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Monthly eDNA Metabarcoding Data From the Coastal North Sea Area Align With Fish Species-Specific Life Cycle Events

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ABSTRACT

Coastal environments serve as essential nursing, feeding, and spawning grounds for commercially and ecologically important fish species, some of which use nearshore habitats as transitional steps in their ontogenetic migration. Understanding fish communities' spatial and temporal dynamics in coastal habitats is fundamental for sustainable ecosystems and fisheries management. Despite the importance of long-term monitoring to obtain information on fish movements and distribution, fine-scale temporal datasets on fish communities remain scarce due to the intense field work required. In the present study, we explored the use of eDNA 12S metabarcoding of seawater samples to monitor fine-scale temporal and spatial patterns in fish communities. In total, 168 samples were collected across 20 sampling campaigns conducted monthly between August 2021 and August 2023 within the 12 nautical miles of the Belgian part of the North Sea. eDNA patterns revealed no marked temporal or spatial patterns at the community levels due to the ubiquitous presence of the dominant Southern North Sea fish species linked to their non-migratory behavior and use of the coastal Belgian waters as nursing and spawning grounds. However, species-specific temporal patterns reflected their reproductive activity and seasonal migrations. Additionally, fish species spatial distribution was consistent with previous beam trawl and eDNA-based surveys conducted within the Belgian part of the North Sea and was mainly driven by the environmental gradient created by freshwater discharge from the Scheldt estuary. Our findings demonstrate that eDNA metabarcoding is a valuable biomonitoring tool and provides insight into fish distribution, migration, and reproductive activity.

1 | Introduction

At the interface between onshore and offshore ecosystems, coastal environments sustain valuable ecosystem services and fishing grounds (Costanza et al. 1996; Knip et al. 2010; Barbier et al. 2011). Each coastal habitat is characterized by its unique substrate composition, hydrodynamic system, and structural characteristics (Koch 2001; Henseler et al. 2019) and the associated species assemblages (Hewitt et al. 2008; Yeager et al. 2011; Henseler et al. 2019). Nearshore environments are essential nursing, feeding, and spawning areas for commercially and ecologically important fish species that rely on such habitats to fulfill their lifecycle (Seitz et al. 2014; Guerreiro et al. 2021). Some species only seasonally use coastal areas as transitional steps in their ontogenetic migration (Seitz et al. 2014; Secor 2015; Perry et al. 2018). Under climate change and intensive fishing pressure, environmental conditions, and fish communities' seasonal migration and dynamics are shifting (Perry et al. 2005;

Engelhard et al. 2014; Donelson et al. 2019). As population centers move, fisheries will need to adapt (Ojea et al. 2020).

Such changes highlight the importance of building knowledge of fish communities' spatial and temporal dynamics in coastal habitats for sustainable ecosystem and fisheries management (Menge and Olson 1990; Stoeckle et al. 2021). Long-term monitoring is needed to understand fish communities' complex dynamics (Berry et al. 2019; Jensen et al. 2022) and obtain information on species (co-)occurrence, movements, and distribution (Sildever et al. 2023). Moreover, achieving fine-scale temporal resolution provides information on the timing of fish lifecycle key moments such as their arrival at feeding, nursing, or spawning grounds. Acquiring and analyzing such data is labor-intensive and time-consuming, highlighting the need to turn towards cost-effective and time-efficient monitoring methods. In addition, traditional catch-based surveys disturb benthic habitats (Depestele et al. 2016) and biological communities by removing fish from the environment (Hilborn et al. 2023). Environmental DNA (eDNA) is a non-invasive sampling method that can provide biodiversity information beyond the capacity of traditional sampling methods (Thomsen and Willerslev 2015; Gilbey et al. 2021).

eDNA has successfully described the spatial and temporal distribution of fish communities in the marine environment (Jeunen et al. 2018; Berry et al. 2019; West et al. 2020) and reflected seasonal turnover in species composition (Stoeckle et al. 2021; Sigsgaard et al. 2017; Cananzi et al. 2022). Even in dynamic nearshore environments where tides drive water movements, the tidal cycle had little influence on eDNA dispersion (Kelly et al. 2018). eDNA is spatially and temporally organized in the marine environment, and the general assumption is that water sampling of eDNA reconstitutes a contemporary picture of fish assemblages and diversity at the sampling location. However, many factors influence eDNA detection probability, and these change seasonally (Troth et al. 2021). The variability in eDNA degradation rates is driven by abiotic and biotic factors fluctuating throughout the year, such as microbial activity, salinity, temperature, and UV light (Collins et al. 2018; Goldberg et al. 2018).

The Belgian part of the North Sea (BPNS) is a shallow and well-mixed environment located in the Southern North Sea characterized by geomorphological, chemical, and physical gradients that shape its ecology. The on-to-offshore gradient results from the influence of river freshwater discharge nearshore and the input of Atlantic water through the English Channel offshore (Lacroix et al. 2004). A strip of low salinity water extends along the Belgian coast, with reducing width towards the Western side (Lacroix et al. 2004). The eastern coastal area is strongly impacted by freshwater discharges, creating an east-to-west salinity gradient. Salinity is a structuring factor of fish communities in coastal areas, as differences in salinity create an ecological barrier for freshwater and marine species distribution (Breine et al. 2011; Koehler et al. 2022). Additionally, a high sedimentology diversity characterizes the bottom of the BPNS: the eastern part of the BPNS is characterized by muddy sediments, and the medium grain size increases until coarse sand towards the West and the 12 nautical miles (NM) limit (Verfaillie et al. 2006). Accordingly, morphology-based surveys have identified a clear horizontal pattern with distinct fish communities nearshore compared to offshore waters over the wider North Sea (Ehrich et al. 2009) and within the BPNS (De Backer et al. 2010, 2022). A transitional community around the 12 NM limit is composed of a mixture of coastal and offshore typical species. Further subdivisions within the coastal fish community with distinct assemblages over the eastern muddy and sandy sediments following epibenthos and macrobenthos distribution were observed (De Backer et al. 2010, 2022). eDNA-based surveys conducted within the BPNS in autumn 2021 (Cornelis et al. 2024; Cananzi et al. 2022; Dukan et al. 2024) revealed spatial patterns of fish communities aligned with beam trawl data. Furthermore, eDNA outperformed beam trawl for uncovering coastal fish diversity, detecting more species, consistent with other marine eDNA-based surveys (e.g., Thomsen et al. 2012; Liu et al. 2022; Veron et al. 2023).

Many species undergo seasonal migration towards nursing, feeding, and spawning grounds or away from unfavorable environmental conditions (Perry et al. 2018). The spatial extent of these migrations depends on the species mobility (Neumann et al. 2013): pelagic species often migrate long distances, while many benthic species only migrate locally (Daan et al. 1990). As such, fish assemblages vary throughout the seasons, particularly in temperate coastal waters (Franco et al. 2006; Llompart et al. 2013; Perry et al. 2018). Overall, fine-scale temporal eDNA datasets remain rare. Few studies have compiled long-term series with weekly to monthly sampling, and their spatial range was limited to one or two sampling sites (Sigsgaard et al. 2017; Stoeckle et al. 2017; Berry et al. 2019; Cananzi et al. 2022; Rivera et al. 2023; Sildever et al. 2023). Within the BPNS, seasonal changes in fish assemblages remain understudied despite the seasonality of structuring environmental factors such as temperature and light (Otto et al. 1990; Sündermann and Pohlmann 2011).

Here, we monitored monthly changes in fish community structure within the 12 NM limit of the BPNS with eDNA metabarcoding. We first explored the eDNA abundance of fish across 20 sampling campaigns conducted from 2021 until 2023 at nine fixed stations. Monthly and seasonal changes were investigated at the community level and for the most abundant fish. We hypothesize that the temporal eDNA metabarcoding patterns will reflect the migratory behavior of commonly found North Sea fishes. Secondly, we investigated spatial eDNA patterns along the nearshore/offshore gradient and the east/west gradient within the 12 NM area of the BPNS. We hypothesize that the different sedimentological characteristics and the associated shifts in benthic fauna may alter fish distribution and associated eDNA patterns in this small area. Understanding fine-scale spatial and temporal eDNA patterns provides valuable information on the accuracy of eDNA for describing fish distribution patterns and is essential for the shift towards non-invasive and cost-effective sampling for fish biodiversity monitoring.

2 | Materials and Methods

2.1 | Study Area

The BPNS, covering 3.454 km^2, is located in the Southern Bight of the North Sea (Belpaeme et al. 2011). It is characterized by shallow depth, with an average depth of 20 m and reaching a maximum depth of 45 m towards the northwest (Ruddick and Lacroix 2006).

The bathymetry features multiple sandbank systems which, together with the influence of the Scheldt estuary, create high sedimentological diversity and complex topography. The eastern coastal zone of the BPNS, near the Scheldt estuary, displays a high mud content, whereas the western coastal zone is characterized by fine sand. Fine sand sediment also occurs more offshore on the eastern side. The grain size increases to medium and coarse sand further offshore (Figure 1; Verfaillie et al. 2006). Macrobenthic, epibenthic, and demersal fish assemblages are strongly associated with sediment types and topography and show similar distribution patterns to each other (Van Hoey et al. 2004; Degraer et al. 2008; De Backer et al. 2010, 2022; Breine et al. 2018).

2.2 | Water Sampling

Water samples were collected monthly within the 12 NM limit of the BPNS between August 2021 and August 2023 as a part of the LifeWatch observatory (https://www.lifewatch.be/). Due to bad weather conditions, no sampling campaigns took place in January, February, May, June 2022, and March 2023. Nine fixed stations cover the near- and mid-shore zones up to 12 NM of the BPNS (Figure 1; Table 1). Samples were taken roughly two meters above the seafloor with Niskin bottles (5 L) mounted on a Conductivity, Temperature, and Depth (CTD) frame. Sub-samples of 2 L were taken and pre-filtered (200 μm mesh nylon filter) from the Niskin bottles into sealed commercial drinking water bottles that were opened just prior to the deployment of the rosette. The Niskin bottles were rinsed with 2 L commercial drinking water before deployment in the next location to reduce potential field contamination between stations. Additionally, the rosette was submerged underneath the water surface for 3 min to flush the bottles with local water at each station. No Niskin negative controls were collected since previous analyses in the same geographical area with the same 3 min subsurface submersion indicated that the carry-over eDNA signal between locations is limited for metabarcoding analyses (Cornelis et al. 2024). In addition, no beam trawling or other fish handling procedures were conducted during these campaigns, further minimizing the risk of fish DNA contaminants in the bottles during eDNA collection. All water samples were stored at −20°C for 2 to ~200 days until further processing. Temperature, salinity, suspended particulate matter (SPM), and nutrient concentrations (ammonium, nitrites, nitrates, phosphates, and silicates) were measured at every time point following the methodology described in Mortelmans et al. (2019) and downloaded from the LifeWatch observatory website (https://rshiny.vsc.lifewatch.be/station-data/; Flanders Marine Institute (VLIZ) 2024). No SPM data were collected between September 2022 and August 2023.

2.3 | eDNA Filtration and Extraction

Sample filtration was performed in a designated laboratory in the ILVO research institute where no other analyses were conducted at the time. To prevent cross-contamination between samples, the tubes of the Masterflex pump were filled with 10% bleach for 1 min and then flushed with 125 mL commercial source water. Each sample was filtered over a 0.45 μm Sterivex-HV Filter (polyvinylidene fluoride (PVDF), with Luer Outlet, Merck—Millipore) using a Masterflex pump (Masterflex

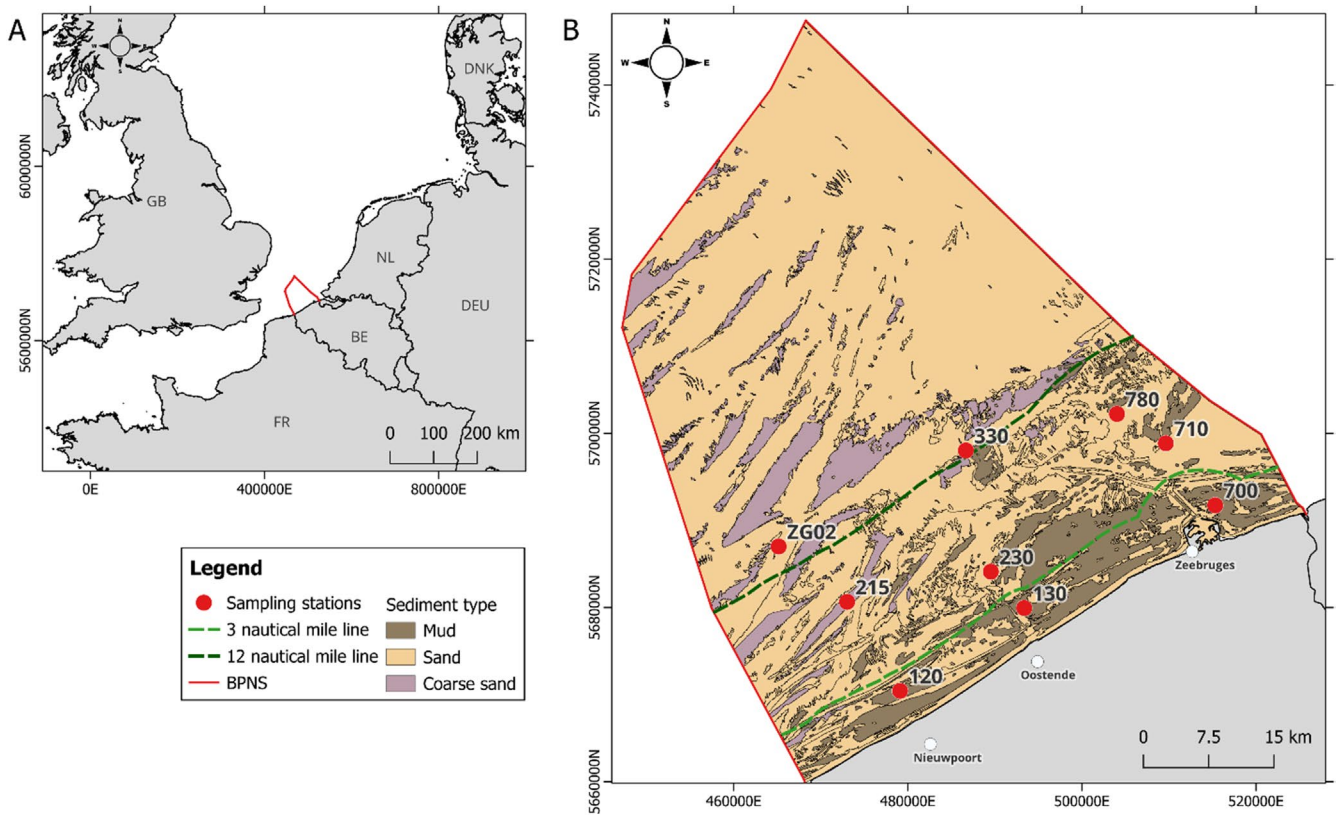

FIGURE 1 | Map of (A) the location of the Belgian Part of the North Sea within the North Sea and (B) the study area. Sampling stations are represented by red dots. The Belgian part of the North Sea is delimited by the red line, the 3 and 12 nautical mile limit by the light and dark green line, respectively. The background is colored according to sediment type.

TABLE 1 | Overview of the sampling stations, including their coordinates, their cardinal position, the distance to shore, the sediment type (Verfaillie et al. 2006), number of timepoints sampled, mean salinity (in practical salinity unit (PSU)), and mean suspended particulate matter (SPM; in mg/L) across all time points for each station.

Station name	Coordinates		Position	Distance to shore	Sediment type	Number of timepoints sampled	Mean salinity (PSU)	Mean SPM (mg/L)
	Latitude	Longitude						
120	51° 11.10′ N	2° 42.07′ E	West	Nearshore	Fine sand	15	33.20	35.94
215	51° 16.60′ N	2° 36.80′ E	West	Mid-shore	Medium/ coarse sand	16	33.40	13.79
ZG02	51° 20.00′ N	2° 30.00′ E	West	Offshore	Medium/ coarse sand	16	33.92	13.4
130	51° 16.25′ N	2° 54.30′ E	Middle	Nearshore	Mud	19	32.08	66.41
230	51° 18.50′ N	2° 51.00′ E	Middle	Mid-shore	Mud	18	32.32	31.10
330	51° 26.00′ N	2° 48.50′ E	Middle	Offshore	Medium/ coarse sand	19	33.75	15.90
700	51° 22.60′ N	3° 13.20′ E	East	Nearshore	Mud	19	30.84	74.11
710	51° 26.45′ N	3° 8.32′ E	East	Mid-shore	Mud	18	32.16	62.44
780	51° 28.27′ N	3° 3.48′ E	East	Offshore	Fine sand	18	32.53	26.36

L/S Variable Speed Pump HV-77910-75), at speed four, until the filter was nearly clogged. All Sterivex filters were sealed with a sterile Luer-lock cap and stored at −20°C for 5 to ~520 days until further processing.

eDNA extraction was performed under a laminar flow cabinet in a PCR-free designated room. Before and after use, a UV light was turned on for 15 min, and all working surfaces were cleaned with 10% bleach followed by 70% ethanol. The eDNA extraction protocol followed a modified version of the Qiagen DNeasy Blood & Tissue kit protocol (Spens et al. 2017). Lysis buffer (800 μL), composed of ATL-buffer (718 μL; Qiagen), Internal Positive Control (IPC; 2 μL (30,000 copies/μL); gBlocks Fragments, Integrated DNA Technologies), and proteinase K (80 μL; 20 mg/mL; Qiagen), was added to each Sterivex filter. Filters were then incubated overnight at 56°C in a rotating incubator (Incubator-Genie, Scientific Industries). After incubation, the lysis buffer was transferred into a 5.0 mL Lobind microtube, and DNA was extracted following the manufacturer's protocol. After washing the extracts, eDNA was eluted in 100 μL TE 1× buffer that had been incubated at 70°C for 10 min. A DNA extraction negative control was included by applying the same protocol to a clean Sterivex filter for samples from batch 2. All eDNA extracts were stored at −20°C for 8 to ~200 days until further processing.

2.4 | Library Preparation and Sequencing

A total of 168 samples, including field samples and 10 negative controls (nine PCR negatives and one extraction negative) were spread over three sequencing runs. All libraries were prepared identically in separate batches (Table 2). Batches 1 and 2 were spread across two Novaseq sequencing runs alongside samples unrelated to the LifeWatch campaigns. Batch 3 consisted of re-sequencing of libraries previously sequenced on a Miseq run to ensure sequencing technology consistency across batches. Additionally, to evaluate the effect of different sequencing runs, six samples from batch 1 and six from batch 2 were re-sequenced with batch 3 on the third sequencing run.

The PCR amplification targeted a 163–185 bp hypervariable region of the mitochondrial 12S ribosomal DNA using MiFish primers developed by Miya et al. (2015). The universal primers (MiFish_U) were degenerated to target Osteichthyes and Elasmobranchii simultaneously (MiFish_U/E_F: 5′-GT(C/T)GGTAAA(A/T)CTCGTGCCAGC-3′, MiFish_U/E_R: 5′-CATAGTGGGGTATCTAATCC(C/T)AGTTTG-3′). Both primers were tagged with a unique sequence of 6 to 10 nucleotides to provide unique barcodes for each eDNA extract. Amplification was carried out in triplicates in 25 μL total volume containing 12.5 μL KAPA HiFi Hotstart 2× ReadyMix, 0.5 μL Bovine Serum Albumin (BSA; 10 mg/μL), 1 μL of both reverse and forward tagged primers (2.5 μM), 7 μL UltraPure water, and 3 μL extracted eDNA. Negative PCR controls (four for batch 1, five for batch 2; four negative controls were added to the Miseq sequencing run, but those were not re-sequenced on the Novaseq run for batch 3 since they contained negligible amount of contamination (between 1 and 6 reads; none assigned to fish ASVs)) were included by adding 3 μL of UltraPure water instead of eDNA extract. The PCR reactions were run on a Bio-Rad T100 thermal cycler with the following cycle: 3 min at 95°C, 40 cycles of 20 s at 98°C, 15 s at 62°C, 15 s at 72°C, and ending with 5 min at 72°C. The PCR products were combined into three pools of uniquely tagged samples, taking 5 μL from each technical replicate. The pools were then purified with CleanNGS beads (CleanNA) by adding 0.8 times the volume of the pools. The pools were placed on a magnetic holder and washed twice with 60 μL ethanol (80%). Finally, the three pools were eluted with 100 μL Tris–HCL buffer (10 mM; pH 8.5) and sent to Admera Health Biopharma Services for PCR-free adaptor ligation followed by sequencing of the three libraries on a Novaseq 2 × 150 bp paired-end run with 15% PhiX.

TABLE 2 | Overview of the three batches, including samples origin (Year and Month), number of LifeWatch samples, and sequence analysis parameters (truncation and decontamination).

Batch number	Campaigns		Number of lifewatch samples	Sequence analysis	
	Year	Months		Truncation	Decontamination
1	2022	April, March, November, December	34	/	microDecon (default parameters)
2	2021	August to December	99	(105:115)	/ (low level of contamination)
	2023	January, February, April to August			
3	2022	July to October	35 47	/	/
	Six samples from RUN1 Six samples from RUN2		12	/	/

2.5 | Bioinformatic Analyses

Sequences from all runs were processed identically, except for the truncation parameters and decontamination step (Table 2). Raw Illumina Novaseq sequencing data quality was checked with FASTQC v0.11.9 (Andrews 2010), and forward and reverse reads were synchronized using Pairfq v0.17 (Staton 2022). Reads were then processed following the pipeline described in Cornelis et al. (2024), including reorientation, demultiplexing, and trimming of paired-reads with cutadapt v4.5 (Martin 2011). After reorientation, paired-reads that did not contain both the forward and reverse primers were discarded, with a maximum error rate of 15%, allowing up to three and four mismatches for the forward and reverse primers, respectively, to account for degenerated primers variability. The remaining reads were demultiplexed based on their unique sample tag with a maximum error rate of zero. Primer sequences and unique barcodes were truncated from the 5′ and 3′ ends of the raw reads with the minimum length fixed at 105. All PCR replicates were then concatenated by summation into a single folder.

Amplicon sequence variants (ASV) were generated following the dada2 workflow adapted for big datasets (https://benjjneb.github.io/dada2/bigdata_paired.html; Callahan et al. 2016) using default parameters except for the truncation of forward and reverse reads based on quality profiles (Table 2) and the error learning algorithm. Sequences from batch 2 were truncated because of a drop in quality of the forward sequences. The error learning algorithm was modified as recommended by Holland-Moritz et al. (2023); (https://github.com/ErnakovichLab/dada2_ernakovichlab) to account for Novaseq binned quality scores. The parameters of the default *errorEstimationFunction*, *loessErrfun*, were modified as follows: monocity was enforced, weight was set to the logarithm of total weight, span to 2, and degree to 1. The three runs were processed independently through the dada2 pipeline up to the chimera removal step. The sequence tables were merged to ensure consistent chimera identification across batches. Taxonomy was assigned in three steps on the merged dataset. First, taxonomy was assigned using a custom-made database (12S reference sequences of 122 North Sea fish species; available at DOI https://doi.org/10.5281/zenodo.13861942; Cornelis et al. 2024) and the naïve Bayesian RDP Classifier (minBoot = 80; Wang et al. 2007). Secondly, unassigned ASVs after the first step were run with the Basic Local Alignment Tool (BLAST), blastn v2.12.0 (Altschul et al. 1990) against the custom-made database. In the third step, unassigned ASVs were subjected to BLAST and the GenBank nt database (from August 2023; qcov_hsps_perc = 75%; perc_identity = 97%). The taxonomy for species with identical 12S sequences was set to genus level (*Alosa alosa* and *Alosa fallax, Raja brachyura, Raja montagui*, and *Raja clavata*) or family level (*Ammodydes tobianus* and *Ammodytes marinus*, *Chelidonichthys lucerna*, *Chelidonichthys cuculus*, and *Eutrigla gurnardus*). One ASV initially assigned to the genus Sardina was resolved to *Sardina pilchardus*, the sole species within this monotypic genus. Other ASVs for which taxonomy could not be determined to species level were classified to family or genus level.

2.6 | Final Filtering

The three batches were decontaminated separately, using their respective PCR and extraction negative controls as recommended for *microDecon* (McKnight et al. 2019). Contaminants in batch 1 were identified and removed using the R-package *microDecon* (*decon*; default parameters; McKnight et al. 2019) on the raw dataset. Samples were sorted into groups of technical replicates (year-month-location combination), the smallest grouping level. We initially employed the same methodology for batch 2, but the negligible contamination level observed in the negative controls introduced bias in the *microDecon* process. The only fish ASV detected was defined as the constant (i.e., an ASV that is entirely contamination and used as the basis for decontamination (McKnight et al. 2019)) and hence removed from the dataset despite its much higher reads count in field samples. Therefore, we decided to retain all ASVs for further analyses. No negative controls were included in batch 3, as it was composed of re-sequencing of libraries. However, negative controls previously sequenced on a Miseq run and corresponding to the library preparation of batch 3 were checked for potential contamination. These negatives yielded no reads for ASV assigned to fish species, ensuring no contamination occurred during library preparation of the libraries re-sequenced with batch 3.

For all samples, only ASVs detected in at least two out of three PCR replicates were kept. This strict filtering improves accuracy in species detection, even though it might remove truly present species (Ficetola et al. 2015; Alberdi et al. 2018; Jensen et al. 2022). We retained only ASVs assigned to the Pisces taxonomic level, and as a final filtering step, ASVs assigned to exotic or freshwater species were removed from the dataset. A Sankey plot illustrating the flow of paired-reads through the bioinformatics processing pipeline is presented in Figure S2; an overview of the number of paired-reads at each step is in Table S2.

2.7 | Ecological and Statistical Analyses

All analyses were performed using R Statistical Software (R Core Team 2021), and all plots were created using R-package *ggplot2* (Wickham 2016).

The eDNA read counts were normalized to account for differences in sequencing depth: the concatenated, unrarefied, and cleaned dataset was double transformed using the R-package *vegan* (*decostand*; Oksanen et al. 2022). First, read counts were converted to relative abundance in each sample (method = total). Then, the relative abundance of each species is scaled to the highest observed relative abundance across all samples (method = max) (Kelly et al. 2019). The normalized read counts are further referred to as the "eDNA Index", an index of read-count proportions scaled from 0 to 1 (Kelly et al. 2019).

To test for a sequencing effect, we compared fish community composition between the same libraries sequenced on different runs. The double-transformed dataset was used to perform Non-Metric Multidimensional Scaling (NMDS) and investigate if the libraries clustered based on run number or samples. We performed a Permutational Multivariate Analysis of Variance (PERMANOVA) with the R-package *vegan* (*adonis*; factor = Run Number, permutation = 9999; Oksanen et al. 2022) to test for significant differences in species composition between runs.

Before ecological analyses, re-sequenced libraries and samples that yielded no reads were removed. All analyses were performed at the species level on the concatenated, double-transformed dataset. We calculated the Bray–Curtis dissimilarity scores (abundance; Bray and Curtis 1957) and the Whittaker dissimilarity scores (presence/absence; Whittaker 1960), which served as input for NMDS plots using the R package *vegan* (*vegdist* and *metaMDS*; Oksanen et al. 2022).

Seasonality was explored in terms of compositional dissimilarity (Bray–Curtis) and species turnover (Whittaker). The species composition differences were tested with PERMANOVA for three grouping variables: Timepoints (levels: 2021_August, 2021_September, 2021_October 2021_November, 2021_December, 2022_March, 2022_April, 2022_July, 2022_August, 2022_September, 2022_October, 2022_November, 2022_December, 2023_January, 2023_February, 2023_April, 2023_May, 2023_June, 2023_July, 2023_August), Seasons (levels: Summer, Autumn, Winter, Spring), and Temperature categories (levels: warm, mild warm, mild cold, and cold). Each Timepoint corresponds to a sampling campaign and is referred to as a month-year combination. Samples were grouped into seasons according to the Northern Hemisphere calendar and into temperature categories according to records of seasonal sea surface mean temperature. The following temperature categories were defined: warm (> 17°C), mild warm (> 12.5°C), mild cold (> 9°C), and cold months (< 9°C) (Figure S2). Each grouping variable was tested using a three-factor PERMANOVA, where Year and Station were included as factors to account for yearly and spatial variation, respectively. To assess the reliability of the statistical significance obtained with PERMANOVA, we performed an analysis of multivariate homogeneity of group dispersions using the R-package *vegan* (*betadisper*, type = centroid, Oksanen et al. 2022). Grouping variables with homogeneous dispersion were then further tested with a pairwise PERMANOVA (*pairwise.adonis2*; Martinez Arbizu 2024) to identify levels of significant difference ($p < 0.05$). Similarity Percentage Analysis (SIMPER; Clarke 1993) was performed using the R-package vegan (*simper*; Oksanen et al. 2022) to identify species contributing the most to pairwise dissimilarities. From the results, species with an average contribution to the dissimilarity between groups higher than 5% were selected for ecological inference. Lastly, we performed an Indicator Species Analysis with the R-package *indicspecies* (*multipath*, during = TRUE; Cáceres and Legendre 2009) to evaluate species association with Season and Temperature categories group levels. The Whittaker Index was used to examine species turnover between timepoints throughout the time series, using the first samples (August 2021) as a reference for each station.

We explored spatial differences in species composition between Stations (levels: 120, 215, ZG02, 130, 230, 330, 700, 710 and 780), in relation to the East–west gradient (levels: east, middle, and west) and Distance to shore (levels: near-, mid-, and offshore). The differences in species composition were tested with one-way PERMANOVA for each factor. Similarly to temporal analysis, we performed an analysis of multivariate homogeneity of group dispersions, followed by a pairwise PERMANOVA for factors with homogenous group dispersions. The analyses were followed by SIMPER and indicator species analyses, and similar criteria were used for ecological inference. Additionally, we fitted the environmental variables (nutrient concentrations, SPM, salinity, and temperature) onto the NMDS using R-package *vegan* (*envfit*; Oksanen et al. 2022) to identify the environmental factors influencing the spatial patterns observed between stations.

3 | Results

3.1 | Environmental Data

Seawater temperature varied seasonally, displaying higher temperatures in summer and lower temperatures in winter (Figure S2). Salinity was lower in late winter and spring compared to summer and autumn (difference of ~4 PSU; Figure S3), while SPM showed no cyclic temporal variation (Figure S4), and their mean values across all timepoints at each station are shown in Table 1. Salinity was slightly higher towards western offshore stations. Conversely, SPM values were higher towards the eastern coastal stations. Nutrient concentration varied over the whole time series and showed higher peaks towards the

eastern coastal side (Figure S5). The sum of nitrites and nitrates concentrations and silicates concentrations displayed a peak in winter each year with decreasing importance as we move towards western offshore stations.

3.2 | Sequencing Results and Taxonomic Diversity

After quality filtering and taxonomic assignment, the three sequencing runs generated 181,165,816 reads and 18,666 ASVs. The dataset was cleaned by removing reads detected as contaminants. Two out of the four PCR negatives of batch 1 yielded 76,744 reads and 5032 reads (Figure S6A). Decontamination resulted in the removal of 23 ASVs, of which 12 were assigned to fish (1,532,348 reads), 1 to humans (2,506,990), 8 were non-assigned (13,704), and 2 were other ASVs (124,400), for a total of 4,177,442 reads. Batch 2 revealed negligible contamination levels (Figure S6B). The extraction negative control produced one read, whereas the third and fifth PCR negatives produced 17 and 7 reads, respectively. The 17 reads in the third PCR control were assigned to a single ASV of *Solea solea* defined as the constant by the *microDecon* algorithm and hence completely removed. However, we decided to retain this ASV since the proportions in the field sample were much higher (up to 260,662 reads).

Filtering out ASVs occurring in only one out of the three PCR replicates for each sample resulted in the loss of 11,717,091 (~6.6%) reads and 16,108 ASVs (~86.3%), including 2417 ASVs assigned to fish. As a result, 31 fish species and one ASV assigned to Order level (Scorpaeniformes) were removed from the dataset. Among these 31 species, 10 were freshwater species (*Alburnoides bipunctatus*, *Alburnus alburnus*, *Barbatula barbatula*, *Gobio gobio*, *Leuciscus leuciscus*, *Perca fluviatilis*, *Pungitius pungitius*, *Squalius cephalus*, *Tinca tinca*, and *Vimba vimba*) and one exotic species (*Gadus chalcogrammus*). Within the remaining 20 species, seven are rarely found in coastal waters (*Cyclopterus lumpus*, *Gymnammodytes semisquamatus*, *Merluccius merluccius*, *Microstomus kitt*, *Pollachius pollachius*, *Trachinus draco*, and *Trisopterus esmarkii*). The detection of the remaining species (Alosa spp., Chelon spp., *Dasyatis pastinaca, Diplecogaster bimaculata, Gasterosteus aculeatus*, *Gobius paganellus*, *Lampetra fluviatilis*, *Lepidorhombus wiffiagonis*, *Lipophrys pholis*, *Parablennius gattorugine*, *Pollachius virens*, *Pomatoschistus microps*, and *Trisopterus minutus*) in only one PCR replicate within samples is not reliable, despite their potential presence within the BPNS. As a final filtering step, only ASVs assigned to the Pisces level were retained, and four freshwater or exotic species were removed from the dataset: *Abramis brama*, *Oncorhynchus mykiss*, *Rutilus rutilus*, and *Sander lucioperca*. The cleaned dataset contained 141,165,672 reads and 51 identified fish species (Table S2). Additionally, some ASVs could only be assigned to genus level (*Liparis* spp. and *Pomatoschistus* spp.), some only to family level (Clupeidae, Gadidae, Pleuronectidae, and Triglidae), and some to Order level (Clupeiformes, Perciformes, and Pleuronectiformes).

The sequencing of the same libraries on different sequencing runs aimed to exclude the possibility that differences between samples might be due to sequencing runs rather than inherent biological differences in species composition. The NMDS ordination revealed that libraries clustered by samples and not by sequencing runs (Figure S7). Additionally, the community composition of libraries of the same sample was almost identical (Figure S8). The PERMANOVA results ($p = 0.1523$; Table S3) confirmed no significant differences in species composition between sequencing runs. Re-sequenced samples were then removed from the dataset for further analysis. After removing the double-sequenced samples, the fish dataset contained 135,782,672 reads and 51 fish species.

3.3 | Temporal Patterns of Fish eDNA

The eDNA of nine species was detected throughout the whole time series: *Merlangius merlangus*, *Sprattus sprattus*, *Clupea harengus*, Ammodytidae spp., *Sardina pilchardus*, *Limanda limanda*, *Solea solea*, *Platichthys flesus*, and *Pleuronectes platessa* (Figure S9). Seven species were mainly detected during spring and summer each year: *Trachurus trachurus*, *Engraulis encrasicolus*, *Scomber scombrus*, *Dicentrarchus labrax*, *Pomatoschistus minutus*, *Trisopterus luscus*, and *Pomatoschistus lozanoi*. The remaining species were only picked up sporadically. Temporal patterns of the eDNA Index over the full time series were plotted for the six species (Ammodytidae spp., *Clupea harengus*, *Limanda limanda*, *Merlangius merlangus*, *Sardina pilchardus*, and *Sprattus sprattus*) with the highest average eDNA Index (Figure 2; Figure S10 for the top 30 species). The eDNA Index of Ammodytidae spp. peaked in spring and *Merlangius merlangus* (Figure 2A) peaked in both spring and autumn (Figure 2D). For *Clupea harengus*, the eDNA Index peaked during autumn, and for *Limanda limanda*, the eDNA Index peaked in autumn and winter (Figure 2B,C). *Sardina pilchardus* was a recurrent species in autumn, though eDNA Index values were lower in 2021 than in 2023, suggesting interannual variation. Additionally, *Sardina pilchardus* appeared at offshore stations in the summer of 2023 (Figure 2E). *Sprattus sprattus* was present throughout the years, with lower eDNA Index values in spring and towards mid-autumn (Figure 2F).

No clustering at the community level was observed for any of the three temporal factors. Samples did not group by timepoints (Figure 3A), by season (Figure 3B) or by temperature category (Figure 3C). Still, the PERMANOVA analysis returned significant results for all three factors (Table 3). Those results are driven by the non-homogeneity of group dispersion for timepoints ($p = 0.000225$; Figure S11) and temperature categories ($p = 0.03157$; Figure S12), whereas seasons group dispersion was homogenous ($p = 0.11$; Figure S13). The statistical differences obtained with PERMANOVA for timepoints and temperature categories, therefore, result from a dispersion effect rather than a time effect and reinforce the observation that there is no clustering at these levels. The pairwise analysis returned significant results for all levels of season (Table S4). SIMPER analysis identified the species contributing the most to differences between seasons (Table S5). Dissimilarities between seasons were mainly due to differences in the eDNA Index of common species such as *Sprattus sprattus*, *Sardina pilchardus*, *Merlangius merlangus*, *Clupea harengus*, and Ammodytidae spp., rather than the presence/absence of different species.

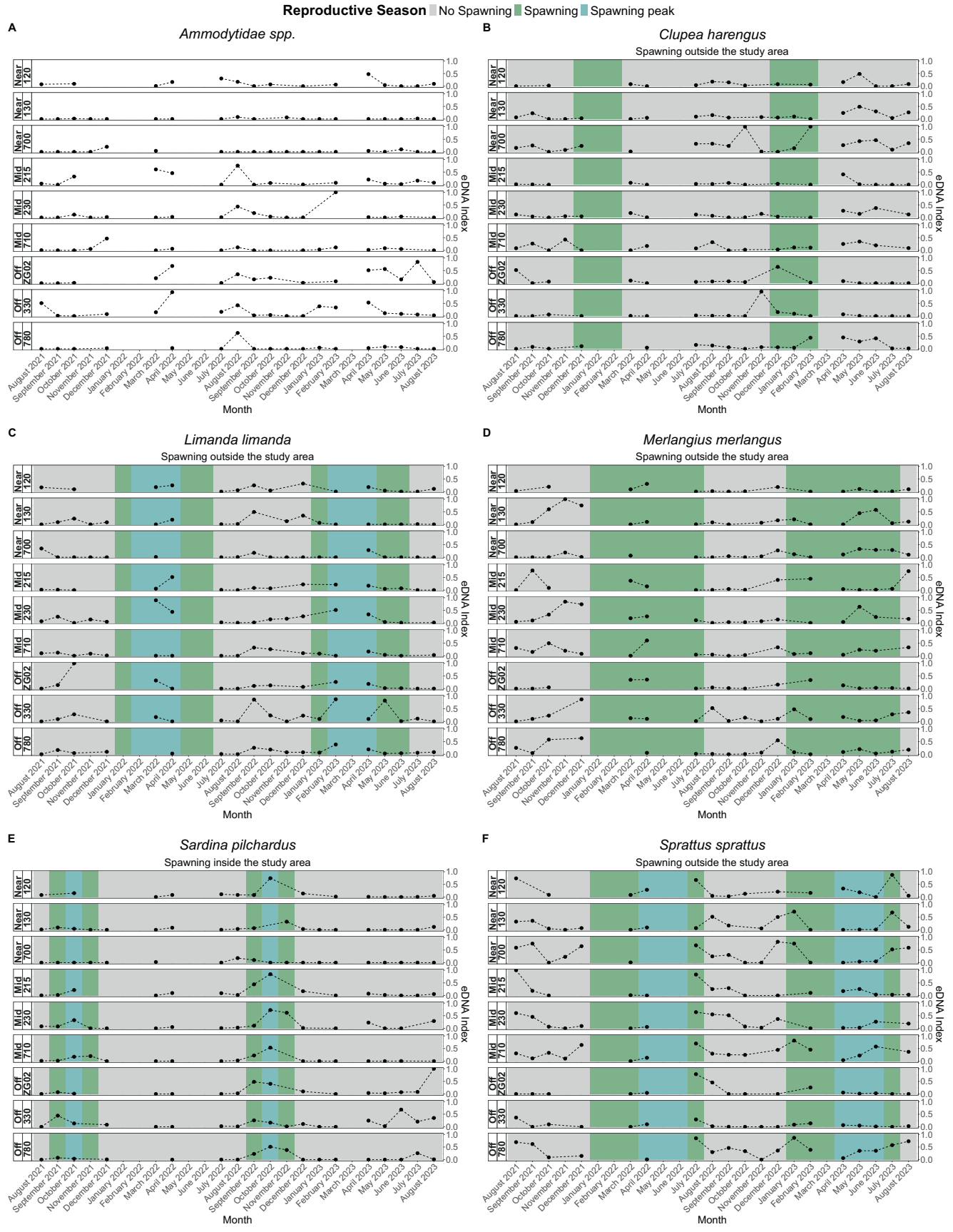

FIGURE 2 | Legend on next page.

FIGURE 2 | eDNA Index values across time points at each station throughout the full times series for the six species with the highest average eDNA Index. The species are ordered alphabetically: (A) Ammodytidae spp., (B) *Clupea harengus*, (C) *Limanda limanda*, (D) *Merlangius merlangus*, (E) *Sardina pilchardus*, and (F) *Sprattus sprattus*. The background is colored according to the reproductive season (gray = No Spawning, green = Spawning, blue = Spawning peak). Species spawning location (inside/outside the study area) is indicated under the species name. The predicted spawning periods and references used are listed in Data S1. The reproductive season was not added for families. Stations are indicated on the left, and organized from nearshore (Near) at the top to offshore (Off) at the bottom.

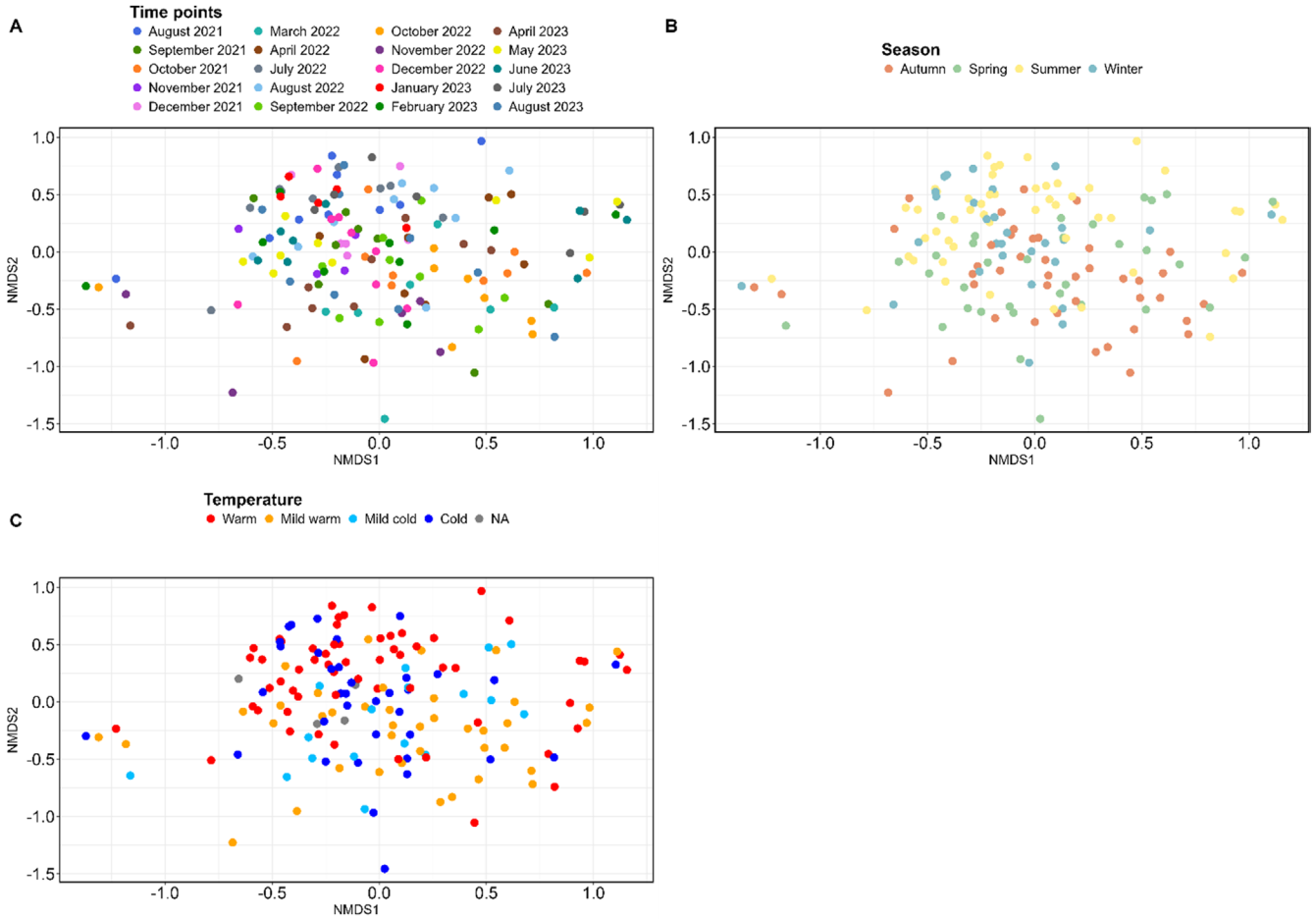

FIGURE 3 | Non-metric multidimensional scaling analysis (NMDS) computed on Bray–Curtis dissimilarity matrix of eDNA Index of fish communities. Dots are colored according to (A) Timepoints, (B) Season, and (C) Temperature categories. Stress = 0.19461; $k = 3$.

The indicator species analysis showed that *Sardina pilchardus*, *Pomatoschistus minutus, Ciliata mustela, Liparis liparis, Agonus cataphractus, and Argnoglossus laterna* were associated with autumn, and *Solea solea* and Ammodytidae spp. were associated with spring (Table S6). *Sprattus sprattus*, *Engraulis encrasicolus*, *Pomatoschistus lozanoi, Trachurus trachurus, and Buglossidium luteum* were associated with summer; whereas Clupeidae spp. was associated with winter.

The Whittaker Index, based on presence/absence, did not reveal any distinct cyclic species turnover between Timepoints (Figure 4). The high turnover observed at stations ZG02, 330, and 780 (offshore stations; Figure 4A–C) is largely influenced by the limited number of species detected in the reference samples from August 2021 (three, five and four species, respectively; Figure S9). The detection of more species at the subsequent timepoints results in high index values and blurs any potential patterns. Conversely, stations 710 and 120 (Figure 4F,G) exhibited an overall lower species turnover. At stations 230, 130, and 700 (Figure 4E,H,I), timepoints between April and August (spring and summer) showed a more similar composition to the reference sample (lower index values) whereas timepoints between September and February (autumn and winter) showed gradually increasing species turnover (higher index values).

3.4 | Spatial Patterns of Fish eDNA

No clear separation was observed between samples from near-, mid-, and offshore stations (Figure 5A) or along the east-to-west transect (Figure 5B). However, for stations, a gradient was discernible along the first axis (Figure 5C). Stations 215, 330, and ZG02 clustered toward the right (green gradient);

TABLE 3 | Results of the Permutational multivariate analysis of variance (PERMANOVA) and the multivariate homogeneity of groups dispersions (BETADISPER) for fish communities for the three temporal factors (Timepoints, Season, and Temperature categories). Year and Station were included in a three-factor PERMANOVA to account for temporal and spatial variations, respectively.

Temporal factors					
Source	**Df**	**SS**	**MS**	**Pseudo-F**	***p***
Timepoints					
PERMANOVA					
Year	2	1.687	0.03346	3.3773	0.0001***
Station	8	5.232	0.10377	2.6187	0.0001***
Timepoints	18	11.534	0.22877	2.7169	0.0001***
Residual	127	31.964	0.63401		
Total	155	50.417	1.00000		
BETADISPER					
Groups	19	0.43484	0.022886	1.9752	0.01598*
Residuals	136	1.60842	0.011827		
Season					
PERMANOVA					
Year	2	1.687	0.03346	3.0620	0.0001***
Station	8	5.232	0.10377	2.3742	0.0001***
Season	3	4.387	0.08701	5.3091	0.0001***
Residual	142	39.111	0.77576		
Total	155	50.417	1.00000		
BETADISPER					
Groups	3	0.05489	0.018295	1.7486	0.1595
Residuals	152	1.59033	0.010463		
Temperature categories					
PERMANOVA					
Year	2	1.687	0.03346	3.0164	0.0001***
Station	8	5.232	0.10377	2.3388	0.0001***
Temperature	4	4.074	0.08082	3.6431	0.0001***
Residual	141	39.424	0.78196		
Total	155	50.417	1.00000		
BETADISPER					
Groups	4	0.11043	0.0276066	2.7721	0.02927*
Residuals	151	1.50374	0.0099585		

Note: Number of permutations: 9999. Significance levels: ***< 0.001; **< 0.01; *< 0.05.
Abbreviations: Df, degree of freedom; MS, mean sum of squares; SS, sum of squares.

stations 130, 230, 700, and 710 clustered toward the left (blue gradient), while stations 120 and 780 (red gradient) were situated in the middle. Environmental factors were fitted onto the ordination to identify environmental factors driving this pattern. The envfit analysis revealed that higher salinity levels were associated with stations 215, 330, and ZG02 (green gradient), whereas SPM, PO_4, SiO_4, and NH_4 were mainly associated with stations 130, 230, 700, and 710 (blue gradient). However, only PO_4 and temperature have a significant influence on the spatial eDNA distribution (Table 4).

PERMANOVA analysis performed on the Bray–Curtis dissimilarity matrix returned significant results for all three factors (Distance to shore, East-to-West position, and Stations) (Table 5).

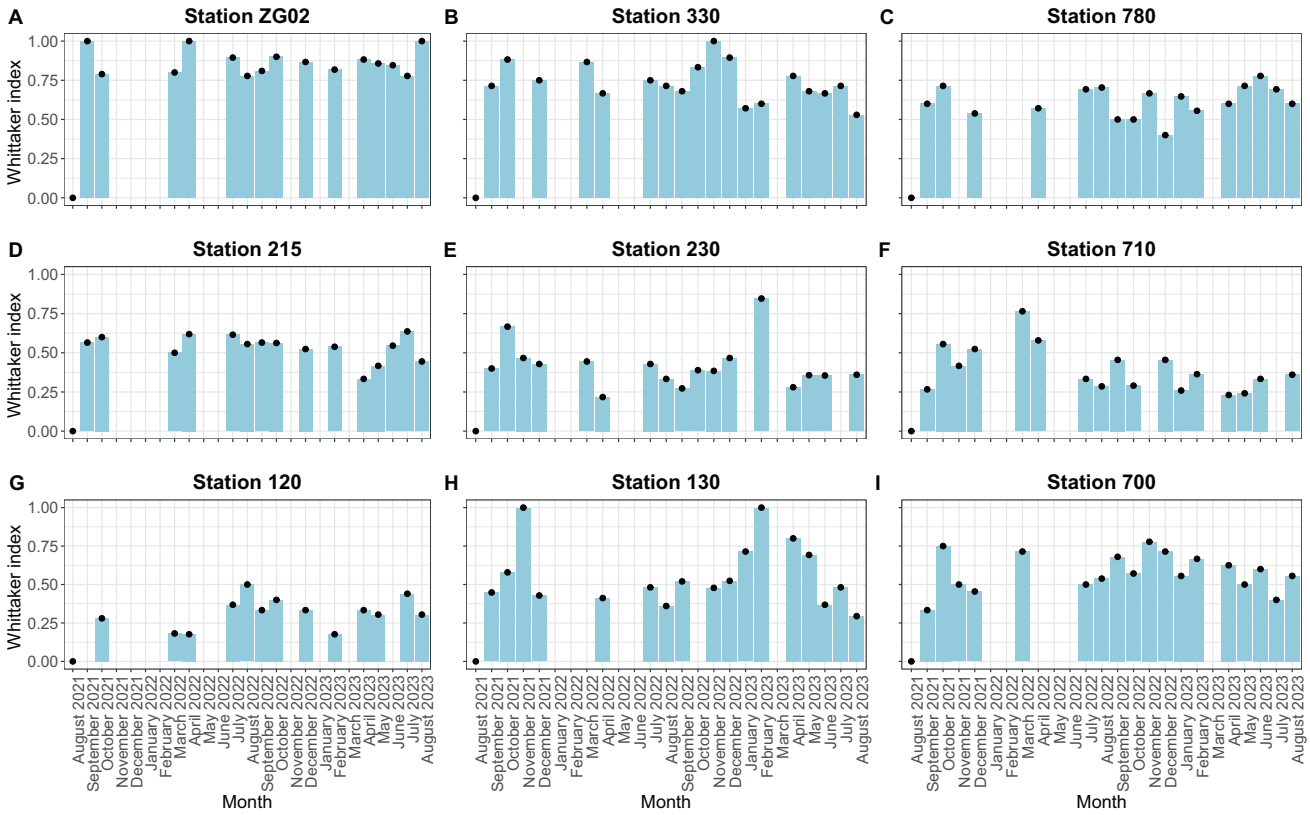

FIGURE 4 | Whittaker Index for each timepoint as compared to the first sample collected in August 2021 at each station. The index quantifies the degree of species turnover between samples: A value of 0 indicates no turnover or change in species composition, and a value of 1 indicates complete turnover or maximum difference in species composition.

However, these results were driven by non-homogeneous group dispersion between stations ($p = 0.01499$; Figure S14) and West-to-East position ($p = 0.006094$; Figure S15), whereas distance to shore group dispersion was homogeneous ($p = 0.1021$; Figure S16).

Along the near- to offshore transect, the pairwise analysis revealed significant differences between the nearshore and both offshore ($p = 2e\text{-}04$; Table S7) and mid-shore ($p = 0.0156$) groups. SIMPER analysis identified the species contributing the most to differences between communities (Table S8). The average dissimilarity was 81.91% and 78.23% between the near- and offshore groups, and the near- and mid-shore groups, respectively. Dissimilarities were explained mainly by differences in the abundance of common species rather than the presence/absence of different species. Indicator species analysis identified three species associated with the nearshore group (*Clupea harengus*, *Dicentrarchus labrax*, and *Pomatoschistus minutus*) and four species associated with the offshore group (Ammodytidae spp., *Engraulis encrasicolus, Trachurus trachurus*, and *Agonus cataphractus*) (Table S9).

Along the east-to-west transect, eastern stations were associated with *Sprattus sprattus* and *Clupea harengus*, whereas western stations were associated with Ammodytidae spp., *Engraulis encrasicolus, Scomber scombrus, Trachurus trachurus, Mullus surmuletus, and Scyliorhinus canicula* (Table S9). At the station scale, ZG02 was characterized by the presence of Ammodytidae spp., *Trachurus trachurus*, and *Scyliorhinus canicula*, whereas *Engraulis encrasicolus* and *Buglossidium luteum* were indicators of stations 215, and *Callionymus reticulatus* and *Gobius niger* of station 330 (Table S9). *Clupea harengus* was strongly associated with station 700. *Pleuronectes platessa* was an indicator for station 120, and *Sprattus sprattus* and *Agonus cataphractus* for station 780.

4 | Discussion

An interplay of environmental factors and biological behavior drives temporal and spatial changes within fish communities. Environmental factors such as temperature, salinity, and nutrient loading fluctuate seasonally and affect the abundance and composition of fish communities by limiting species distribution within favorable conditions (Baptista et al. 2019; Getz and Eckert 2023). Temporal changes have been successfully explored with eDNA in coastal fish communities (Sigsgaard et al. 2017), in estuaries (Stoeckle et al. 2017), around coastal islands (Berry et al. 2019; Nhat et al. 2024), in a lagoon (Cananzi et al. 2022), and in a lake (Rivera et al. 2023). Within the BPNS, we did not observe marked temporal patterns in the coastal fish communities. However, changes in eDNA abundances were observed at the species level, associated with ontogenetic migrations and biological needs.

4.1 | eDNA Revealed Low Temporal Species Turnover in the Coastal Belgian Part of the North Sea

The absence of temporal patterns in the coastal fish community composition (Figure 3) is explained by the absence of

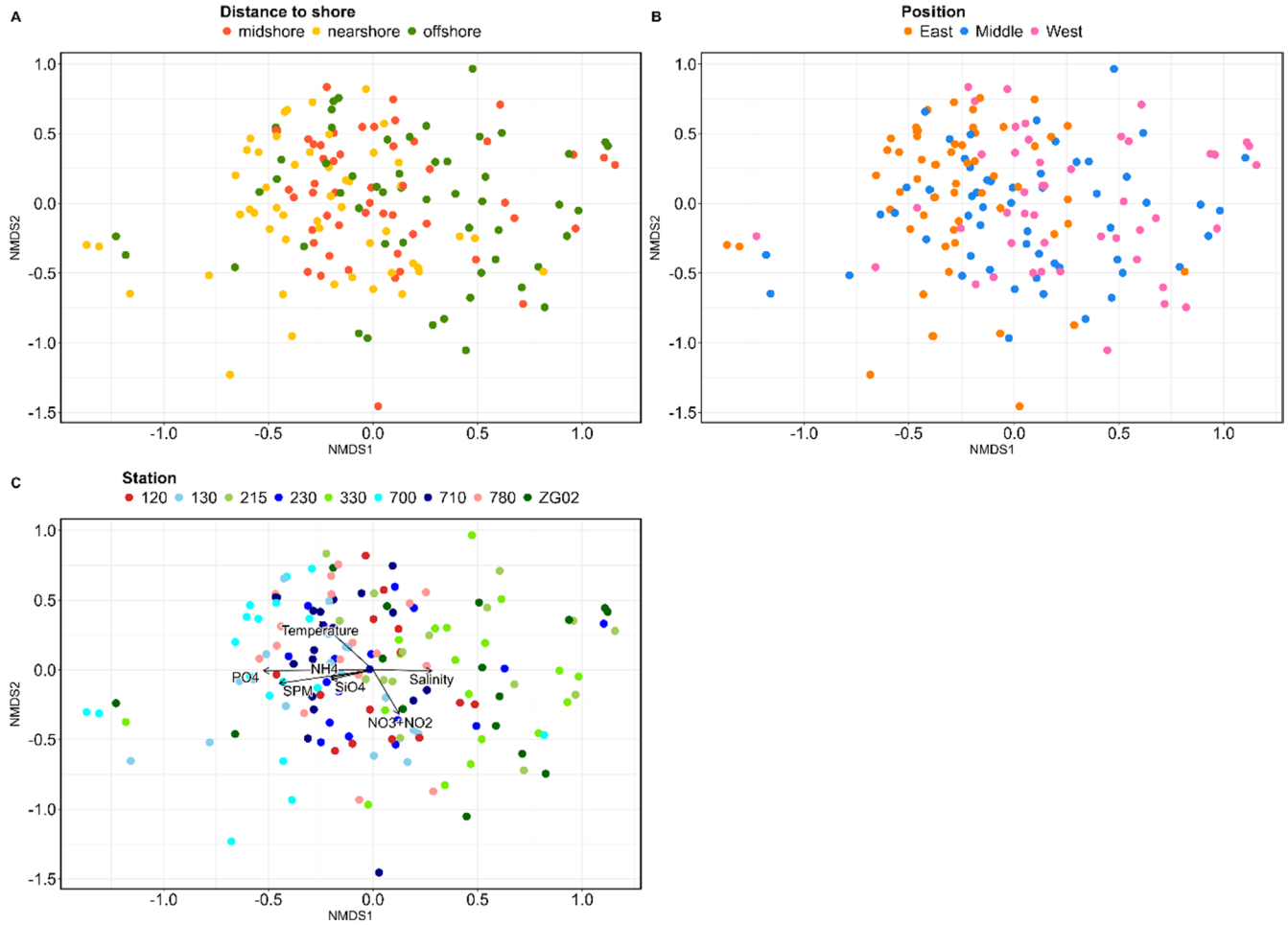

FIGURE 5 | NMDS computed on Bray–Curtis dissimilarity matrix of eDNA Index of fish communities. Dots are colored according to (A) Distance to shore, (B) East-to-West position, and (C) Stations and fitted environmental variables, including salinity, temperature, phosphate concentrations (PO4), suspended particulate matter (SPM), Silicate concentrations (SiO4), Ammonium concentrations (NH4), and Nitrites and nitrates concentrations (NO3 + NO2). Stress = 0.19461; $k = 3$.

TABLE 4 | Output of the envfit function showing the relationship between the environmental factors and the first and second axis of the Non-Metric Multidimensional Scaling (NMDS1 and 2, respectively) ordination. Factor labels: NH_4 = ammonium, $NO_3 + NO_2$ = nitrates and nitrites, PO_4 = phosphate; SiO_4 = silicates, SPM = suspended particulate matter.

Factors	NMDS1	NMDS2	R2	*p*
NH_4	−0.96830	0.24979	0.0433	0.397
$NO_3 + NO_2$	0.36722	0.93013	0.1174	0.063
PO_4	−0.99997	0.00800	0.2741	0.001***
Salinity	0.99970	0.02433	0.0793	0.140
SiO_4	−0.94651	0.32269	0.0446	0.346
SPM	−0.94651	0.32269	0.0446	0.356
Temperature	−0.59123	−0.8065	0.1847	0.01**

Note: Number of permutations: 9999. Significance levels: ***< 0.001; **< 0.01; *< 0.05.

Abbreviations: *p*, significance of the relationship; R2, proportion of variance explained by the ordination axes.

marked species turnover (Figure 4) and the persistent presence of dominant species throughout the whole timeseries (Figure S9). Only coastal eastern stations showed temporal variation between summer/spring months and autumn/winter months in terms of species presence/absence (Figure 4). The eastern BPNS is under the influence of the Scheldt freshwater discharge, which varies in intensity seasonally. The Scheldt is a rain-fed river (Meire et al. 2005), with freshwater discharge reaching a peak in winter (180 m^3/s) and then decreasing towards summer (60 m^3/s) (Baeyens et al. 1998). Such changes in estuarine outflow modify environmental conditions and lead to seasonal fluctuations in seawater temperature, salinity, and nutrient loadings (Jiang et al. 2023; Nhat et al. 2024). SPM did not vary seasonally within the coastal zone of the BPNS (Figure S4), while seawater temperature showed clear temporal variation (Figures S2, S3, and S5, respectively). The temporal changes were more marked at nearshore and eastern stations compared to the western and offshore stations, reflecting the influence of river freshwater discharge nearshore. Nutrient concentration peaks (nitrites and nitrates, and silicates) occurred between early winter (November/December)

TABLE 5 | Results of the Permutational multivariate analysis of variance (PERMANOVA) and the multivariate homogeneity of groups dispersions (BETADISPER) for fish communities for the three spatial factors (Station, Distance to shore, and Position).

Spatial factors					
Source	**Df**	**SS**	**MS**	**Pseudo-F**	***p***
Station					
PERMANOVA					
Station	8	5.309	0.10531	2.1628	1e-04***
Residual	147	45.107	0.89469		
Total	155	50.417	1.00000		
BETADISPER					
Groups	8	0.16167	0.0202092	2.5796	0.01154*
Residuals	147	1.15164	0.0078343		
Distance to shore (Near-, Mid-, Offshore)					
PERMANOVA					
Distance to shore	2	1.456	0.02887	2.2746	1e-04***
Residual	153	48.961	0.97113		
Total	155	50.417	1.00000		
BETADISPER					
Group	2	0.03582	0.0179078	2.6255	0.07567
Residuals	153	1.04358	0.0068208		
Position (West, Middle, East)					
PERMANOVA					
Position	2	2.407	0.04775	3.8357	1e-04***
Residual	153	48.009	0.95225		
Total	155	50.417	1.00000		
BETADISPER					
Groups	2	0.08831	0.044154	5.2775	0.006076**
Residuals	153	1.28009	0.008367		

Note: Number of permutations: 9999. Significance levels: ***< 0.001; **< 0.01; *< 0.05.
Abbreviations: Df, degree of freedom; MS, mean sum of squares; SS, sum of squares.

and late spring (May/June) in 2022 and 2023, corresponding to the rainy season and subsequent higher water discharge from the Scheldt. Additionally, the peak concentrations decreased as we moved offshore and westward. Such changes in environmental conditions were reflected in the species turnover: species composition in terms of presence/absence was more similar in summer and spring months as indicated by the lower Whittaker index values (in reference to August 2021), compared to winter and autumn months during the peak rainy season displaying higher index values. We tested whether the absence of seasonal patterns and species turnover was due to the removal of rare species subsequent to the filtering by PCR replicates. The same analyses were run on an unfiltered dataset from which only exotic and freshwater species absent from the Scheldt estuary were removed. The results did not reveal clearer seasonal patterns or species turnover, confirming our results that fish communities within the coastal area of the BPNS, as detected by eDNA, do not show seasonality.

4.2 | eDNA Detected Species-Specific Temporal Variation Reflecting Their Biology in the Study Area

The eDNA concentration is the result of an interplay between transport and degradation processes on the one hand, and on biological factors of the species on the other hand (Barnes and Turner 2016). In addition, PCR bias associated with the metabarcoding protocol may distort species-specific patterns. Nevertheless, multiple studies based on eDNA metabarcoding have demonstrated that species-specific trends align with a priori expectations derived from literature on lifecycle, migration, and habitat use (Port et al. 2016; Hallam et al. 2023;

Carvalho et al. 2024). This is because the transport of eDNA in the marine environment is limited (Port et al. 2016; O'Donnell et al. 2017; Murakami et al. 2019), even in highly dynamic areas and sites under tidal influence (Kelly et al. 2018) and because eDNA decay rates in temperate coastal waters are not significantly different between seasons (summer/winter; Collins et al. 2018). Moreover, proportional indices of amplicon reads capture trends in taxon biomass with high accuracy, thereby reducing the effects of PCR bias in the metabarcoding protocol (Kelly et al. 2019). The temporal eDNA patterns in the coastal area of the BPNS observed in the current study further support the link between eDNA and the biology of the species.

The nine fishes detected throughout the time series (Figure S9) are widespread within the wider BPNS (*Limanda limanda*, *Merlangius merlangus*, *Pleuronectes platessa*, and *Sardina pilchardus*) or in the coastal waters (*Sprattus sprattus*, *Clupea harengus*, *Solea solea*, and *Platichthys flesus*) based on beam trawl data (Ehrich et al. 2009; De Backer et al. 2022) and eDNA data (Cornelis et al. 2024; Dukan et al. 2024). Despite being usually associated with the offshore area of the BPNS (between the 12 NM limit and the offshore limit of the BPNS; De Backer et al. 2022), Ammodytidae spp. was a prevalent taxon within the 12 NM zone of the BPNS in our eDNA data. The Southern North Sea and the Belgian coastal waters serve as important spawning grounds and provide coastal nurseries for these persistent species (Knijn et al. 1993; Ellis et al. 2012). The ubiquitous presence of these dominant species can thus be explained by their non-migratory behavior within the BPNS and the use of the BPNS as a spawning or nursing ground.

The indicator species further support the link between eDNA and reproductive behavior. Indicator species of summer (*Buglossidium luteum*, *Engraulis encrasicolus*, *Pomatoschistus lozanoi*, *Sprattus sprattus*, and *Trachurus trachurus*) are all summer spawners using the Belgian coastal waters as spawning grounds (Hamerlynck 1993; Knijn et al. 1993). Similarly, autumn spawners (*Sardina pilchardus* and *Agonus cataphractus*) were strong indicators of the autumn season. *Sardina pilchardus* is a warm-water species known to spawn in the English Channel (Coombs et al. 2006, 2010) and seawater temperature is an important factor altering the intensity and phenology of its spawning season (Abdelouahab et al. 2020). Autumn sea surface temperatures in the BPNS align with *Sardina pilchardus*' optimal spawning temperature range between 13°C and 15°C (overall range: 10°C–17°C; Stratoudakis et al. 2007). *Agonus cataphractus* spawning is known to occur in late autumn or early winter but has been reported to start as early as October (Wheeler 1969). Their occurrence in autumn might also point to an inshore spawning migration because their lifecycle usually relies on estuaries (Elliott et al. 1990). Spring was associated with *Solea solea*, a spring spawning species for which the BPNS is an important spawning ground. *Solea solea* migrates inshore in spring before their peak spawning activity in April/May in coastal areas (Knijn et al. 1993).

Next to spawning activities, the indicator species analyses also illustrated migration patterns of coastal fishes. *Liparis liparis*, *Pomatoschistus minutus*, and *Ciliata mustela* were indicators of autumn. The latter is known to reach coastal feeding grounds in between overwintering and summer spawning and feed on *Pomatoschistus minutus* among other prey (Knijn et al. 1993). Similarly, *Liparis liparis* undergoes seasonal migration as it migrates from offshore waters late summer towards estuaries where its abundance is known to peak in winter (Henderson and Holmes 1990). Additionally, Ammodytidae spp. were detected as an indicator of spring. As seawater temperatures rose in spring, Ammodytidae spp. emerged from the seabed where they buried in sandy bottoms and moved along sandbank edges in large schools to feed (van der Kooij et al. 2008).

The temporal occurrence of *Dicentrarchus labrax* and *Scomber scombrus* in spring and summer can be linked to migration towards feeding grounds for the first one and as a transitional migration step for the second one in summer (Eltink et al. 1986; de Pontual et al. 2023). The sporadic appearance of the remaining species agrees with their migration movements using the BPNS as a transitional step (*Gadus morhua*, *Mullus surmuletus*, *Mustelus asterias*, *Salmo salar*, and *Belone belone*) or their preference for offshore waters (*Buglodissium luteum* and *Echiichthys vipera*) (Knijn et al. 1993; Brevé et al. 2016).

Temporal patterns and changes detected in fish eDNA Index were mainly related to their reproductive behavior. eDNA concentration peaks can concur with the reproductive period of fish species (Collins et al. 2022) and can be up to 20-fold higher than outside of the reproductive period (Wacker et al. 2019). Our results are consistent with these observations and with previous studies (Bylemans et al. 2017; Ratcliffe et al. 2021; Tsuji and Shibata 2021) showing that eDNA detection is strongly associated with the reproductive activity of fishes. Aggregation activity, spawning events, the presence of fertilized eggs and nearby egg-producing adults, and larvae are all potential sources of eDNA. Intact eggs do not seem to shed eDNA (Takeuchi et al. 2019; Wilder et al. 2023), but their presence in the water column might still influence the eDNA peak either directly by their presence on the filter or indirectly by leakage of DNA from dead eggs (Ostberg and Chase 2022). Accordingly, eDNA cannot disentangle the exact reproductive stage of the species. Therefore, eDNA patterns can be correctly interpreted and linked to species lifecycle events only if the species biology in the studied area is well known and documented.

4.3 | eDNA Did Not Reveal Marked Spatial Patterns in Fish Community Structures

Previous studies have successfully described spatial patterns in fish community structure using eDNA across various marine habitats, including oceanic waters (Jeunen et al. 2018; Fraija-Fernández et al. 2020), reefs (West et al. 2020; Valdivia-Carrillo et al. 2021), lagoons (Cananzi et al. 2022), and even within the BPNS (Cornelis et al. 2024; Dukan et al. 2024). The latter two eDNA studies picked up the coastal, transitional (around 12 NM), and offshore fish communities described with beam trawl data (De Backer et al. 2022). In dynamic nearshore environments, eDNA-based surveys have detected fine-scale patterns in fish community distribution (O'Donnell et al. 2017 (4 km transects); Yamamoto et al. 2017 [47 stations over 11 km^2]; West et al. 2021 [252 stations over 140 km^2]). Within the 12 NM area of the BPNS,

a shallow (19.2 m at the deepest station), well-mixed and tidal environment, our eDNA results did not reveal clear spatial patterns. At this spatial scale, 12 years of beam trawl data divided the coastal fish community into an eastern muddy community dominated by *Pomatoschistus* spp., *Pleuronectes platessa*, and *Solea solea*, and a widespread fine sand community dominated by *Limanda limanda*, *Merlangius merlangus*, *Callionymus lyra*, and *Pomatoschistus* spp. (De Backer et al. 2022). Although these dominant species were detected in our eDNA data (Table S2), the two communities were not observed in our eDNA data, even when subsetting the data for only the autumn samples and when adding mud/sand as a factor (Figure S17). None of these species were listed as indicator species for the west/east gradient (Table S9). Indicator species for the eastern stations were *Sprattus sprattus* and *Clupea harengus*, whereas western stations were dominated by Ammodytidae spp., *Engraulis encrasicolus, Scomber scombrus, Trachurus trachurus, Mullus surmuletus*, and *Scyliorhinus canicula*. The discrepancy can be explained by the fact that beam trawling targets demersal fish, while eDNA data also include pelagic fishes. Moreover, the sampling design of our study is probably less suited to infer small-scale spatial resolution patterns since only one bottle was collected at nine stations evenly spread across the 12 NM zone.

4.4 | Species-Specific eDNA Patterns Reflected the Environmental Gradient Created by the Estuarine Outflow

Species-specific eDNA patterns identified indicator species associated with specific environmental conditions and consistent with eDNA and beam trawl observations along the distance to shore transect, east-to-west gradient, and even stations. For instance, *Dicentrarchus labrax*, *Clupea harengus*, and *Pomatoschistus minutus* were indicator species of the nearshore area, aligning with previous observations within the BPNS (Dukan et al. 2024), except for *Dicentrarchus labrax*. Our temporal scale included summer months during which *Dicentrarchus labrax* fed along the Belgian coast, whereas cited observations were based on autumn samples. *Sprattus sprattus and Clupea harengus* were associated with the eastern side of the BPNS. Both species, primarily described as oceanodromous, have adapted to a marine estuarine opportunist strategy (Guelinckx et al. 2006). Observations within the Scheldt estuary (Maes et al. 1998) and other North Sea estuaries (Teal et al. 2009) support this behavior. As such, *Clupea harengus* was strongly associated with the most nearshore and eastern station (700).

Conversely, Ammodytidae spp., *Engraulis encrasicolus*, *Agonus cataphractus*, and *Trachurus trachurus* were indicators of the offshore zone, corresponding to the transition zone of full BPNS scale studies. Ammodytidae spp. here most likely consists of two species, *Ammodytes tobianus* and *Hyperoplus lanceolatus* based on the long-term beam trawl dataset from the BPNS (De Backer et al. 2022). Consequently, the indicator status for this taxon may be questionable. Nevertheless, Ammodytidae spp. and the other species were mostly observed in the transition zone and offshore waters with beam trawl (De Backer et al. 2022) and eDNA surveys (Dukan et al. 2024), except for *Agonus cataphractus*, which was more characteristic of the coastal area and fine sand sediment. In our results, *Agonus cataphractus* was an indicator of station 780 situated over fine sand sediment and closer to the shore than other offshore stations.

The western side of the BPNS was associated with species typical of transition/offshore waters and with a preference for sandy substrates. Ammodytidae spp. are closely associated with sandy substrates and usually inhabit turbulent areas such as the edges of sandbanks (Reay 1970; Wright et al. 2000). Accordingly, Ammodytidae spp. were an indicator species of station ZG02 located within the slope and gullies of the Flemish Banks system. *Scyliorhinus canicula*, also an indicator of station ZG02, preys on Ammodytidae spp. among other organisms (Knijn et al. 1993). Similarly, *Buglossidium luteum*, an indicator of station 215, is a transition/offshore species (De Backer et al. 2022), which prefers sandy sediment (Teal et al. 2009). The presence of *Engraulis encrasicolus*, an indicator of station 215, in the Southern North Sea is associated with water influx through the English Channel (Knijn et al. 1993; Alheit et al. 2012). Their occurrence mostly towards the western offshore side might be linked to the populations in the English Channel. Situated over the coarse sand sediment, station 330 was associated with the presence of *Callionymus reticulatus*, an offshore species (De Backer et al. 2022) that buries in the sand (Knijn et al. 1993) and *Gobius niger*, a species identified as transitional (Dukan et al. 2024).

According to Menge and Olson (1990), the relative importance of structuring factors depends on the spatial scale. At the regional and local scales, species assemblages are mainly under the influence of interactions between biological processes and physical factors (Ellis et al. 2000; Callaway et al. 2002; Barletta et al. 2005). Estuarine communities are structured mainly by salinity and temperature (Jaureguizar et al. 2004; Barletta et al. 2005), whereas coastal communities are strongly influenced by depth, temperature, and substrate (Ellis et al. 2000). Within the 12 NM limit of the BPNS, fish communities were shaped by an interplay of environmental parameters. Higher salinity was associated with western offshore stations (Figure S3), under the influence of Atlantic water through the English Channel offshore (Lacroix et al. 2004). Conversely, eastern nearshore stations were associated with high suspended particulate matter levels (Figure S4), which strongly affect turbidity. Turbidity can have both negative and positive effects on fish, depending on its intensity and the fish species (Utne-Palm 2002). The distribution of certain fish species is partially determined by turbidity (Cyrus and Blaber 1987). Additionally, eastern stations were associated with higher nutrient loadings (mainly PO4, and to a lesser extent NH4 and SiO4; Figure S5). Nutrient availability positively affects planktonic organisms, an important food source, consequently impacting fish communities (Cermeño et al. 2006). Lastly, at the station scale, habitats are created along the sedimentological gradient and are associated with distinct fish species as supported by the indicator species.

4.5 | Considerations for Proper Negative Controls During eDNA Samples Collection

Although negative controls are a fundamental practice in eDNA research, comprehensive and standardized guidelines on their collection, processing, analysis, and reporting are lacking

(Takahashi et al. 2023). In our study, we did not include field and filter negatives primarily due to logistic constraints associated with conducting monthly sampling campaigns over 2 years and processing a large number of samples. Field controls aboard research vessels may involve sampling the Niskin bottles (Cornelis et al. 2024) or the collection bottles (Gold et al. 2021). It remains to be shown whether Niskin control samples are meaningful to take. The Niskin bottles cannot be sterilized onboard the vessel, and a small number of eDNA molecules in the Niskin control samples will most likely be overamplified since this is the only template available during the PCR (Cornelis et al. 2024). Moreover, Niskin bottles mounted on a CTD rosette are submerged in local seawater for 3 min at each location to allow for CTD calibration, which may completely eliminate carry-over eDNA from the previous location. However, this requires empirical evidence. Another type of control to assess contamination in the field involves sampling sterile distilled water from the collection bottles used to subsample the Niskin bottles (e.g., Gold et al. 2021). When subsequent filtering occurs on the vessel, this step also includes possible contamination (e.g., eDNA molecules in the tubings, filters, caps). Our seawater collection procedure consists of opening a sealed plastic bottle and pouring the commercial drinking water in the Niskin bottle just prior to Niskin deployment, closing the empty commercial water bottle until the Niskin rosette is back on deck, collecting a 2 L subsample from the Niskin in the drinking water bottle, closing the bottle, and storing it in the freezer at −20°C. A valid field control for our sample collection procedure could have been to empty a commercial bottle and fill it with drinking water from a second, newly opened commercial drinking water bottle onboard the vessel, which would mimic our handling procedure in the field. Although we consider it very unlikely that fish eDNA contamination can occur considering the tiny opening of the drinking water bottle (ca 2 cm in diameter) and the lack of any fishing activity onboard the vessel during our sampling campaigns, the addition of such a field control in our future campaigns represents an improvement to our eDNA field collection protocol. The lack of filter controls to verify contamination during the filtering step in our study represents a shortcoming, which was driven by the large number of different filtering events weeks apart from each other over the 2-years timeframe. As with DNA and PCR control samples, during each filtering event, a filter negative should be included. For our large-scale temporal study, the addition of field and filter control samples would have increased the number of samples for lab processing and sequencing by 20%, which means that associated costs and effort should be clearly anticipated at the beginning of temporal studies. A possible solution may lie in taking control samples only during a subset of the sampling campaigns (e.g., Collins et al. 2022) or filter events; however, we argue that this does not say anything about possible contamination in the non-sampled campaigns and would create a false sense of tackling contamination issues. Finally, many eDNA studies do not even report the number of ASVs/reads in the negative controls of the lab procedures and perhaps often do not even sequence them because of a lack of visible PCR product, suggesting that there is room for improvement in how data from control samples is reported. In all, there is an urgent need for guidelines that define which types of negative controls should be taken, how many of each type should be collected, and how the resulting sequencing data should be reported to ensure robust and reliable eDNA results.

5 | Conclusion

eDNA-based surveys offer a non-invasive and cost-effective method to obtain long-term and fine-scale temporal monitoring data, providing crucial information on key moments of fish lifecycles. We monitored monthly temporal and spatial changes in eDNA of fish over 2 years within the 12 NM limit of the BPNS using 12S metabarcoding. Our results revealed no distinct spatial patterns in coastal fish communities but unveiled species-specific spatial distribution consistent with traditional beam trawl and eDNA-based surveys conducted within the BPNS. Species distribution highlighted the importance of the freshwater input from the Scheldt estuary and the resulting environmental gradient (nutrient loading, salinity and SPM) in shaping fish communities. While no temporal patterns were observed at the community level due to the lack of marked species turnover, eDNA effectively reflected known biology, reproductive activity, and migration patterns of Southern North Sea species. Our findings underscore the potential of eDNA metabarcoding in monitoring fish reproductive activities and migrations, offering valuable insights for ecosystems and fisheries management.

Author Contributions

M.L. performed the laboratory work, analyzed, and interpreted the data, and wrote the manuscript. I.C. helped with the bioinformatic processing of the data. S.M. helped process the samples in the laboratory. J.M. organized the sampling surveys and sample collection. H.P. acquired funding. S.D. designed the study, acquired funding, interpreted the data, and helped write the manuscript. All authors critically reviewed the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All raw demultiplexed data and associated metadata generated are deposited in the Sequence Read Archive (SRA; NCBI) under the BioProject accession code PRJNA1131323. Species occurrence data is available on GBIF (DOI: https://doi.org/10.15468/kg56dz) and includes all the species detected in the concatenated data after decontamination. All scripts used for data processing and analysis, and the custom database are available on Zenodo (https://doi.org/10.5281/zenodo.13861942).

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Data S1:** edn370175-sup-0001-DataS1.docx. **Data S2:** edn370175-sup-0002-DataS2.docx.