is known regarding short-term temporal variation that spans hours to days. To address this, we filtered marine water samples from a single coastal site in Denmark every hour for 32 h. We used metabarcoding to target both fish and broader eukaryote diversity and evaluated temporal changes in this marine community. Results revealed variation in fish species richness (15–27) and eukaryote class richness (35–64) across the 32 h of sampling, and we further evaluated sampling efforts needed to reach different levels of diversity saturation. Relative read frequency data for both fish and eukaryotes indicated a clear diel change in community composition, with different communities detected during daylight versus dark hours. The abundance signals in our data reflected biological variation rather than stochastic variation, since replicates taken at the same hour were more similar to each other than those taken at different hours. Our compositional results indicated a dynamic community, rather than a static pool of eDNA—even across a few hours. The fish data showed a daily pattern of relative species abundances, and the uncoupling of fish and broader eukaryote data suggest that variation in eDNA profiles across a single day can provide valuable information reflecting diel changes, at least for highly mobile organism groups. However, our results also point to several pitfalls in current eDNA experimental design, in which samples are taken over large areas without relative time-consistency or short-term replication. Our findings shed new light on short-term variation in coastal eDNA and have wide implications for experimental study design and for incorporating temporality into project conceptualization for future aquatic biodiversity monitoring.

1 | INTRODUCTION

Over the past two decades, environmental DNA (eDNA) has become established as a useful tool to study both contemporary and ancient biodiversity (Taberlet et al., 2018; Thomsen & Willerslev, 2015), using a variety of sources such as freshwater (e.g., Ficetola et al., 2008; Sigsgaard et al., 2015; Thomsen, Kielgast, Iversen, et al., 2012), seawater (e.g., Sigsgaard, Nielsen, Carl et al., 2017; Thomsen, Kielgast, Iversen, et al., 2012), and sediments (e.g., Giguet-Covex et al., 2014; Pansu et al., 2015; Willerslev et al., 2003). In aquatic ecosystems, detection of eDNA was initially used to investigate biodiversity on small, local scales (e.g., Thomsen, Kielgast, Iversen, et al., 2012), but the potential of the approach as a more cost-efficient and less invasive monitoring tool has led to an increase in studies whose inferences are made on regional and even national scales (Agersnap et al., 2022; Carraro et al., 2020; West et al., 2021). The use of eDNA for studying biological patterns over larger spatial scales further necessitates knowledge of eDNA dynamics at the temporal scale—particularly in lotic and marine ecosystems where eDNA can potentially be transported across long distances.

Temporal changes in community compositions are key to understanding complex community dynamics, and eDNA sampling designs that focus on temporal changes have become more common (e.g., Salter, 2018; Stoeckle et al., 2018; Uchii et al., 2017). While it has been established that eDNA composition changes with seasonal species turnover in fish communities (Sigsgaard, Nielsen, Carl et al., 2017; Stoeckle et al., 2017), there currently are very limited data on short-term variation spanning hours to days (Bálint et al., 2018). Degradation time and transport of eDNA are not fully understood in natural aquatic environments, but several studies have documented rapid degradation over hours to days in temperate water samples (e.g., Andruszkiewicz et al., 2017; Ely et al., 2021; Sigsgaard, Nielsen, Bach et al., 2017; Strickler et al., 2015; Yamamoto et al., 2016). Because of rapid degradation, the general assumption is that sampling aquatic environments provides a contemporary picture of biodiversity at that site, but the exact temporal scale remains unknown.

Sampling for an eDNA study is often carried out at varying times of the day (mainly during the day, but occasionally also during the night, e.g., Thomsen et al. (2016)), despite potential daily or hourly changes in species composition at the sample site. If a weak eDNA signal can disappear over just a few hours (Andruszkiewicz et al., 2017; Collins et al., 2018; Saito & Doi, 2021; Tsuji et al., 2017), this could strongly influence results in cases where species have diel variation in abundance patterns. A snorkeling study performed in the same area as the current study showed that demersal (near-bottom) fishes were more abundant during the day, and benthic (associated with or on the bottom) and pelagic (in the water column) fishes were more abundant at night (Holm-Hansen et al., 2019). Furthermore, there can be pronounced differences in species observations on a day-to-day basis. If the signal from eDNA is contemporary, such changes would be reflected in eDNA sampled on an hourly basis. With a few exceptions (e.g., Ely et al., 2021; Kelly et al., 2018; Sengupta et al., 2019), the influence of sampling eDNA at different times of day has been mostly neglected in aquatic eDNA studies, and broad inferences are often made without attention to sampling time. As eDNA detections rely on stochastic observation processes, an ongoing challenge is to distinguish the signals arising from stochasticity (PCR, sequencing variation, and heterogeneous distribution of eDNA in the water) with those representing actual biological variation (e.g., diel species behavior). Despite this stochasticity, several studies have inferred meaningful abundance patterns from eDNA read count data (e.g., Laporte et al., 2021; Salter et al., 2019).

Here, we investigated the degree of short-term variation in eDNA composition using marine samples collected hourly at a single sampling site over 32 h by comparing temporal abundance patterns inferred from relative read counts. We focused both on fishes (a group whose ecology and daily activity patterns of most species are well-known), as well as on general eukaryote community composition. We hypothesized that the species composition detected from eDNA samples would vary from hour to hour and would reflect known diel variation of the detected species. Such fine-scale temporal resolution would have wide implications for future aquatic biodiversity monitoring. As resources are always limited, we also examined how much sampling effort would be needed to capture most of the taxonomic diversity at the study site, given short-term variations in eDNA composition.

2 | MATERIALS AND METHODS

2.1 | Study site, sampling, and weather conditions

We collected surface water samples from Skovshoved Harbour (55°45′39″N, 12°35′59″E), north of Copenhagen, Denmark, on September 11–12, 2017 (Figure 1). The sampling site is a stone pier, and the water depth was approximately 2 m. The adjacent area is mostly shallow with a mix of sandy and rocky bottom and patches of eelgrass (*Zostera* spp.) and seaweeds, having a maximum depth of ~6 m within a 1.5 km radius from our sampling site and a salinity of ~12 PSU (Burchard & Kristensen, 2002). The sampling site experienced only ±0.1 m changes in tidal height throughout the sampling, and we thus disregarded this aspect in our analysis. Sampling was carried out during relatively stable weather conditions (Text A, Figure S1). Sunset was at 19.37 h on day one and sunrise at 06.36 h on day two. Sampling was initiated at 10.00 h on the September 11, and three samples were collected every hour for 32 consecutive hours (96 total filters). For each individual sample, we filtered 1 L of water through sterile 0.22 μm Sterivex-GP filters (Merck Life Science). With continuous sampling carried out across 32 h, the second occurrence at the same hour for sampling was denoted with a "2." prefix. Two field blanks were collected (11.00 h and 17.00 h on September 11) by filtering 0.5 L of bottled mineral water to account for possible airborne DNA or other sources of contamination. All samples were stored in a cooler bag with ice

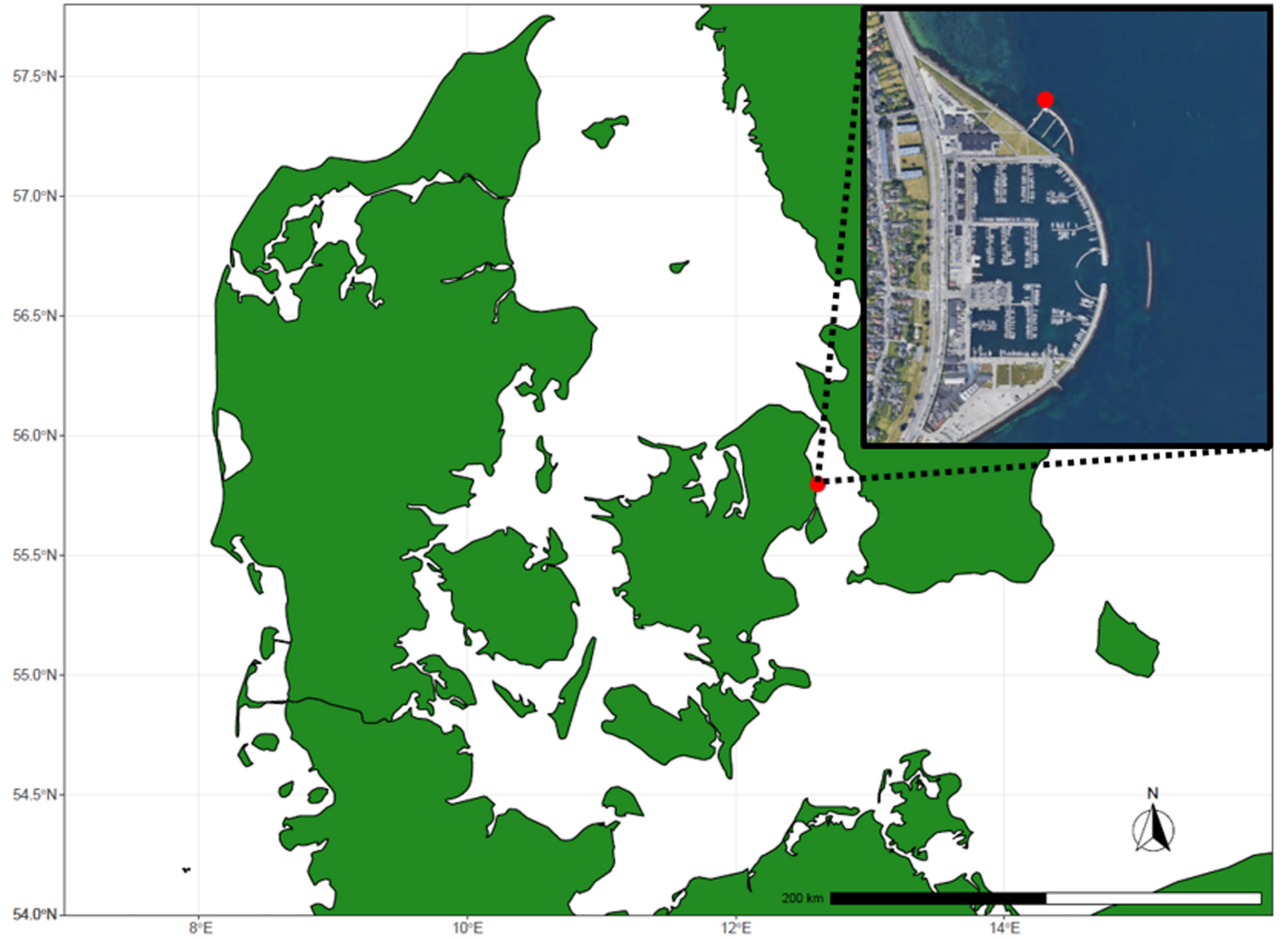

FIGURE 1 Map of Denmark with red circle denoting the sampling site at Skovshoved Harbour. Right-corner insert is a satellite image from Google Maps with the red circle detailing the exact sampling point at the northern pier. Satellite map downloaded September 13, 2021

packs immediately after filtering and were transferred to a −20°C freezer upon completion of sampling.

2.2 | DNA extraction

The eDNA samples were extracted using the DNeasy® Blood & Tissue kit (Qiagen) in a clean laboratory facility dedicated for working with samples having low DNA concentration at the Department of Biology, Aarhus University. We used four times more AL buffer and proteinase K compared to the manufacturer's protocol and an incubation time of 3 h. Spin columns were incubated with elution buffer AE (2*60 μl) over two rounds at 37°C for 10 min for a final volume of 120 μl (Sigsgaard, Nielsen, Bach et al., 2017). All 96 individual samples were extracted individually, after which we mixed 20 μl aliquots of each of the three hourly replicates into new pooled samples (3*20 μl, now 32 pooled samples). The filtered samples that were pooled were then denoted with a number for the time point when the sample was taken and a suffix "P" for pooled. This numbering was used throughout the manuscript and in the figures and tables. For each round of extraction, an extraction blank was included, and all extracted samples were stored at −20°C.

2.3 | PCR amplification

We used DNA metabarcoding targeting the mitochondrial 12S rRNA gene for fishes and the nuclear 18S rRNA gene for a broader range of eukaryote taxa. For each pooled sample, we ran four PCR replicates targeting fishes using the forward primer MiFish-U-F (5′-GTCGGTAAAACTCGTGCCAGC-3′) and the reverse primer MiFish-U-R (3′-GTTTGACCCTAATCTATGGGGTGATAC-5′), which amplify 163–185 bp of the 12S rRNA gene of the mtDNA (Miya et al., 2015). The same setup of four PCR replicates was applied when targeting eukaryotes, this time using the primers 18S_allshorts forward (5'-TTTGTCTGSTTAATTSCG-3') and 18S_allshorts reverse (5'-CACAGACCTGTTATTGC-3'), which amplify ca. 110 bp of the 18S rRNA gene (Taberlet et al., 2018). Note that we omitted the "T" at the 5'-end of the reverse primer compared to the original description by Guardiola et al. (2015), which was omitted to better equilibrate the melting temperatures of the two primers.

To keep track of the effect of biological replicates versus hourly variation, we also included three sets of individual triplicate samples in the metabarcoding setup (from 12.00 and 20.00 h on September 11, as well as from 04.00 h on September 12). With 52 different primer twin-tags (2–3 N's and a unique sequence of six nucleotides (De Barba et al., 2014)), we individually tagged 32 pooled samples (one per hour), nine non-pooled replicate samples (see above), two field blanks, and eight extraction blanks. We used the final tag for a positive control as well as for four PCR blanks. Both PCR setups (i.e., with "MiFish-U" and "18S_allshorts") were run with 10 μl HotStarTaq Master Mix (Qiagen, Cat. no. 203445), 10 μl ddH_2O, 1 μl primer mix (5 μM forward and 5 μM reverse), 1 μl BSA (Bionordica, Cat. no. B9000S), and 3 μl DNA template. The thermocycler conditions for the fish setup were set to an initial 15 min of denaturing at 95°C, followed by 50 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 5 min. The thermocycler conditions for the broader eukaryote setup were set to an initial 15 min of denaturation at 95°C, followed by 40 cycles of 94°C for 30 s, 45°C for 30 s, and 72°C for 30 s. Products were verified on 2% agarose gels stained with GelRed™. The PCR products were combined into four pools per primer set, each containing one PCR replicate per sample (excluding the positive control), and were then purified using Qiagen's MinElute PCR purification kit. A double amount of PCR product was used for the samples with weak bands on the gel, indicating low DNA yield, compared to samples with strong bands. We followed the manufacturer's protocol for purification with the exception that samples were incubated with the elution buffer (2*20 μl EB) over two rounds of 37°C for 10 min, and included a purification blank.

2.4 | Library building and Illumina sequencing

A total of eight libraries (four from each primer set) were prepared using the TruSeq DNA PCR-free LT Sample Prep kit (Illumina), with an input of ca. 750 ng of purified PCR product from each pool, as measured with a Qubit HS DNA Kit (Thermo Fisher Scientific). Libraries were then sequenced on an Illumina NovaSeq 6000 by Novogene using 150 PE sequencing and requesting 10 Gb of output per library.

2.5 | Extraction, amplification, and sequencing of fish tissue for reference database

We extracted DNA from tissue samples for 41 species of fishes sampled in or near Denmark, which did not at the time have complete sequences available in GenBank for the 12S rRNA region that the MiFish-U primers target. The samples from these species (Table S1) were obtained from the Natural History Museum of Denmark, Copenhagen. DNA extractions were carried out using the E.Z.N.A.® Tissue DNA Kit (Omega Bio-tek) according to the manufacturer's protocol.

For the museum vouchered samples, we set up PCR reactions using forward primer MiFish-U-F and reverse primer MiFish-U-R (both untagged). The PCRs were performed using 10 μl HotStarTaq Master Mix (Qiagen, Cat. no. 203445), 12 μl ddH_2O, 1 μl primer mix (5 μM forward and 5 μM reverse), 1 μl BSA (Bionordica, Cat. no. B9000S), and 3 μl DNA template in a 1:10 ddH_2O dilution. Thermocycler conditions for the tissue sample setup were set to an initial 5 min denaturing at 95°C, followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 1 min, and a final extension of 72°C for 5 min. Products were verified on 2% agarose gels stained with GelRed™. Resulting PCR products (diluted 1:2 with ddH_2O) were then sent to be purified and commercially Sanger-sequenced (Sanger & Coulson, 1975; Sanger et al., 1977) by Macrogen Europe (https://dna.macrogen-europe.com/eng/), where they were sequenced twice in both forward and reverse directions. Resulting sequence chromatographs were *de novo* assembled and manually inspected for errors using Geneious v. 10.6.2 (Kearse et al., 2012). For colloquial names and authorities of species mentioned throughout the manuscript, we refer to FishBase (https://www.fishbase.se; Froese & Pauly, 2021).

2.6 | Data filtering and analysis

Raw sequencing reads were demultiplexed, trimmed, and filtered using the MetaBarFlow pipeline (Sigsgaard et al., 2022). The specific workflow and scripts used in this study are available upon request. We specified a minimum read length of 100 bp for the fish data and a minimum of 90 bp for the broader eukaryote data. Sequencing reads were searched against a local version of the GenBank nucleotide (nt) database (downloaded November 24, 2020) using BLASTn, specifying up to 500 hits, 90% query coverage, and 80% sequence similarity. For the broader eukaryote data, the BLASTn settings also included a *negative_gilist* (search term: "environmental samples [organism] OR metagenomes [organism]"), downloaded from the National Center for Biotechnology Information (NCBI) GenBank on January 25, 2021. This list was incorporated to exclude ~18 M entries of unspecific hits from GenBank in the search parameters.

For the fish dataset, reads were also searched against the newly generated sequences from tissue samples, using Geneious (Kearse et al., 2012). We then updated the best hit for the fish reads if the newly generated sequences provided an equal or higher percentage of sequence identity compared to hits from the GenBank nt database. If we encountered ambiguity in best matches, we assigned a last common ancestor identification to the read. Hits with a query coverage of <100 or similarities below 98% and hits not matching fishes were filtered out.

For the broader eukaryote dataset, we evaluated all hits at the class level, as taxonomic resolution in many cases does not allow for discrimination below this level. We also filtered out hits with a query coverage of <100 and similarities below 98%.

Both datasets were then filtered for taxa that occurred in a higher read count in field controls, extraction controls, or PCR controls than in any seawater sample. Detected taxa were required to be present in at least two out of four PCR replicates of a sample, for

the hit to be retained. This improved certainty in taxon detections, although it may have removed some taxa that were truly present, but rare (Alberdi et al., 2018; Ficetola et al., 2015). We then performed species accumulation curves on individual PCR reactions per sample. To address the influence of differential sample sequencing depth, we rarefied the final, aggregated sample reads to the median read number across all the individual PCR replicates using the R-package *ROBITools* (v0.1). This step removed a large proportion of sequencing reads, but alleviated bias associated with differential sequencing depth. We used the nine individual biological replicate samples that were also included in the sequencing to evaluate the effect of pooling and to test whether we could distinguish technical variation from real biological signals. These nine samples were omitted from subsequent analyses.

Based on the species that were detected in pooled samples, we inferred species and class accumulation curves for the fish data and the broader eukaryote data, respectively. Nine nonlinear regression models (Arrhenius, Gleason, Gitay, Lomolino, Asymp, Gompertz, Michaelis–Menten, Logis, and Weibull, all using the *specaccum* function of the R-package *vegan* (v.2.5–6; Oksanen et al., 2019)) were fitted to the two datasets. The best-fit model selected from the lowest AIC value was used to extrapolate the accumulation curves to 100 samples using the *predict* function. This allowed us to calculate the observed richness (R_O) and extrapolated richness (R_E) and infer the expected proportion of species/classes detected by taking fewer samples for both R_O and R_E.

Whittaker dissimilarity scores (Whittaker, 1960), Bray–Curtis dissimilarity scores (Bray & Curtis, 1957), and input for non-metric multidimensional scaling (NMDS) plots were calculated using the *vegdist* and *metaMDS* functions of the R-package *vegan* (v.2.5-6; Oksanen et al., 2019). These indices were used to quantify beta diversity among samples and represent proportional species turnover (Whittaker, presence/absence) and compositional dissimilarity (Bray–Curtis, abundance), respectively. All plots were created using *ggplot2* (v3.2.1; Wickham, 2016).

3 | RESULTS

3.1 | Fish barcode generation

We successfully generated barcodes for the 12S rRNA region targeted by the MiFish-U primers for 40 of 41 fish species (166–181 bp). Although we attempted to resequence *Brama brama*, we were not able to generate a barcode for this species. Furthermore, we here released 17 barcodes of Danish fish species that were generated by Valentini et al. (2016), but which were not previously deposited in GenBank. The forward primer Tele02 (5'-AAACTCGTGCCAGCCACC-3', Taberlet et al. (2018)) and the reverse primer teleo_R (5'-CTTCCGGTACACTTACCATG-3') were used to generate these barcodes. The resulting barcode overlapped with the MiFish-U barcode, but was much longer (658–664 bp), and did not cover the entirety of the latter. Five of the 17 species were also amplified with the MiFish-U primers in the current study. All barcodes and information on vouchered specimens (Table S1) were deposited in NCBI GenBank (accession numbers MW995331–MW995387). The reference sequences generated by Margaryan et al. (2021) ($n = 42$) were also used for our taxonomic assignment.

3.2 | Fish dataset

We generated between 30.17 and 56.05 M reads per library (average of 41.55 M reads), and after initial filtering, 0.36–3.13 M reads per pooled sample were retained (average of 1.59 M reads, $n = 32$). Individually sequenced filter samples ($n = 9$) each produced 1.40–2.56 M reads (average of 1.88 M reads). Extraction blanks ($n = 8$) produced 0–38,904 reads (average of 12,844 reads), whereas field blanks ($n = 2$) produced 126 and 7201 reads, respectively. The four PCR blanks produced 226 reads in all. In total, we found 4341 amplicon sequence variants (ASVs) across all samples. PCR blanks and field blanks indicated no noteworthy contaminants. Extraction blanks yielded three ASVs belonging to *Alburnus alburnus* and *Gobio gobio*, neither of which were detected in any seawater samples, and *Leuciscus idus*, which was also detected in seaweater samples but at lower sequence counts. These three ASVs were removed from the data. Two reads (a single ASV) of *Platichthys flesus* were detected in the extraction blank, but this ASV was retained as the highest read count found in a seawater sample was ~30,000.

Rarefaction curves of individual PCR reactions indicated that sequencing depth was sufficient (Figure S2) to reach a species occurrence plateau, and species accumulation curves indicated that four PCR replicates adequately represented the diversity within each sample (Figure S3).

After removing species that solely occurred in single replicates within a sample, and after rarefying reads to the median value of the individual PCR replicates (1,425,184 reads per sample), a total of 41 fish species were found (pooled and individual samples). Venn diagrams of species presence (Figure S4A) and stacked bar plots of relative read frequencies (Figure S5A) supported a match between individual samples and their corresponding pooled sample. A few species found in the individual samples were not found in the corresponding pooled samples (i.e., *Belone belone*, *Clupea harengus*, and *Pholis gunnellus* for 4P, *C. harengus*, *Cyclopterus lumpus*, *Syngnathus typhle*, and *Pomastochistus microps* for 12P, and *B. belone*, *Nerophis ophidion*, *Salmo trutta*, *Sprattus sprattus*, *Syngnathus rostellatus*, *S. typhle*, and *Trachurus trachurus* for 20P). Similarly, a few species occurred in the pooled samples, but not in the corresponding individual samples (i.e., *N. ophidion* for 04P, *Pungitius pungitius* and *P. gunnellus* for 12P, and *Aphia minuta* for 20P). We provide an overview of these inconsistencies in Text B, Supp. Info.

Species richness varied from 15 to 27 species (Figure 2a, Table S2), with 40 different species found in the pooled samples across the 32 h of sampling (Table S2). The second day of sampling yielded fewer species compared to the first day. On average, 4.41 fewer species were detected on day two, when comparing hours 10–18 on day

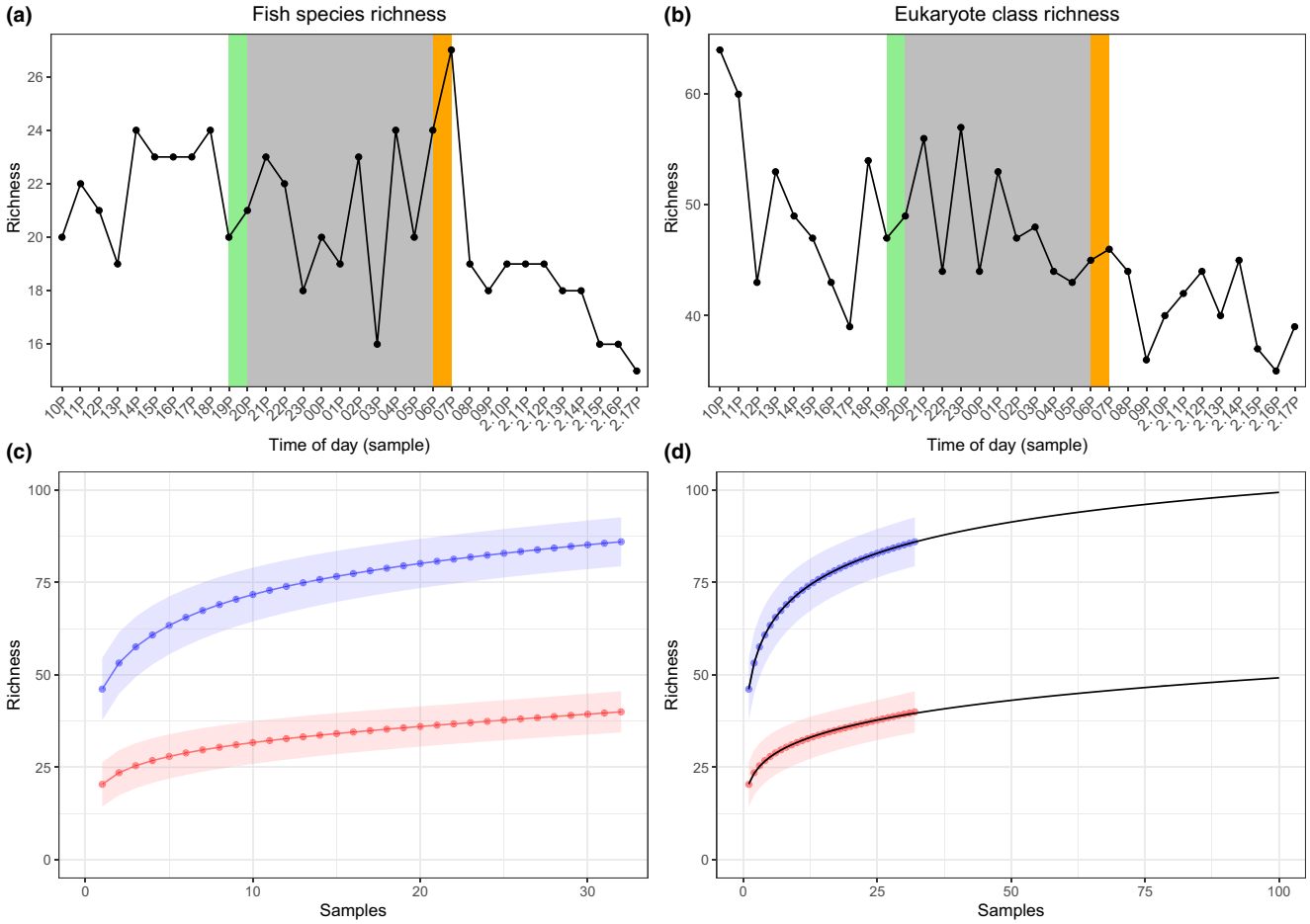

FIGURE 2 (a) and (b) richness plots throughout the 32 h of sampling, where the suffix "P" denotes pooled samples. (a) Fish species richness and (b) eukaryote class-level richness. Green color indicates sunset (dusk), gray indicates dark hours (night), and orange indicates sunrise (dawn). (c) and (d) Species/class accumulation curves. (c) Species accumulation curve for fish (red) and class accumulation curve for eukaryotes (blue). (d) Extrapolation of species/class accumulation curves based on the best-fitting Arrhenius (fish data, red) or Weibull (eukaryote, blue) models to 100 samples. Shaded areas (blue or red) denote 95% confidence interval based on the unconditional standard deviation

one with hours 08–17 on day two (daylight hours). The three species having the highest relative read frequencies across all samples were *Pomatoschistus flavescens*, *Ctenolabrus rupestris*, and *Gasterosteus aculeatus*. Likely reflecting the extensive sampling at a single site, we detected several rarer species, including *T. trachurus*, *Piaractus brachypomus* (pirapitinga), *Rutilus rutilus*, *Lesueurigobius friesii*, *C. lumpus*, *Liparis montagui*, *Hippoglossoides platessoides*, and *Microstomus kitt* (Figure S6A).

The species accumulation curve indicated that even a few samples were sufficient for approaching saturation of fish diversity (Figure 2c). When extrapolating the curve to 100 samples, using Arrhenius as the best-fitting model, we found an estimated R_E of 49.21 species (Figure 2d). This is only ~23% higher than our R_O at 32 samples. For this specific sampling site, we covered ~50% of the R_O and ~41.5% of the R_E by taking a single sample. In order to cover 80% of the R_O, ~11 samples would need to be taken, whereas ~30 samples would need to be taken to cover 80% of the R_E using 100 samples as an estimator of the total diversity at the site. Ten species were detected in all 32 pooled samples, whereas 16 species were detected in >90% of the samples (Figure S6A, Table S2).

Bray–Curtis dissimilarities were lower for individual samples taken at the same hour compared to those taken at different hours (Figure 3a,b, Wilcoxon rank sum test, $p < 0.05$). Bray–Curtis dissimilarity scores between the pooled samples varied from 0.09 to 0.76 (average 0.35), with sample 2.13P being the most distinct across the 32 h of sampling (Figure 4a). Interestingly, the Bray–Curtis dissimilarity matrix (Figure 4a) clearly indicated a shift in community composition between day and night, with the hours between 19.00 and 06.00 displaying greatest dissimilarity. This trend was not as clear with the presence/absence data and Whittaker dissimilarities (Figure S7A).

Several tendencies in the relative read frequency data (Figure 5a) reflected the known biology of the species in question. For example, *C. rupestris* and *Symphodus melops* were less abundant during the night hours (Figure 6a), when Ammodytidae (sand lances) and *Anguilla anguilla* were more abundant (Figure 6b). *Pomatoschistus*

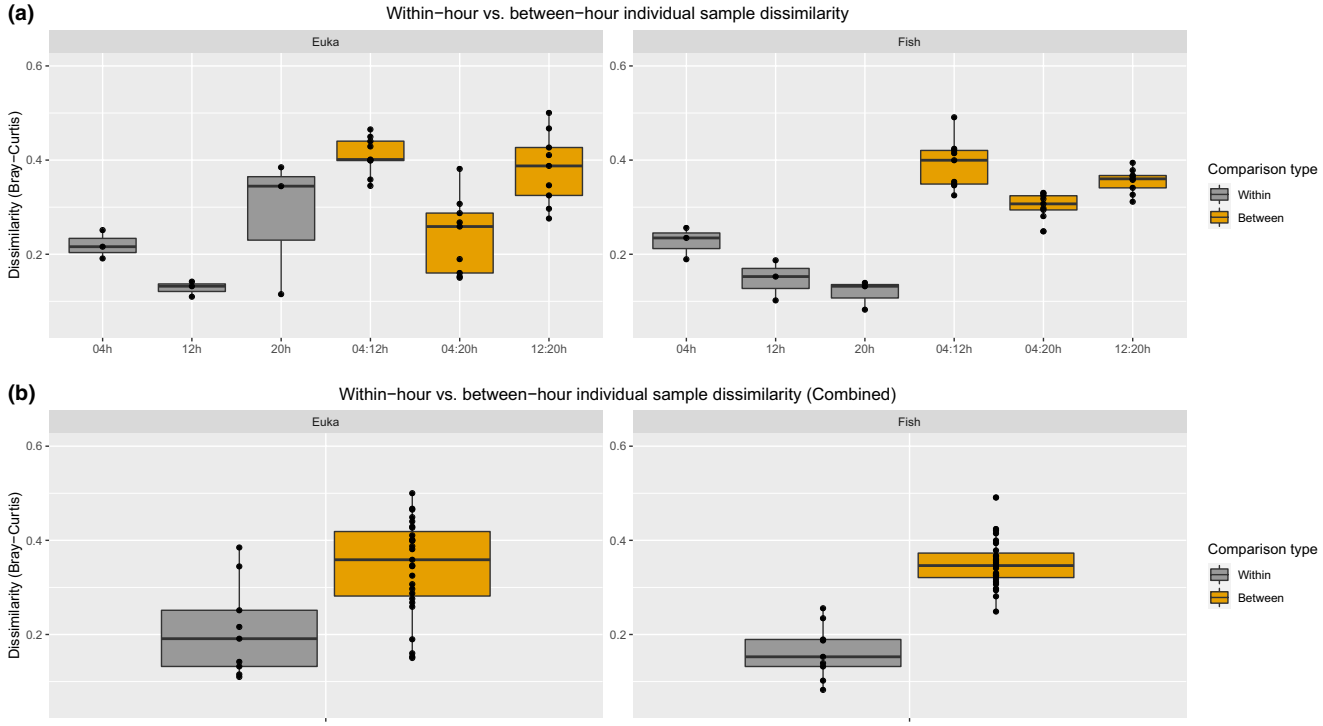

FIGURE 3 Comparison of Bray–Curtis dissimilarities (abundance data) obtained for within-hour individual biological replicates (gray, $n = 3$ per hour) and between-hour individual biological replicates (orange, $n = 9$ per hour). Dots represent actual data points. "Euka" refers to the eukaryote dataset, and "Fish" refers to the fish dataset. The three time points where individual biological replicate samples were sequenced in parallel with pooled samples were 04.00 h (day two), 12.00 h (day one), and 20.00 h (day one). (a) All samples and pairwise combinations compared and (b) combined data for the three time points. Based on (b), both the fish and eukaryote dataset show significantly lower within-hour dissimilarity than between-hour dissimilarities (Wilcoxon rank sum test, $p < 0.05$)

flavescens and *Zoarces viviparus* remained somewhat constant in their relative eDNA contributions throughout the day. The relative contribution of *Pollachius virens* eDNA peaked exactly during dusk and dawn hours (Figure 6c). *Gasterosteus aculeatus* eDNA peaked at 07.00 and 13.00 h on day two, most likely indicative of schools passing by (Figure 6d). The sudden, high proportion of *G. aculeatus* eDNA is also what explains sample 2.13P as being the most distinct (Figure 4a). For more details on the individual species contributions, all relative contributions are included per species throughout the 32 h in Figure S8.

Non-metric multidimensional scaling plots of the presence/absence data (Figure 7a) and particularly relative abundance data (Figure 7b) revealed a clear differentiation between species communities during daylight hours and dark hours, as well as between day one and day two, although only 8 h of sampling were carried out on day two. Samples taken during dusk hours were also highly differentiated from the rest of the samples.

The hourly intervals between samples and their dissimilarity values displayed a positive correlation in linear regression (adjusted $R^2 = 0.07$, $p < 0.01$, Figure 8a). This indicates that the fish communities were more dissimilar across time. However, when plotting a "loess" trend line using the fish data, we observed a bell-shaped trend in data points, suggesting that samples returned to a more similar state indicating a diel pattern (Figure 8a). This pattern was not present in the presence/absence data using Whittaker dissimilarity values (Figure S9).

3.3 | Eukaryote dataset

We generated between 47.06 and 82.94 M reads per library (average 69.24 M), and following initial filtering 0.84–7.32 M reads per pooled sample were retained (average of 3.51 M reads, $n = 32$). Individual samples ($n = 9$) produced 4.00–6.03 M reads (average of 4.63 M reads). Extraction blanks ($n = 8$) had 136–3.15 M reads (average of 0.52 M reads), whereas field blanks ($n = 2$) yielded 25,398 and 4.16 M reads, respectively. The single tag used for the four PCR blanks had 79,765 reads. In total, we found 19,339 ASVs across all the samples, although just 4728 ASVs passed the 98% similarity filter. PCR blanks, extraction blanks, and most notably field blanks detected a large variety of broader eukaryotes, and the list of filtered out ASVs along with explanations can be found in Text C.

Rarefaction curves for individual PCR reactions, with eukaryote data classified to class level, indicated that sequencing depth was sufficient (Figure S10) to reach a plateau. Accumulation curves indicated that four PCR replicates were sufficient to cover the diversity within each sample at the class level (Figure S11). After filtering out classes solely found in single replicates in a sample, and after

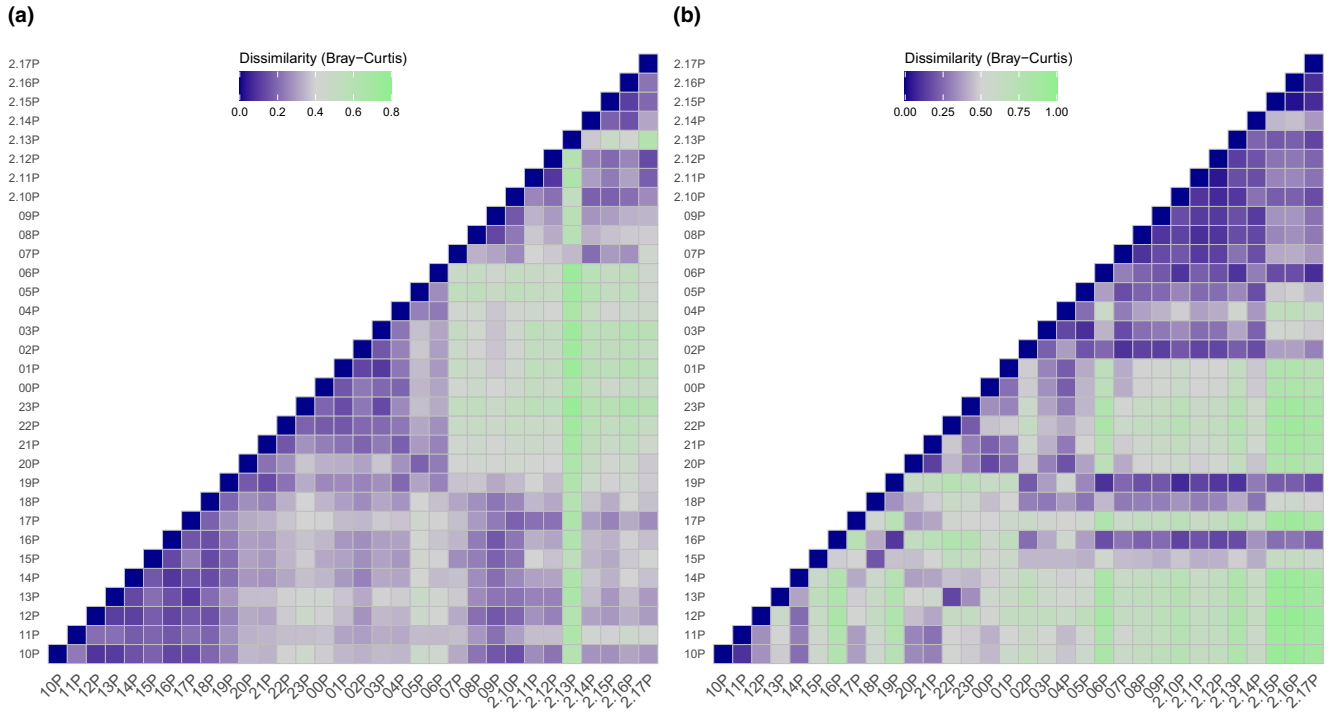

FIGURE 4 Matrices of sample-by-sample Bray–Curtis dissimilarities, an index for quantifying the compositional dissimilarity between samples. The prefix "2." denotes sampling at day two, and the suffix "P" denotes pooled samples. (a) Fish data and (b) eukaryote data

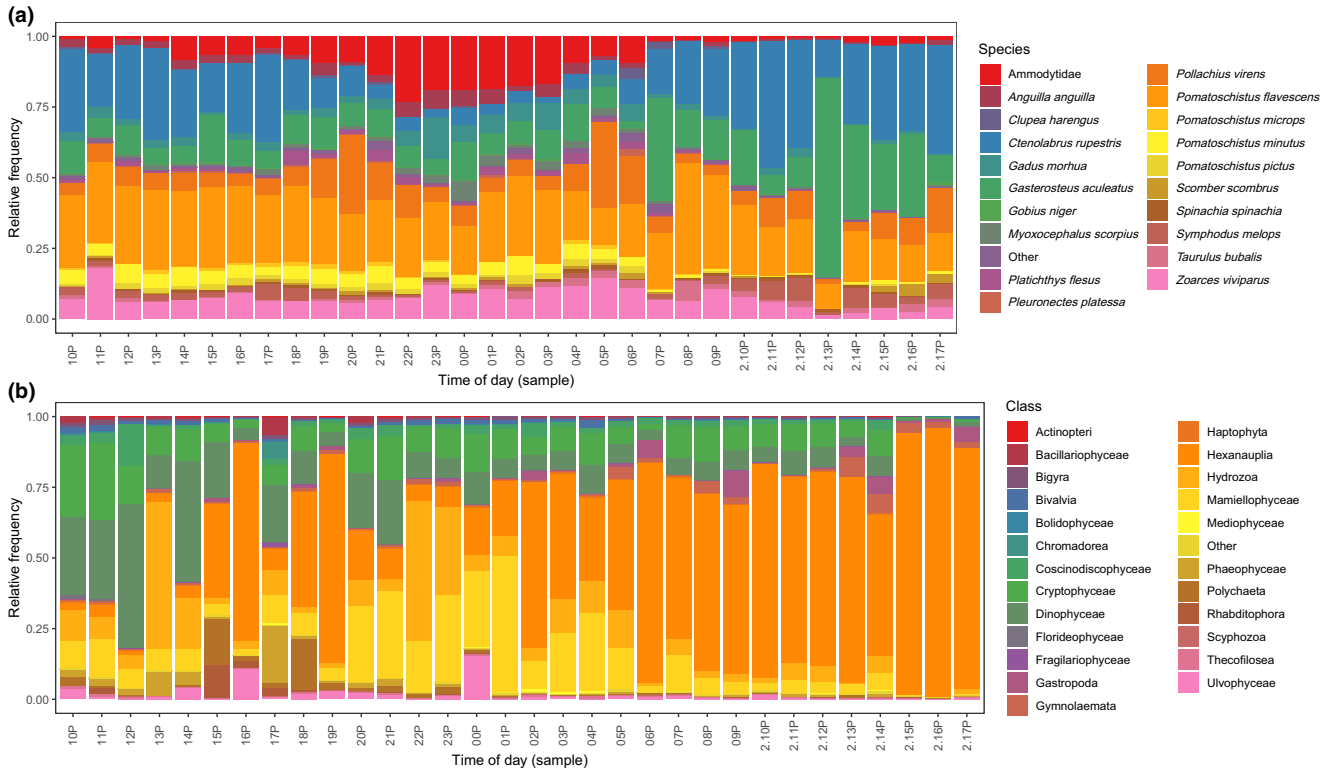

FIGURE 5 Stacked barplots showing relative frequency of reads for (a) the 20 most abundant fish species throughout the 32 h of sampling and (b) the 24 most abundant eukaryote classes throughout the 32 h of sampling. The remaining, less abundant fish species/eukaryote classes are here grouped as "Other." The prefix "2." denotes sampling at day two, and the suffix "P" denotes pooled samples

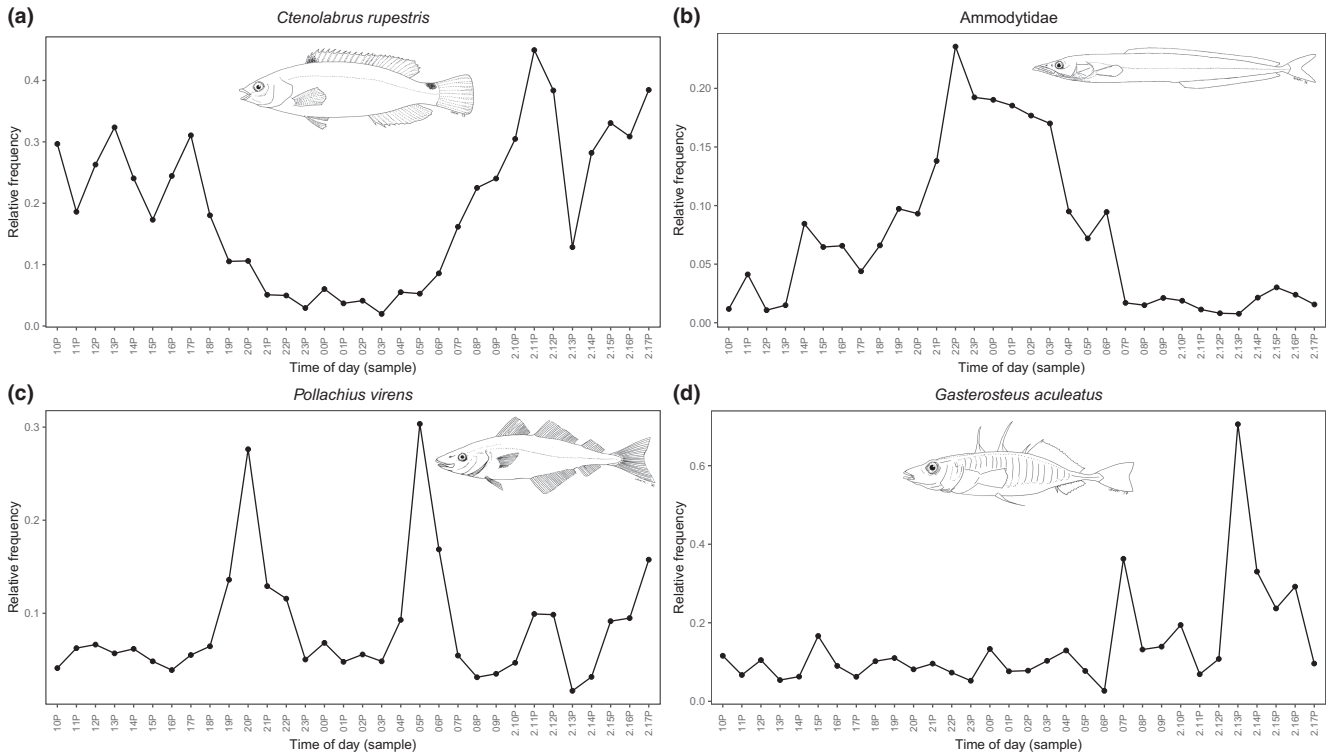

FIGURE 6 Relative frequency of occurrence throughout the hourly sampling of eDNA for (a) *Ctenolabrus rupestris*, a species known to be most abundant during the daytime, (b) Ammodytidae, a family known to be most abundant during the night, (c) *Pollachius virens*, a species known to come to shore during dusk and dawn to feed, and (d) *Gasterosteus aculeatus*, a species known for its schooling behavior. Drawings are not scaled, drawings by SWK

rarefying the reads to the median value of the individual PCR replicates (3,203,920 reads), 88 classes were discerned. Comparisons of class presence (Figure S4B) and relative read frequencies (Figure S5B) showed an overlap between individual samples and their corresponding pooled samples, although not as much as with the fish data. This was particularly evident for the quantitative data, in which pooled samples showed a more pronounced deviation from the individual samples. Several classes were detected in individual samples, but not in their corresponding pooled sample, and similarly, a few classes were detected in the pooled samples but not from the individual samples. A detailed walkthrough of these inconsistencies can be found in Text D, Supp. Info.

Richness varied from 35 to 64 classes (Figure 2b, Table S3), with 86 different classes found in the pooled samples throughout the 32 h of sampling (Table S3). Similar to the fish dataset, the number of eukaryote classes found also decreased on day two in comparison with day one of sampling. On average, ~10 more classes were detected on day one, comparing hours 10–18 on day one with hours 08–17 on day two (daylight hours). Relative read frequencies were highly dominated by Hexanauplia, with Dinophyceae (dinoflagellates), Mamiellophyceae (groups of unicellular green algae), Cryptophyceae (cryptophytes), and Hydrozoa (hydrozoans) also showing high abundances.

The class accumulation curve indicated that even a few samples appeared to be enough for approaching saturation of eukaryote diversity (Figure 2c). When extrapolating the curve to 100 samples, using Weibull as the best-fitting model, we found an estimated R_E of 99.35 classes (Figure 2d). This was only ~15.5% higher than our R_O at 32 samples. For this specific sampling site, we covered ~53.7% of the R_O and ~46.5% of the R_E by taking a single sample. In order to cover 80% of the R_O, ~8 samples would be required, whereas ~19 samples would be required to cover 80% of the R_E, provided that 100 samples was used as an estimator of the total diversity at the site.

We also found lower Bray–Curtis dissimilarities for individual samples taken at the same hour compared to samples taken at different hours for the broader eukaryote data (Figure 3a,b). While still significant (Wilcoxon rank sum test, $p < 0.05$), this was not as clear-cut as with the fish marker, mainly because samples taken at 20 h were quite dissimilar. Bray–Curtis dissimilarity scores between pooled samples varied from 0.03 to 0.96 (average 0.48), with samples 12P and 13P being the most distinct from other samples across the 32 h of sampling (Figure 4b). The Bray–Curtis dissimilarity matrix (Figure 4b) indicated large shifts in community composition over short time periods, primarily driven by the onset of the complete Hexanauplia dominance (from 02.00 h and onwards). When relying on class-level presence/absence data and Whittaker dissimilarity values, samples 10P, 11P, and 2.16P were the most dissimilar, driven primarily by high richness (10P and 11P) and low richness (2.16P), respectively (Figure S7B).

Across the 32 h of sampling, the eukaryote community composition showed drastic changes in read frequencies (Figure 5b).

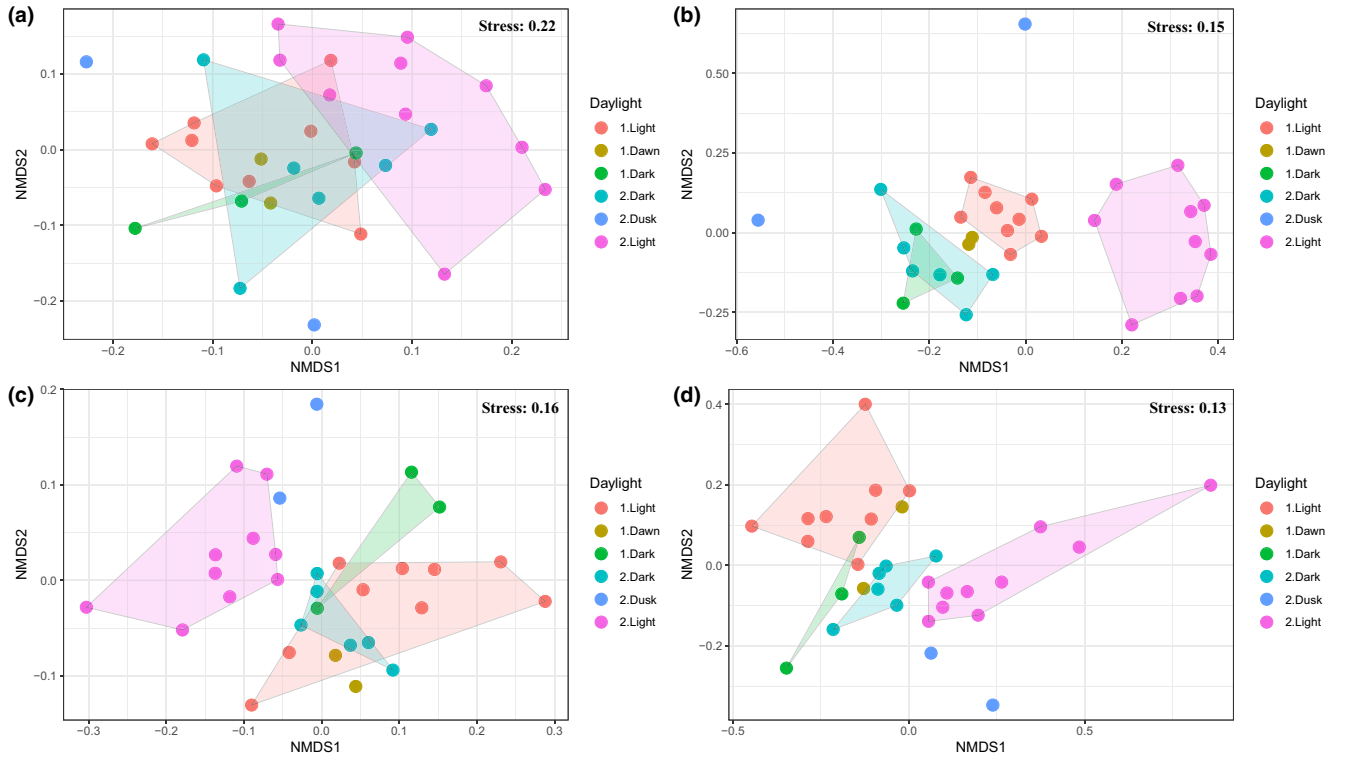

FIGURE 7 Non-metric multidimensional scaling (NMDS) plot of distances between eDNA samples from (a) presence/absence fish data, (b) fish abundance data, (c) presence/absence eukaryote data, and (d) eukaryote abundance data. Daylight groupings are 10P-18P (1. Light), 19P-20P (1. Dawn), 21P-23P (1. Dark), 00P-05P (2. Dark), 06P-07P (2. Dusk), and 08P-2.17P (2. Light)

Hexanauplia varied from constituting ~1.7% of the reads at 12.00 h on day one to >95% of the reads at 16.00 h on day two, completely outnumbering otherwise abundant classes, including Cryptophyceae, Dinophyceae, and Hydrozoa, on day two. Another noteworthy aspect was how abruptly large changes appeared in the data. For example, Hydrozoa declined from ~52% at 13.00 h to ~2% at 15.00 h on day one. Likewise, Ulvophyceae was present at relatively high frequencies at 16.00 h and 00.00 h on day one (~11% and ~15%, respectively), but constituted <1% of the reads at the next sampling time. For more details on the individual class contributions, all relative contributions are included per class throughout the 32 h in Figure S12.

The NMDS plots (Figure 7c,d) indicated a gradual community transition in which the light hours of day one and day two communities were quite distinct, with the dark hours grouping mostly with the light hours on day one (presence/absence data, Figure 7c) or in between the light hours of the 2 days (abundance data, Figure 7d). Similar to the fish dataset, the hours around dusk were also distinguishable in the broader eukaryote dataset.

Using linear regression, positive correlation occurred between the hourly intervals among samples and their dissimilarity (adjusted $R^2 = 0.27$, $p < 0.01$, Figure 8b). This indicates that the eukaryote communities became more dissimilar over time. The loess trend line did not indicate a bell shape as in the fish dataset, suggesting that samples simply became more and more dissimilar over the sampling period (Figure 8b).

4 | DISCUSSION

Detection of environmental DNA has gained momentum in aquatic research for species monitoring and biodiversity inference, and its potential applications and limitations are still being investigated. Here, we demonstrate by employing a standardized sampling scheme how even hourly differences in sampling time can affect the number of detected taxa and community compositions of seawater samples. No major weather changes occurred during the 32 h of sampling, with relatively stable winds, currents, and little rain, making it difficult to ascribe any of the community changes to environmental factors. We demonstrated biologically meaningful trends in our quantitative read frequency data, which were not due to technical variation, and thus likely indicate biological signals.

4.1 | Fish dataset

The fish diversity at Skovshoved Harbour is well-known from both snorkel visual census (Holm-Hansen et al., 2019) and eDNA (Sigsgaard, Nielsen, Carl et al., 2017). Holm-Hansen et al. (2019) found 28 species at Skovshoved during a 15-month snorkeling survey and Sigsgaard, Nielsen, Carl et al. (2017) detected eDNA from 29 species in 26 pooled samples spanning one year (32 species when including snorkeling survey). Here, we found 41 species over just 32 h of sampling, with the reference database being expanded

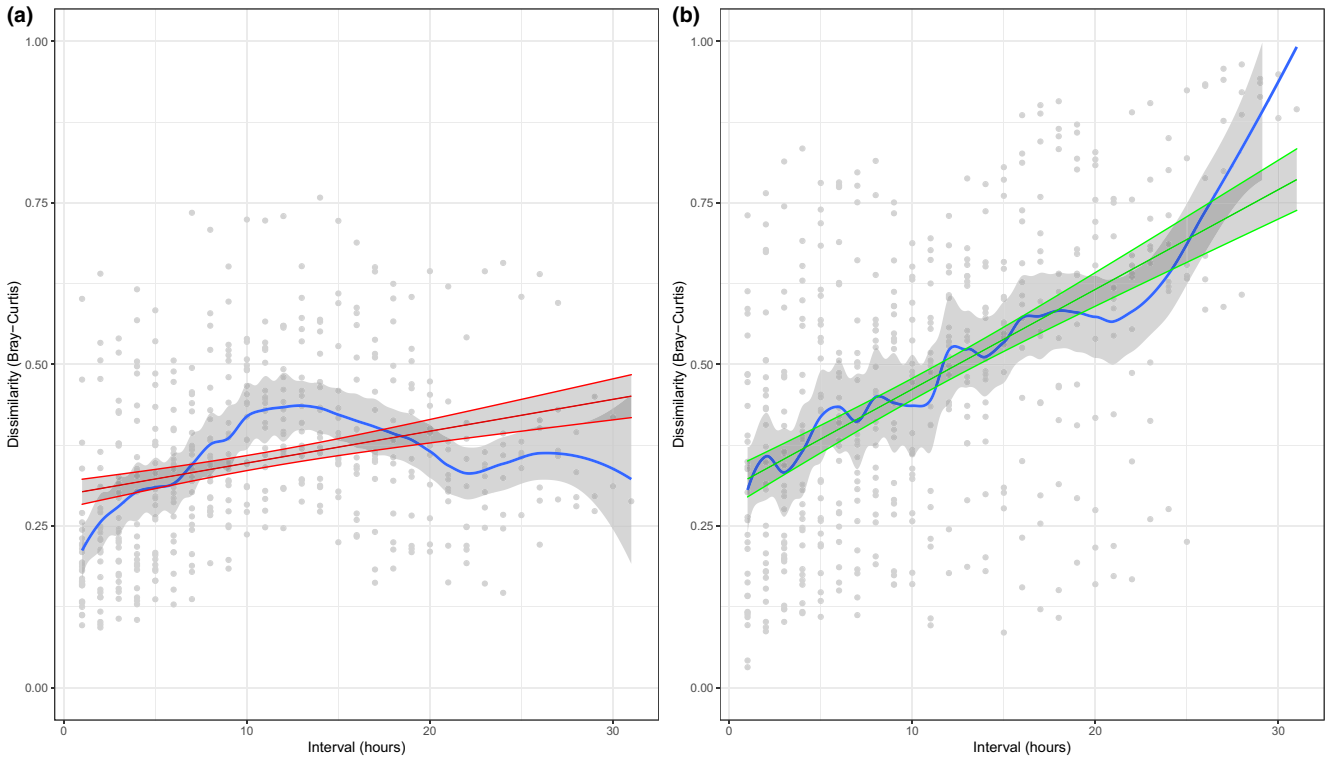

FIGURE 8 Scatterplots of Bray–Curtis dissimilarities between all pairs of pooled samples, plotted by their respective hourly intervals. Red and green lines depict significant linear regressions (red line: adjusted $R^2 = 0.07$ and $p < 0.001$, green line: adjusted $R^2 = 0.27$ and $p < 0.001$) with 95% confidence intervals showing increasing dissimilarity over time. Blue lines and shaded (gray) areas represent a "Loess" trend line fitted to the data points with 95% confidence intervals. (a) Fish data and (b) eukaryote data. Note the uncoupling between fish and eukaryote trend lines, indicating a diel signal in fish communities but not in the eukaryote composition. Also note the uncertainty associated with decreasing data points moving from left to right

in the meantime (including the reference sequences from this study). The MiFish primers also enabled better taxonomic resolution for Gadidae and Pleuronectidae, which alone accounted for six of the additional species detected compared to the findings from Sigsgaard, Nielsen, Carl et al. (2017). However, we note that the MiFish primers are not ideal with regard to the fish family Syngnathidae, for which primer bias is known (Nester et al., 2020).

Finding 41 different species could point to short-term eDNA sampling being adequate for broad scale coverage of the fish diversity from a specific coastal site in Denmark, but some of the species detected here have not previously been documented at this site. We discuss these findings in detail in Text E, Supp. Info. Although all detected species occur in Denmark and most of them have been observed at the sampling site, DNA input from both deeper waters and potentially river runoff from Mølleåen may explain the additional species. Aquatic eDNA samples can contain eDNA shed from species that are absent from the immediate surroundings, but which inhabit areas in close proximity to the sampled location (Carraro et al., 2020; Jensen et al., 2021; Sigsgaard et al., 2020). When attempting to infer exact species richness at a single site, this issue is exacerbated during short-term sampling, resulting in more rare species detections as well as species from surrounding areas. Another inherent risk is the possibility of detecting eDNA from species not present at the sampling location, but originating from secondary predation by either fishes or birds in their waste products or food droppings (Guilfoyle & Schultz, 2017; Jerde, 2021). Regardless, we observed a high detection of species that are known to regularly inhabit these waters. When sampling extensively at a single site, as done here, it appears as if a few samples are sufficient for obtaining eDNA from the most abundant fish species (Figure 2c, Figure S6), and we thus recommend that sampling intensity should depend on the desired coverage of the rarer species at the site.

Species richness was highest during dawn hours (06.00–07.00 h), and there seemed to be increased activity during the dusk hours (19.00–21.00 h), when greater species richness was detected compared to the preceding hour (Figure 2a, Table S2). This may indicate that the nocturnal and diurnal species overlap in activity in shallow waters during dusk and dawn. Dusk and dawn are often preferred times for recreational fishing, and the greater species richness may be explained by predatory species searching for food and prey species trying to hide, which the rocky sampling site is well-suited for.

The quantitative data revealed *P. flavescens*, *C. rupestris*, and *G. aculeatus* as the three species exhibiting the highest relative abundances, in accordance with previous snorkeling surveys (Holm-Hansen et al., 2019). We detected a clear compositional dissimilarity pattern between samples, indicating that species composition differs especially between night and day (Figure 4a). The quantitative data (Figure 5a) revealed some interesting tendencies regarding

diel variation, which to a large degree reflect the individual species' biology (see, e.g., www.fiskeatlas.dk/artstekster for the biology of marine Danish fishes). Holm-Hansen et al. (2019) reported that *P. flavescens*, *C. rupestris*, and *S. melops* were all more abundant during the daytime. This trend is clearly visible in our data for *C. rupestris* and *S. melops*, but less so for *P. flavescens*. *Pomatoschistus flavescens* appeared relatively stable in its eDNA contribution throughout the 32 h of sampling, which could indicate that these may not be leaving the coastal area during the night, but perhaps instead hide in nearshore rocky habitats for protection. Holm-Hansen et al. (2019) also reported that *A. anguilla*, *C. harengus*, *Gadus morhua*, *Myoxocephalus scorpius*, and *Taurulus bubalis* were more frequently encountered at night. The levels of eDNA detected from *A. anguilla*, *G. morhua*, and *M. scorpius* reflected this nocturnal trend, whereas *T. bubalis* started to become more abundant around 01.00 h and onwards, peaking at 08.00 h. The eDNA signal from *C. harengus* appeared to be more sporadic, with its main abundance around dusk (06.00–07.00 h). Ammodytidae was most abundant during the night and *P. virens* peaked during dusk and dawn hours, when juveniles are known to come to shore during the autumn. *Gasterosteus aculeatus* peaked at 07.00 h and 13.00 h (day two), when rises in abundance accounted for >25% and >50% of the relative frequencies, respectively (Figure 3a). Given the schooling behavior of this species, this marked difference may be due to schools passing by during those hours. The abundance signals from the eDNA data reflected known diel variation in the fish community. Our eDNA profiles showed that fish communities grouped according to both time of day and day of sampling, which was more pronounced in the abundance data (Figure 7a,b). We interpret this as a true biological signal, reflecting diel variation in fish species behavior (Figure 8a) and stochasticity of species occurrence between days.

4.2 | Eukaryote dataset

Although the fish diversity at Skovshoved is well-studied, there is little information on the short-term dynamics of broader eukaryote community compositions, although these are increasingly targeted in eDNA studies (e.g., Holman et al., 2019; Kelly et al., 2017; Stat et al., 2017). We detected 88 eukaryote classes (including individual samples), which overall represented a broad diversity of metazoans, algae, protists, and fungi. Our quantitative data revealed a progressive dominance of the crustacean class Hexanauplia with other groups such as Hydrozoa, Dinophyceae, Cryptophyceae, and Mamiellophyceae also being relatively abundant. Although we limited this taxonomic resolution to class level, Hexanauplia likely consists primarily of DNA from copepods in the water masses and barnacles attached to substrates around the harbor. Ostracoda (seed shrimps), Echinoidea (sea urchins), and Ophiuroidea (brittle stars) were detected sporadically until 03.00 h, after which their signals disappeared, perhaps because of the progressive onset of Hexanauplia dominance in the water masses (which might have dominated the templates for PCR and sequencing). Similar to the fish data, taking just a few samples appeared sufficient to characterize the dominant eukaryote classes at the site (Figure S6B, Table S3), supported by the fact that 28 classes were detected in all 32 pooled samples.

Although we applied a standardized experimental setup, which should enable direct comparison among samples, we caution that quantitative inference with the 18S primer set is not necessarily robust. This was evident in our comparison between individual and pooled samples (Figure S5B), in which the pooled sample from 12.00 h deviated from the average of the individual samples. This can be explained by stochasticity in individual PCR reactions, given the highly complex sample composition. It may also be explained by differences in target locus copy numbers among eukaryote classes (Krehenwinkel et al., 2017) or by primer bias (Polz & Cavanaugh, 1998). However, target locus copy number and primer bias should remain consistent across sampling times and are unlikely to skew the relative contribution of detected classes. It is also possible that our filtering of reads had uneven impact on sample compositions, particularly as we required a 98% similarity score for an ASV to be retained, which caused ~75% of the ASVs to be filtered out. This points to a deficiency in GenBank reference sequences, which is to be expected when working broadly with eukaryote groups, as, for example, only ~1% of marine fungi are thought to have been identified (Gladfelter et al., 2019). Populating the reference database would aid this issue for future studies and make quantitative comparisons more trustworthy. We urge the continuous taxonomic identification, description, and barcoding of microeukaryotes as a priority.

We observed a dynamic taxonomic composition, where the only samples without high dissimilarity were those having extreme dominance of Hexanauplia (from 02.00 h onwards). We find it likely that the predominantly planktonic life mode of the broadly targeted eukaryotes represents a more dynamic and unpredictable community than the fish. The dark hours appeared to be a transition between days for the eukaryote community, rather than being an actual diel variation signal as seen in the fish data (i.e., the difference in transitional direction between daylight groupings in Figure 7c,d compared to 7a,b). We did not find evidence of the eukaryote community composition returning to a previous state (Figure 8b). However, with the ongoing development of primer sets having higher taxonomic resolution while targeting eukaryotes broadly (Wangensteen et al., 2018), or through the usage of multiple parallel primer sets targeting various eukaryotic groups, future studies might be able to identify other eukaryote taxa exhibiting diel variation.

4.3 | Implications for future eDNA monitoring and study design

Our results indicated that a single sample taken at any given time is not sufficient to represent the diversity at a coastal sampling site. However, the remarkable repeatability in detecting the dominant taxa for both datasets indicates that taking a few samples is sufficient for characterizing the prevalent taxa at each site. Nearly all eDNA

studies have focused on multiple sample sites, in order to monitor the diversity across a geographical area. However, significant biases can occur if sampling is carried out at different times during the day across sites. This is particularly true if taxa with known diel variation in behavior are present. Short-term replication appears important for elucidating the complete contemporary diversity at a coastal site, but requires many hours in the field and many more samples to be processed. However, with the advent of automated eDNA samplers (Hansen et al., 2020), the field work could be significantly reduced in the future. Furthermore, temporal differences in sampling across large spatial scales could be eliminated by involving citizen scientists (Agersnap et al., 2022).

Environmental DNA is at present commonly used for monitoring elusive (Boussarie et al., 2018; Mauvisseau et al., 2017), cryptic (Agersnap et al., 2017; Port et al., 2016), rare (Sigsgaard, Nielsen, Bach et al., 2017; Weltz et al., 2017), endangered (Sigsgaard et al., 2015), and invasive species (e.g., Miralles et al., 2016; von Ammon et al., 2019; Wood et al., 2017) in aquatic environments. We argue that eDNA sampling for such monitoring should carefully consider the bias associated with short-term temporal variation, at least when targeting taxa that show short-term temporal variation and in places with large tidal changes (Kelly et al., 2018). Kelly et al. (2018) investigated tidal eukaryotic community changes using a replicated eDNA setup with four samplings per site across 28 h and primarily ascribed the changes in community composition to changes in physiochemical water mass characteristics, and not the tidal action per se. However, the studied taxa were primarily planktonic eukaryotes, which would be expected to drift with water. We detected an uncoupling between the fish data and eukaryote data, which could reflect that fish species actively move around according to species-specific diel cycles, whereas less motile eukaryotes may simply fluctuate in abundance depending on water movement. Naturally, there can also be very different behavioral patterns within and among eukaryote classes, meriting improved taxonomic resolution.

Our data indicate a highly dynamic community rather than a static pool of eDNA over the 32 h of sampling. This interpretation warrants additional investigation to provide insight into short-term repeatability of eDNA-detected communities, especially as we only included one night of sampling. In hindsight, it would have been extremely beneficial to sample throughout at least one additional night, to investigate a potential cyclic component between night and day, as seen in seasonal eDNA sampling (Sigsgaard, Nielsen, Carl et al., 2017), and as suggested in our fish data (Figure 8a). Future studies should focus on how many consecutive days are required at a sampling site to elucidate when and if cycles of diversity profiles occur, and whether nearby locations display similar patterns. Sampling over multiple nearby locations might reveal cyclic diversity profiles being repeated across time and space. It would also be interesting to test a similar setup at high diversity locations such as coral reefs or mangroves, which may exhibit more diverging eDNA profiles over time.

The compositional changes discerned in short-term eDNA sampling calls for critical thinking of study design. Broad-scale inferences based on eDNA are frequently carried out without attention to sampling time and short-term variation in the species dynamics of the water column. Our study showed that median individual sample dissimilarity was consistently lower than median dissimilarities between pooled samples throughout the 32 h of sampling, using both Whittaker and Bray–Curtis dissimilarities (Figure S13–S14), although our setup was not ideal for testing this statistically. Also, median dissimilarities were lower when comparing within-hour versus between-hour individual samples using Whittaker dissimilarity (Figure S15), and particularly so when using Bray–Curtis dissimilarity (Figure 3). This suggests that for aquatic eDNA studies, stochastic variation across samples is less of a concern and focus should be placed on the dynamics of the species composition targeted.

Few eDNA studies report the exact time of sampling. At least for coastal biodiversity studies, this represents a bias, as diel and day-to-day variation is evidently reflected in eDNA samples. However, it also represents a unique opportunity, as such short-term temporal change would be difficult to acquire data for using traditional fishing methods, such as gill nets, traps, and trawling. In conclusion, we advocate that short-term replication and diel variation in species behavior should be carefully considered in aquatic biomonitoring, especially when large-scale inferences are made from eDNA sampling.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

PFT and PRM conceived the ideas for the project. HC, EES, PFT, PRM, SA, SWK, WBL, MES, and YX carried out sampling in Skovshoved Harbour. MAK, HC, and PRM contributed tissue samples for the reference database. MRJ performed the laboratory work. MRJ analyzed the data with input from MDPA and PFT. MRJ led the manuscript writing with assistance and input from all authors. All authors contributed to the manuscript drafts and approved the final version.

DATA AVAILABLITITY STATEMENT

The raw sequencing data and tag files for demultiplexing are available on Dryad. The data can be accessed via https://doi.org/10.5061/dryad.q2bvq83kx. Any enquiries should be directed to the corresponding author.

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