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ORIGINAL ARTICLE

Environmental DNA for monitoring the impact of offshore wind farms on fish and invertebrate community structures

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Abstract

To reach the renewable energy targets set by the European Commission, a tenfold expansion of the installed offshore wind farms is needed. Since the construction of offshore wind farms may affect local soft-sediment fauna, an efficient monitoring technique is needed to monitor the potential effects on the marine ecosystem. Here, we assess whether eDNA metabarcoding is a suitable alternative to monitor fish and epibenthos biodiversity in these difficult to access marine habitats. Water sampling and trawl surveys were conducted in parallel in 12 coastal and 18 offshore sites, the latter located inside and outside two offshore wind farms in the Belgian part of the North Sea. 12S eDNA metabarcoding retrieved 85.7% of the fish species caught in the beam trawls, whereas the COI eDNA metabarcoding only identified 31.4% of the epibenthic invertebrate species. Furthermore, the 12S marker resulted in an additional detection of 26 unique fish species, whereas the COI marker detected an additional 90 invertebrate species. Spatial patterns in alpha diversity recovered with eDNA metabarcoding were not significantly different from those observed with morphological determination. Significant differences were found in fish and invertebrate community structures between the coastal, transition and offshore zones as well as on the smaller wind farm scales, which agreed with the morphological beam trawl data. Indicator species found with morphological beam trawl monitoring for each of the three zones (coastal, transition, offshore) were also detected with 12S eDNA metabarcoding, and the latter method detected an additional 31 indicator species. Our findings show the need for adequate quality control of the obtained species lists and reveal that 12S eDNA metabarcoding analyses offers a useful survey tool for the monitoring of fish communities in offshore wind farms, but the used COI assay did not adequately capture the epibenthic communities as observed with beam trawl data.

KEYWORDS
bottom trawl surveys, environmental impact assessment, false positives, North Sea, shallow marine environment

1 | INTRODUCTION

The European Commission has set the renewable energy targets to generate 300 GW of offshore wind energy by 2050 (European Commission, 2020), which implies a tenfold expansion of the currently installed 30 GW (WindEurope, 2022). Construction of offshore wind farms (OWFs) introduces hard substrates in soft sediment environments and can cause changes that may affect local soft-sediment fauna at a variety of spatial scales (Ashley et al., 2014; Lindeboom et al., 2011; Raoux et al., 2017). Many fish species and other megafauna are attracted to the introduced hard structures for food and shelter, the so-called artificial reef effect (Degraer et al., 2020). Additionally, fisheries are excluded from most OWFs in Europe, which is another potential effect at play to induce changes on the soft-bottom assemblages (Coates et al., 2011; Handley et al., 2014). Given the future expansion of OWFs, it is important to enable monitoring of the potential effects on the ecosystem in an efficient way.

Beam trawl monitoring, if allowed by the wind farm owners, is commonly used to assess the effects of OWFs on fish and epibenthic invertebrates (Buyse et al., 2022; Lindeboom et al., 2011; Vandendriessche et al., 2015). However, beam trawl surveys disturb the sampled habitats and cause harm to the organisms (van Denderen et al., 2015). Moreover, the datasets resulting from these beam trawl surveys are often limited in spatial and temporal coverage, because they are time consuming, labour intensive, expensive, require taxonomic expertise and good weather conditions (Andruszkiewicz et al., 2017; Gold, Sprague, et al., 2021). Environmental DNA (eDNA) is organismal DNA released into the environment by organisms and may be present in a cellular or extracellular form (Bohmann et al., 2014). Methods based on eDNA could offer a very useful and efficient alternative monitoring tool. Due to its ease of sampling and non-destructiveness, eDNA metabarcoding may increase the spatial and temporal coverage of surveys for monitoring marine biodiversity. Recent studies have, furthermore, demonstrated that taxonomic profiling based on eDNA metabarcoding and morphological determination are compatible but not exclusive, since both methods yield more information when used together (Sigsgaard et al., 2017; Stat et al., 2019; Valdivia-Carrillo et al., 2021; van Bleijswijk et al., 2020). In shallow systems that are subjected to tidal and along-shore currents, eDNA metabarcoding is also able to distinguish spatial patterns in fish and invertebrate communities from diverse marine habitats (soft sediment, kelp, seagrass, rocky reefs, open water) within a spatial scale of less than five kilometers (Jeunen et al., 2019; Port et al., 2016). The use of eDNA for monitoring fish and plankton communities near a floating offshore windfarms has potential, but ground truthing with morphological data is needed (Hestetun et al., 2023). Whether eDNA monitoring could be used for monitoring a shallow, well mixed area dominated by a soft sediment habitat and for monitoring the impact of fixed OWFs, an artificially introduced hard substrate in an otherwise soft sediment habitat, has hitherto not been explored.

Within the Belgian part of the North Sea (BPNS), a semi-enclosed shelf sea with a total area of only 3.454 km^2 (~0.5% of the North Sea), an area of 238 km^2 was designated at the north-east border for offshore wind energy (Belgisch Staatsblad, 28/03/2014). Since the end of 2020, this OWF area is fully operational and has an installed capacity of 2.26 GW generated by a total of 399 wind turbines (Degraer et al., 2022). Since the construction start of the OWF area, an environmental beam trawl survey took place, using a Before/After—Control/Impact design in the two oldest OWFs to investigate the effects on the soft-sediment epibenthos and demersal fish communities (De Backer et al., 2020). This revealed small but significant OWF effects with an increased abundance of four common fish species *Callionymus lyra*, *Echiichthys vipera*, *Buglossidium luteum* and *Pleuronectes platessa* within one OWF. Moreover, an expansion of the reef effect was indicated based on an increase of hard substrate-associated species such as *Pisidia longicornis*, *Cancer pagurus*, *Loligo vulgaris* and *Mytilus edulis* (De Backer et al., 2020). Furthermore, epibenthos and fish communities of the wider BPNS were recently described based on a long-history of beam trawl surveys related to environmental monitoring programs (De Backer et al., 2022). There is a clear distinction between coastal and offshore fish and epibenthic communities, and for fish a third community is identified as the transitional community around the 12 nautical mile zone. The latter consists of a mixture of coastal and offshore fish species, and appears to be the most species rich community (De Backer et al., 2022). The spatial distribution of these epibenthos and fish communities is strongly associated with sediment properties and sand bank topographies along the onshore-offshore gradient (De Backer et al., 2022).

This in-depth local ecological knowledge make the BPNS an excellent study area to investigate whether 12S and COI eDNA metabarcoding are able to describe respectively, the fish and invertebrate communities in agreement with those previously described by the beam trawl surveys both on the wider BPNS and the OWF scale. To this end, seawater samples for eDNA metabarcoding were collected in 12 coastal and 18 offshore sampling locations in parallel with conventional beam trawl samples for morphological determination (Figure 1). The offshore samples were taken inside and outside the OWFs C-Power (near the transition zone) and Belwind (fully offshore) to study the effect of OWFs. Our goals were to: (i) compare the fish and invertebrate species identified by eDNA metabarcoding and by morphological determination of beam trawl samples, and (ii) investigate whether eDNA metabarcoding is able to capture differences in species diversity and community structures on a wider BPNS scale (coastal, transition, offshore) and a smaller OWF scale (inside and outside two OWFs). The combined analyses of eDNA metabarcoding and morphological determination for fish and invertebrates in this study allows to evaluate whether eDNA metabarcoding could form a less destructive monitoring method to obtain robust and accurate biodiversity measurements in offshore wind farms for fish and epibenthic invertebrates.

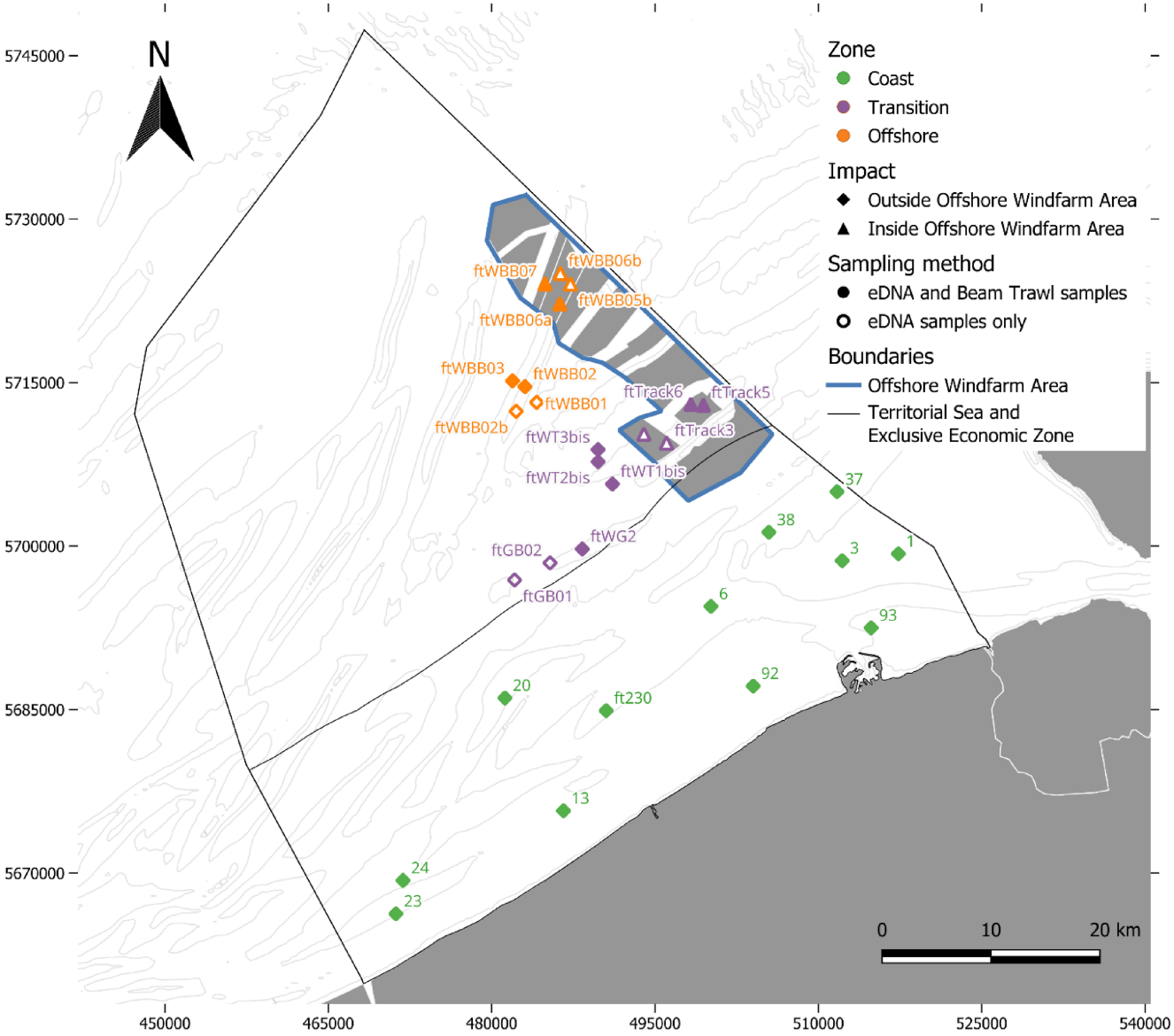

FIGURE 1 Map of the sample locations within the BPNS. Samples were taken in three different zones: coast (green), transition (purple) and offshore (orange). Locations inside the offshore wind farms in the transition (C-Power) and offshore (Belwind) zones are marked by triangles (▲/△). Locations outside the offshore wind farms are marked by diamonds (◆/◇). The filled symbols (◆/▲) mark the locations where seawater samples and beam trawl samples were collected in parallel. The open symbols (◇/△) mark the locations where only seawater samples for eDNA metabarcoding were collected.

2 | MATERIALS AND METHODS

2.1 | eDNA sample collection

During two different field campaigns in September and November 2021, a total of 12 coastal and 18 offshore locations, situated within and outside the OWFs C-power (transition zone) and Belwind (offshore zone), were sampled for seawater, later on followed by beam trawl sampling (see 3.2) (Figure 1). The coastal locations were sampled in triplicate during the September field campaign with the research vessel Simon Stevin using a Niskin carousel. The offshore locations and one coastal location (ft230) were sampled in November 2021 with the research vessel GeoOcean V. During this campaign five biological replicates were taken by successively lowering one Niskin bottle five times. One exception was the coastal site ft230, where only three biological replicates were taken.

At each location, seawater was collected at 1 m above the seafloor using a 10-liter Niskin bottle. From each 10-liter Niskin bottle, a subsample of 2 L was collected in clean commercial plastic drinking water bottles, using a sterilized 200 μm mesh nylon prefilter to remove bigger pieces of debris. Between locations, the Niskin bottles were rinsed with 2 L commercial source water. Nine Niskin control samples were taken by collecting commercial source water from the Niskin bottles after they were carefully rinsed using 2 L commercial source water, also using the prefilter. The water samples were either

immediately filtered on board (GeoOcean V) or stored in the dark at −20°C (Simon Stevin) until further processing.

On board and in the lab, the collected water samples were filtered in a separate room, where no fish or DNA samples were handled. Each sample was filtered over a 0.45-μm Sterivex polyvinylidene fluoride (PVDF) filter (Sterivex-HV Filter, with Luer outlet, Merck – Millipore) using a Masterflex pump with double pumphead until the filter was nearly clogged or until 1 L was filtered. After filtering, the Sterivex filters were sealed with two sterile Luer-lock™ caps at the in- and outlet of the filter capsule, and stored at −20°C until further processing. Between locations, the tubes of the Masterflex pump were flushed with 10% bleach and with 125 mL commercial source water. Six negative filter controls were included by filtering source water over a blanco 0.45-μm Sterivex filter.

2.2 | Beam trawl sampling

At each location, trawling took place immediately after eDNA sampling. Due to technical difficulties, no beam trawl samples for morphological determination were taken at the transitional locations ftGB01, ftGB02, ftTrack2 and ftTrack3, and the offshore locations ftWBB01, ftWBB02b, ftWBB05b and ftWBB06b. On the RV Simon Stevin (September), a 6-m wide beam-trawl was used, while on the GeoOcean V (November), the beam trawl was 8-m wide. Both beam-trawls targeted epibenthos and mainly smaller fish and younger year classes as they had a cod-end mesh size of 22 mm and were equipped with a bolder chain in front of the ground rope. The net was towed for 15 min (8-m trawl, GeoOcean V) or between 15 and 30 min (6-m trawl, RV Simon Stevin) with the current at an average speed of 3 (6-m trawl) or 4 (8-m trawl) knots over the ground. No significant difference was observed between the surface of the thawed area between the two campaigns (averages of $13{,}514 \pm 3705\,m^2$ and $15{,}446 \pm 2068\,m^2$ for the RV Simon Stevin and GeoOcean V campaigns, respectively; *t*-test: $t = 2.14$, $df = 10$, $p = 0.06$). To accommodate for the differences between the two sampling campaigns, the morphological count data were Hellinger transformed. All fish and epibenthic species were counted and identified to species level when possible. For some species (e.g. Gobiidae, Actinaria, Bryozoa, etc.), identification to species level is challenging on board and therefore they were identified to a higher taxonomic level.

2.3 | eDNA extraction

DNA extraction of the sampled Sterivex filters was conducted in a laminar flow cabinet in a PCR-free designated room. Before and after use, a 15 min UV-treatment was applied and all surfaces were successively cleaned with 10% bleach and 70% ethanol.

The Sterivex filters were incubated overnight at 56°C in a rotating incubator (Incubator-Genie, Scientific Industries), with 800 μL lysis buffer (718 μL ATL buffer [Qiagen], 80 μL Proteinase K [Qiagen] and 2 μL gBlocks® fragments IPC [1/10,000] [Integrated DNA Technologies]). After transferring the lysis buffer into a 5.0 mL LoBind tube (Eppendorf), extraction was performed using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. After washing, eDNA was eluted in two steps in a total volume of 100 μL TE buffer (70°C). Three extraction negative controls were included by applying the same protocol on blanco 0.45-μm Sterivex filters. In total 144 filters were extracted. The obtained eDNA extracts were subsequently quantified with the QuantiFluor® dsDNA System (Promega), according to the protocol provided, and stored at −20°C until further processing.

2.4 | Library preparation

A one-step amplification protocol was used for library preparation. The PCR amplification was performed using fusion primers (Sigma Aldrich), which contained the template specific primer sequence and a unique barcode tag of 6 to 10 nucleotides. The PCR reactions were performed in triplicate in a total volume of 25 μL containing 12.5 μL KAPA HiFi Hotstart 2x ReadyMix (Roche), 0.5 μL Bovine Serum Albumin (BSA) (10 mg/μL), 1 μL of each primer (2.5 μM), 7 μL UltraPure™ water (Invitrogen™) and 3 μL extracted eDNA. Six (12S) and eight (COI) PCR negative controls were included by replacing the extracted eDNA with 3 μL of UltraPure™ water.

The 12S target sequence was amplified using the MiFish primers developed by Miya et al. (2015) which target a 163–185 bp region of the mitochondrial 12S rDNA. The universal forward and reverse primer pair (MiFish_U) were degenerated to simultaneously target Osteichthyes and Elasmobranchs (MiFish_U/E_F: 5′-GT(C/T)GGTAAA(A/T)CTCGTGCCAGC-3′, MiFish_U/E_R: 5′-CATAGTGGGGTATCTAATCC(C/T)AGTTTG-3′). The COI target sequence was amplified using the mlCOIintF and jgHCO2198 primers designed by Leray et al. (2013). These primers target a 313 bp fragment of the COI gene that is especially suited to distinguish between metazoan species.

The reactions were run on a Bio-Rad T100™ thermal cycler and began with 3 min of denaturation at 95°C, 40 cycles of denaturation for 20 s at 98°C, annealing for 15 s at 62°C and elongation for 15 s at 72°C, and ended with a final elongation step of 5 min at 72°C for the 12S barcode. The COI barcode was amplified with an initial denaturation of 3 min at 95°C, 40 cycles of denaturation for 30 s at 98°C, annealing for 30 s at 54°C and elongation for 30 s at 72°C, and ended with a final elongation of 5 min at 72°C. From each of the technical replicates, a subset was quality checked on the Bioanalyzer (2100 Bioanalyzer, Agilent) according to the protocol provided with the Agilent DNA 7500 Kit.

In the post-PCR lab, the three PCR replicates were combined into separate pools. For the 12S barcode each of the 150 uniquely indexed samples from each PCR replicate were pooled into three separate pools. For COI only 98 unique indexed primer pairs were available for sample tagging. The 41 samples, including eight negative controls, from the September field campaign were prepared with samples for another project. The 111 samples, including 17

negative controls, from the November field campaign were prepared in two separate 96-well plates. The three PCR replicates were then pooled into nine separate pools so that samples with identical barcode tags could be distinguished by the use of unique adapter tags for each pool. From each PCR product 5 μL (12S) or 15 μL (COI) was added into a 1.5 mL LoBind tube (Eppendorf). Each pool of uniquely tagged PCR products was purified using magnetic CleanNGS beads (CleanNA), by adding 1x (12S) or 0.8x (COI) of the total volume of the pool. On the magnetic holder the beads were washed twice with 60 μL of 80% ethanol. Elution of the purified PCR-products was performed in 100 μL (12S) or 50 μL (COI) of 10 mM Tris–HCl buffer at pH 8.5. After purification, the three 12S and nine COI pools were quality checked with the Bioanalyzer.

At the Admera Health Biopharma Services (NJ, USA) the PCR-pools were ligated with the Illumina TruSeq adapters and pooled into one 12S pool and two COI pools. The 2 × 300 bp paired-end sequencing of the three pools was performed using three flowcells on the Illumina MiSeq platform. The raw sequencing data was demultiplexed for each of the technical PCR replicates.

2.5 | Bioinformatic processing

The quality of the raw Illumina MiSeq sequencing reads was verified with FASTQC v0.11.9 (Andrews, 2010). The paired-end reads were then reorientated, demultiplexed and trimmed by using Cutadapt v3.5 (Martin, 2011) according to the adapted demultiplexing script from the meta-fish-pipe v1.0 bioinformatics module (Collins et al., 2021; Liu et al., 2022). After reorientation, with a maximum error rate of 15%, paired reads that did not contain both primers were discarded. The remaining paired reads were demultiplexed by using their unique sample tag with a maximum error rate of zero, followed by trimming of both primer sequences and the removal of reads shorter than 105 nucleotides. After demultiplexing, DADA2 v.1.20.0 (Callahan et al., 2016) was used for denoising, dereplication, merging, and removing of chimeric reads from the demultiplexed sequences. The taxonomic assignment of the resulting ASV sequences was performed against a custom made reference database using RDP classifier (Wang et al., 2007) in DADA2 (Callahan et al., 2016) with a minimum bootstrapping support of 80. The 12S database contained 115 unique reference sequences of 122 fish species that have been detected in beam trawls during environmental monitoring campaigns of ILVO on the BPNS. The GEANS COI reference database for macrobenthos was complemented with sequences from BOLD and MIDORI (Leray et al., 2022; GenBank release 237, 08/08/2022) and contained 56,089 sequences from 53,178 unique species. ASVs that remained unassigned at species level with RDP were successively run with BLASTn v2.12.0 (Altschul et al., 1990) against the custom made reference databases and the GenBank nucleotide database (from October 2022). Sequences were assigned to a species if there was ≥97% sequence identity and a query coverage of at least 75%. The chosen query coverage threshold was set to be less stringent in order to explore ASVs belonging to other kingdoms than Animalia for both marker genes. However, all 12S ASVs assigned to Animalia at the species level had a query coverage above 95%, while for COI, only four ASVs had a query coverage below 95% (85%, 92%, 92% and 93%). Some closely related fish species have identical 12S sequences and are thus only assigned to family or genus level. However, for two pairs of two species (*Merlangius merlangus* and *Melanogrammus aeglefinus*; *Limanda limanda* and *Hippoglossoides platessoides*) an exception was made. The ASVs assigned to either of the four species were assigned to *Merlangius merlangus* (19 ASVs) and *Limanda limanda* (13 ASVs), respectively, because the majority of these ASVs had high read counts and *Melanogrammus aeglefinus* and *Hippoglossoides platessoides* are very rare in the BPNS. For the 12S eDNA metabarcoding, only ASVs that were assigned to marine species belonging to the Pisces order were used for further analysis. 12S eDNA metabarcoding also identified five freshwater fish species (*Acipenser gueldenstaedtii* × *Acipenser baerii*, *Alburnus alburnus*, *Oncorhynchus mykiss*, *Oreochromis niloticus* and *Squalius cephalus*) that were excluded from further analysis. For the COI eDNA metabarcoding only the ASVs assigned to species and genus level and belonging to the kingdom Animalia, excluding the Chordata, were kept. After taxonomic assignment, the raw count table was cleaned by removing all the ASVs identified as contaminant by microDecon using the Niskin controls, and filter, DNA extraction and PCR negative control samples (McKnight et al., 2019). Before running microDecon, the 12S and COI eDNA metabarcoding datasets were divided into two subsets. Subset 1 contained the samples and negative control samples from the September field campaign (RV Simon Stevin), and subset 2 contained the samples and negative control samples from the November field campaign (GeoOcean V). We used the standard parameters of microDecon and grouped the samples by location (McKnight et al., 2019). After decontamination, the count data of three PCR-replicates of each sample were concatenated, using summation.

2.6 | Statistical analysis

All data analyses were carried out with the cleaned and non-rarefied data. Statistical analyses were performed using R v4.1.3 (R Core Team, 2014) and the vegan community ecology package v2.6-4 (Oksanen et al., 2007).

To compare fish and invertebrate species detected by 12S and COI eDNA metabarcoding with the fish and invertebrate species detected during the beam trawl surveys, a Venn diagram was constructed in R using VennDiagram v1.6.20 (Chen, 2022). The Venn diagrams were constructed using the presence/absence data from the morphologically determined beam trawl samples and the non-rarefied and cleaned eDNA metabarcoding data.

The observed species richness was used to compare the alpha diversity patterns obtained from eDNA metabarcoding and morphologically determined beam trawl samples. At four locations in

the transition (ftGB01, ftGB02, ftTrack2 and ftTrack3) and offshore (ftWBB01, ftWBB02b, ftWBB05b and ftWBB06b) zones no beam trawl samples were collected. Hence, only the 12S and COI eDNA metabarcoding data from locations at which the abundance data from morphologically determined beam trawl samples were available, were selected. In addition, for eDNA metabarcoding the number of biological replicates at each location differed between the coast (3 biological replicates) and the transition and offshore zone (5 biological replicates). Since a higher number of replicates may result in the detection of more species, the three biological replicates with the highest number of reads were selected for each location in the 12S and COI eDNA metabarcoding data respectively (Data S1, Sample selection). Since the beam trawl does not target pelagic fish species, the observed species richness of the 12S eDNA metabarcoding data and morphological fish catch data were also calculated with only the demersal and bathydemersal fish species. The observed species richness was calculated for each sample, and averaged across the three zones (coast, transition and offshore) and visualized with a box plot using phyloseq v1.42.0 (McMurdie & Holmes, 2013) and ggplot2 v3.4.0 (Wickham, 2016). To investigate the differences in the observed species richness between the three sampling zones (coast, transition, offshore) and the method used (eDNA metabarcoding and morphological determination), a two-way Analysis of Variance (ANOVA) was performed, on the generalized linear model with Poisson distribution, with sampling zone and method as main factors and 'zone * method' as the interaction factor. A subsequent Levine's and Shapiro–Wilk test were used to test the assumptions of homogeneity of variances and normality, respectively. A post-hoc Tukey HSD (Honestly Significant Difference) test was conducted to look at pairwise significant differences. The analysis of the alpha diversity patterns has also been performed after coverage-based rarefaction, of the eDNA metabarcoding and morphological determination datasets, using the function phyloseq_coverage_raref of the metagMisc package (Mikryukov, 2018) (Appendix S2: Rarefaction).

To analyze the beta-diversity, the non-rarefied and cleaned eDNA metabarcoding datasets (12S and COI) were double transformed by converting the read counts to the relative abundance in each sample (using total in the decostand function) followed by scaling the relative abundances of each ASV to the highest observed relative abundance across all samples (using max in the decostand function) (Kelly et al., 2019). This index of eDNA-read proportions has been shown to better represent (semiquantitatively) the observed fish community data (Guri et al., 2023). The two morphologically determined datasets (fish and invertebrates) were standardized and transformed using Hellinger (square root of the relative abundance per location) using the decostand function (Legendre & Gallagher, 2001). The Hellinger transformation is well suited for species abundance data, to make variability of the species abundances comparable. To analyze the differences in fish and invertebrate community structures between the sampling locations a distance matrix based on the Bray-Curtis method was generated with the transformed eDNA metabarcoding and morphologically determined data. First, the differences in fish and invertebrate community structures were tested at a larger scale, between the three sampling zones (coast, transition and offshore), using a permutational multivariate analysis of variance (one-way PERMANOVA, adonis 2, 9999 permutations) with one factor, the sampling zone. The one-way PERMANOVA analysis was followed by a companion multivariate homogeneity of group dispersions test (BETADISPER) and pairwise multilevel comparison test to detect the contribution of the levels to the statistical significance (function pairwise.adonis2; 9999 permutations, *p*.adjust.m = "bonferroni", PAIRWISEADONIS package v0.0.1) (Martinez Arbizu, 2020). To determine the species that are associated with each of the three zones an Indicator Species Analysis (multipatt, func = "IndVal.g", duleg = TRUE and 9999 permutations) was carried out using the indicspecies package v1.7.12 (De Cáceres et al., 2010). To visualize this, the relative read abundances of all detected fish species and 50 invertebrate species with the highest relative read abundances were visualized in two separate heatmaps to determine which species are associated with the three zones. The heatmaps were constructed with ggplot2, based on the cleaned, non-rarefied and double transformed 12S and COI eDNA metabarcoding datasets. In each heatmap, the fish and invertebrate species were clustered according to the hierarchical cluster analysis using the ward.D agglomeration method in hclust from the stats package v4.2.2 (Murtagh & Legendre, 2014).

Next, potential differences in fish and invertebrate community structures at a smaller scale, inside and outside the OWF area, were analyzed using a two-way PERMANOVA (adonis 2, 9999 permutations), with sampling area (transition vs. offshore) and overall OWF effect (inside vs. outside OWF) as main factors. Homogeneity of group dispersions test (BETADISPER) and pairwise multilevel comparison tests were performed as described above. Due to the low number of beam trawl samples taken inside the OWFs no PERMANOVA analysis was performed with the morphologically determined data. The species community structures were also visualized using a non-metric multidimensional scaling (NMDS) with two dimensions ($k = 2$).

3 | RESULTS

3.1 | Data overview and taxonomic assignment

For the metabarcoding data generated via the 12S marker, an average of 2,296,092 ± 904,588(SD) reads per PCR pool remained after filtering, merging and removal of the chimeric sequences. This translated to an average of 15,106 ± 15,233(SD) reads per sample and a total of 3415 ASVs. After taxonomic assignment, only 350 ASVs (10.2%) were assigned to 63 marine fish species at species level (Figure S1). However, they represented the majority (78.7%) of the reads (Figure S2). A total of 431,000 reads, represented by 345 ASVs, were also present in the negative controls, the majority of which were detected in the Niskin (343,479 reads, 79.7%) and negative filter (85,819 reads, 19.9%) controls (Figures S1 and S2). In the Niskin controls 95.5% of the reads (from 127 ASVs) were assigned to fish species, in the negative filter controls this decreased

to 16.6% of the reads (from 20 ASVs). In the negative controls taken during eDNA extraction and PCR amplification only 0.6% of the reads (from 9 ASVs) were assigned to fish species. After removing contaminant ASVs, a total of 3250 ASVs remained of which 309 were assigned to 62 fish at species level. An additional 22 ASVs remained unassigned at fish species level due to the low taxonomic resolution of the 12S barcode between four groups of closely related fish species (*Ammodytes marinus*, *Ammodytes tobianus* and *Hyperoplus lanceolatus*; *Chelidonichthys cuculus*, *Chelidonichthys lucerna* and *Eutrigla gurnardus*; *Alosa alosa* and *Alosa fallax*; *and Chelon ramada and Chelon labrosus*). A total of 6 ASVs were assigned to Gadidae sp. by DADA2, however no assignment to species level was received by BLASTn against our own reference database or GenBank.

For the COI marker, a total of 12,364,150 reads remained after filtering, merging and removal of the chimeric sequences. For samples collected in November at location ft230 and the transition and offshore zones, an average of 38,280 ± 70,450(SD) reads per sample remained. An average of 14,359 ± 13,541(SD) reads remained per coastal sample collected in September. Together, these raw reads were allocated to 9701 ASVs, of which only 481 ASVs (5.0%) received a taxonomic assignment (Figure S3). Of these 481 ASVs, 255 ASVs were assigned to 108 species belonging to the kingdom Animalia, representing 2,152,456 reads (17.4%). The second most common kingdom was the Chromista (1,741,086 reads, 14.1%). However, the majority of the reads did not receive a taxonomic assignment (8,048,842 reads, 65.3%) (Figure S4). A total of 9907 reads were detected in the negative control samples, 99.5% of which were detected in the Niskin controls, in which 28.7% of the reads were assigned to Animalia, 63.2% of the reads were assigned to other kingdoms, and 8.1% of the reads did not receive a taxonomic assignment. After removal of contaminant ASVs, a total of 9601 ASVs remained of which 230 ASVs were assigned to 106 unique invertebrate species, 19 ASVs were assigned to invertebrates to genus or family level, since no identification to species level was received after the full taxonomic assignment procedure. In addition, COI eDNA metabarcoding was also able to detect 22 unique fish species spread over 25 ASVs.

3.2 | eDNA metabarcoding versus morphological determination of beam trawl samples

Morphological determination of beam trawl samples resulted in the detection of 42 fish species across all sampling locations, of which 36 species (85.7%) could also be retrieved via eDNA metabarcoding (Figure 2). The six species that could not be detected with the 12S marker were: *Alosa alosa*, *Ammodytes tobianus*, *Chelidonichthys lucerna*, *Eutrigla gurnardus*, *Hyperoplus lanceolatus* and *Zeus Faber* (Figure 2). However, eight ASVs were assigned to Ammodytidae and six ASVs to the Triglidae at family level, and two ASVs to *Alosa* and two ASVs to *Chelon* at genus level. In addition,

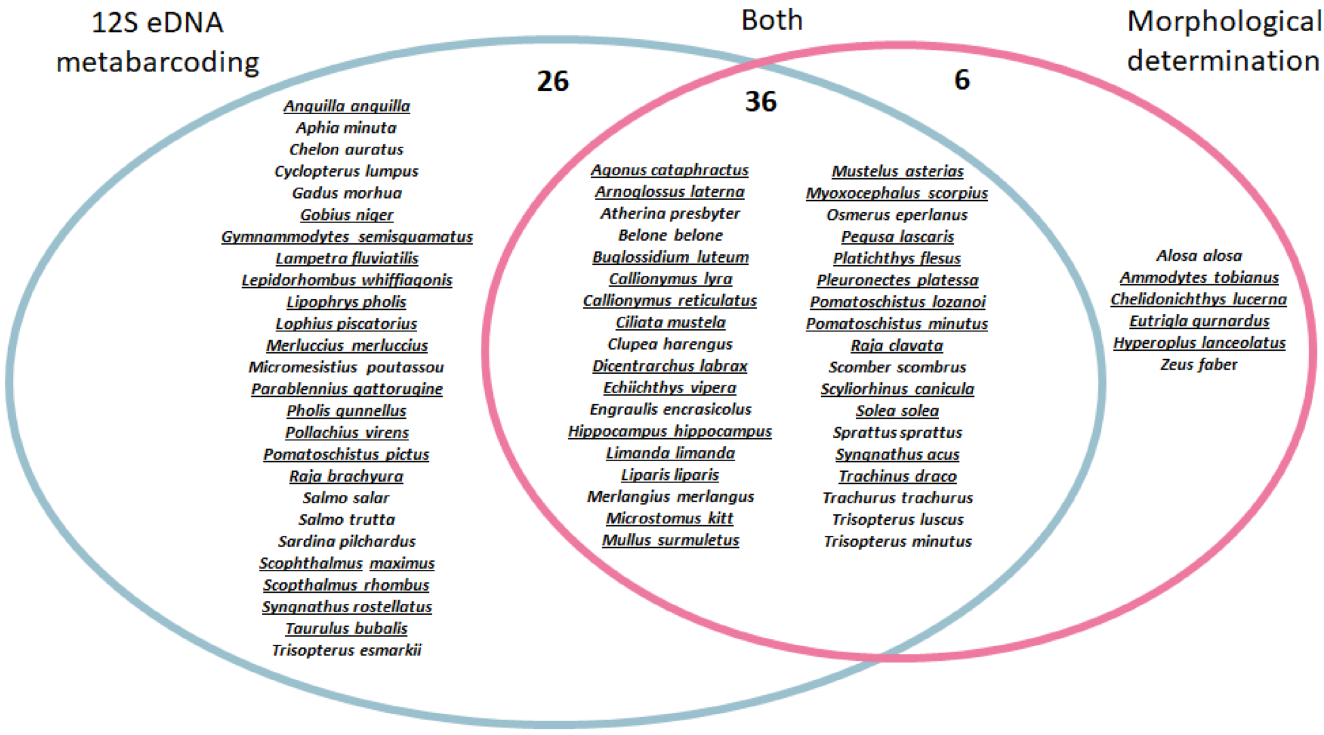

FIGURE 2 Overview of the fish species detected with eDNA metabarcoding and morphological determination of beam trawl samples. The Venn diagram shows the overlap between the eDNA data (blue) and the trawl surveys (red) of the coastal and offshore waters combined. Both methods were able to detect 36 species, 26 species were only detected by eDNA metabarcoding and six species were only detected by morphological determination of beam trawl samples. All demersal and bathydemersal fish species are underlined.

26 fish species were only detected through 12S eDNA metabarcoding (Figure 2).

Via morphological determination of beam trawl samples, 51 invertebrates were identified to species level in the epibenthos fraction (Figure 3). COI eDNA metabarcoding detected 16 (31.4%) invertebrate species in common with beam trawl surveys, whereas 35 species were unique to the beam trawl data (Figure 3). COI eDNA metabarcoding, on the other hand, detected an additional 90 unique invertebrate species. Most of them were Annelida (25 species), Cnidaria (24 species) and zooplankton species (15 species), which are not sampled by the beam trawl. The COI marker was also able to identify 22 fish species, of which *Ammodytes marinus*, *Chelidonichthys lucerna*, *Hyperoplus lanceolatus* and *Zeus faber* could not be detected with the 12S marker.

compared to morphological determination. 12S eDNA metabarcoding detected between 5 and 35 fish species per sample, while for the morphological determination method this was between 6 and 18 fish species per sample. No significant differences in the number of fish species were observed between the three zones (two-way ANOVA: $p=0.42$; two-way ANOVA Table S1.1). When only considering the demersal fish species, the main factors 'method' and 'zone' were not significant (two-way ANOVA: $p=0.26$, $p=0.81$; two-way ANOVA Table S2.1).

For the invertebrates the interaction effect 'zone * method' was not significant (two-way ANOVA $p=0.12$; two-way ANOVA Table S3.1). The posthoc analysis, showed no significant difference between the observed species richness (two-way ANOVA Table S3.2).

3.3 | Alpha diversity patterns in eDNA metabarcoding and morphologically determined beam trawl samples

For the observed species richness, no significant interaction effect 'zone * method' was observed for all fish species or for the demersal fish species only (two-way ANOVA resp. $p=0.63$ and $p=0.98$, Figure 4; two-way ANOVA Tables S1.1 and S2.1). When including all fish species, the main factor 'method' was significant (two-way ANOVA: $p<0.001$; two-way ANOVA Table S1.1) with a higher number of fish species detected with 12S eDNA metabarcoding

3.4 | Fish and invertebrate community structures at BPNS scale

Morphological determination and eDNA metabarcoding both showed that the zone significantly affected the fish and invertebrate eDNA community structures (one-way PERMANOVA: $p<0.001$; one-way PERMANOVA Tables S1.1, S2.1, S3.1 and S4.1). Pairwise tests indicated that the fish community structures differed between all three sampling zones: coast, transition and offshore (pairwise tests $p<0.001$; one-way PERMANOVA Tables S1.2 and S2.2). The invertebrate community structure differed significantly between the

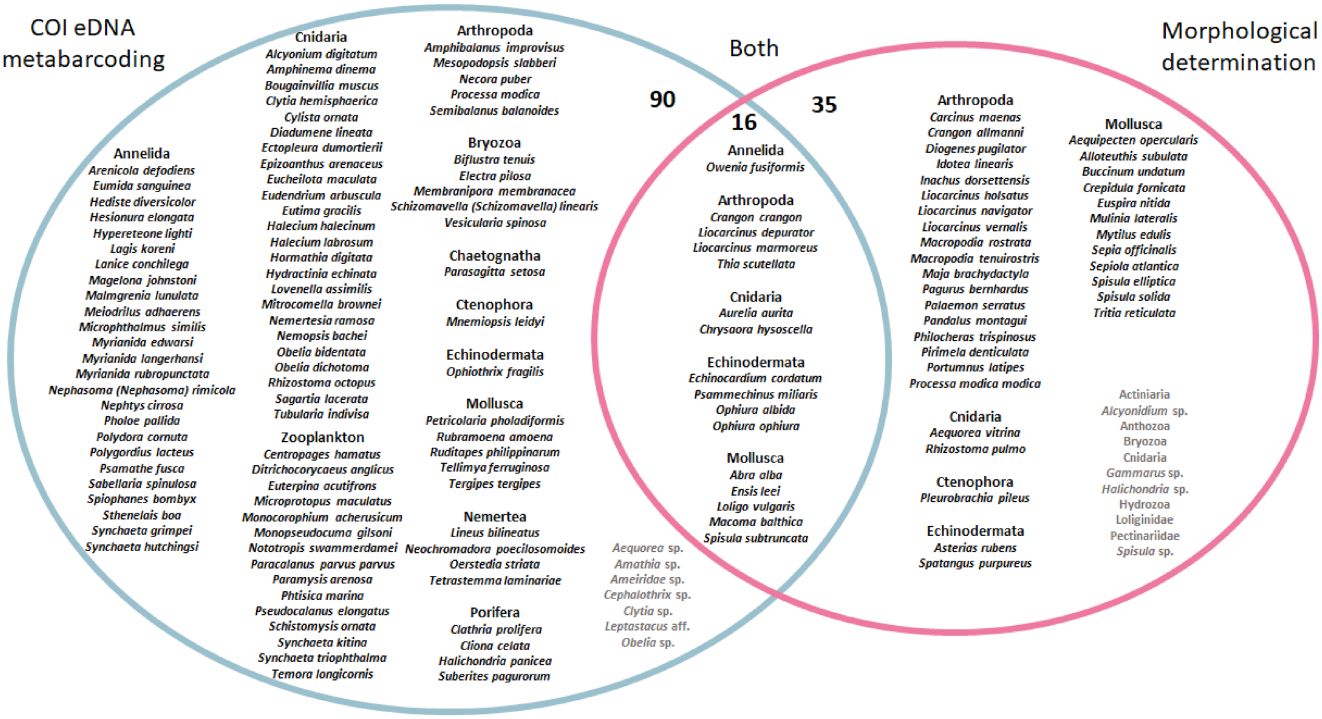

FIGURE 3 Overview of the invertebrate species detected with eDNA metabarcoding and morphological determination of beam trawl samples. The Venn diagram shows the overlap between the eDNA data (blue) and the trawl surveys (red) of the coastal and offshore waters combined. Both methods were able to detect 16 species, 35 species were detected by trawling and 90 species were only detected by eDNA metabarcoding.

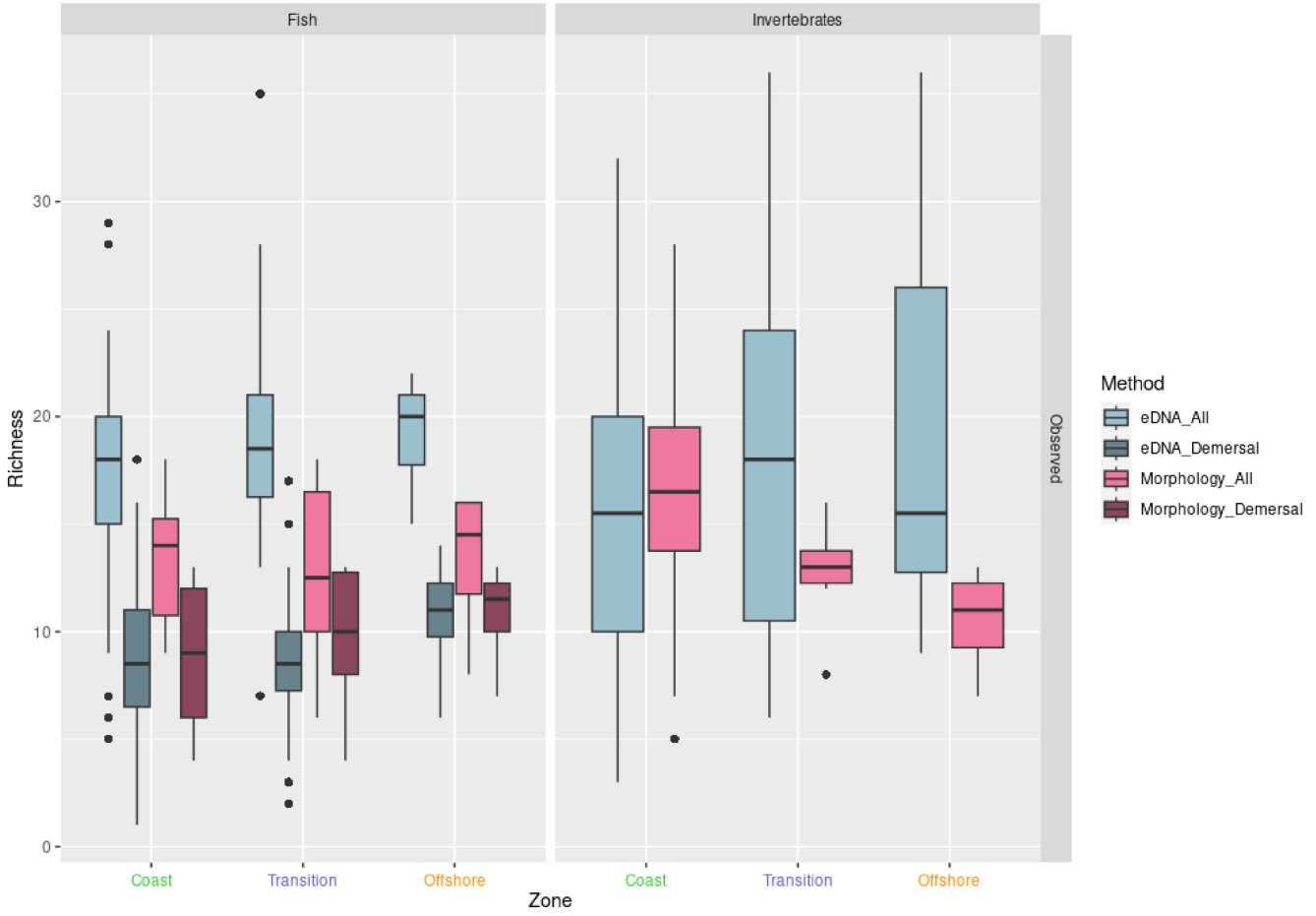

FIGURE 4 Observed species richness in each of the three zones. The observed species richness in each zone was calculated based on the fish (right) and invertebrate (left) species detected by morphological determination (red hues) and eDNA metabarcoding (blue hues). The lighter colors refer to the species richness when all fish and invertebrate species are included and the darker colors refer to the observed species richness when only the demersal fish species are considered. The box is drawn from the first quartile to the third quartile and the black line represents the median. The whiskers represent the values larger and smaller than 1.5 times the third and first quartile. The black dots are the outliers that lie beyond the range of the whiskers.

coast and the transition and offshore zone (one-way PERMANOVA: pairwise tests $p < 0.001$; one-way PERMANOVA Tables S3.2 and S4.2). No significant difference was observed between the transition and offshore zone (one-way PERMANOVA: pairwise tests $p > 0.05$; one-way PERMANOVA Tables S3.2 and S4.2) in the COI eDNA metabarcoding and morphological determination dataset. Although the PERMANOVA results from the 12S and COI eDNA metabarcoding dataset and the morphological determined fish dataset could both be affected by non-homogenous dispersion of the samples (betadisper: $p < 0.001$ for 12S and COI and $p = 0.003$ for fish; one-way PERMANOVA Tables S1.3, S2.3 and S3.3), the betadisper plot supports a distinct separation of the clusters per zone (Figures S5.1, S5.3 and S5.5).

The NMDS-plots, based on the Bray–Curtis dissimilarity matrix of the double transformed eDNA metabarcoding and Hellinger transformed morphologically determined data, showed that the coastal samples are well separated from the transition and offshore samples for all datasets (Figure 5). It is important to note that the coastal samples were collected during a different campaign than the transition and offshore samples, which may result in a confounding effect of the sampling campaign on the observed spatial pattern. However, one coastal location was sampled during the November campaign, and all three samples of this location were also separated from the transition and offshore samples (Figure S6). Moreover, data from autumn 2022 in which all samples from the three zones were collected during the same campaign show the same spatial clustering of coastal locations (Dukan et al., submitted). The invertebrate species detected by morphological determination also showed a clear separation of the clusters between the beam trawl samples taken in the transition and offshore zone. For the two eDNA datasets, there was no clear segregation between the samples collected in the transition and offshore zone (Figure 5).

The Indicator Species Analysis found consistently more species in the 12S eDNA metabarcoding dataset compared to the morphological identification of beam trawl samples, and included four of the five indicator species found in the morphological beam trawl dataset (Data S1, Indicator Species). In the coastal zone *Sprattus sprattus* was identified as an indicator species by both methods, while, 12S eDNA

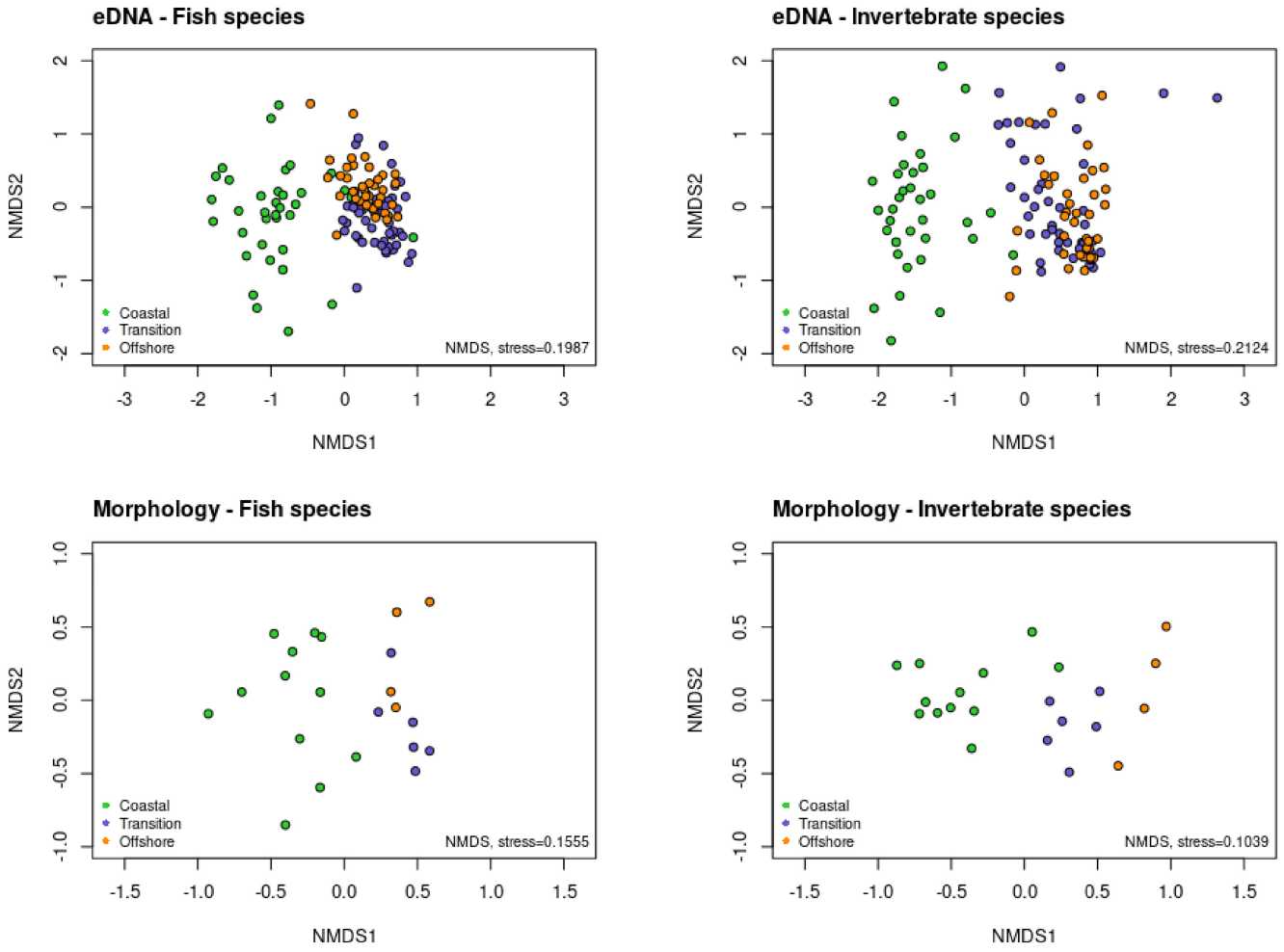

FIGURE 5 NMDS plot of the Bray-Curtis dissimilarities from eDNA metabarcoding and morphological determination based data of taxonomic profiles of the three sampling zones. The NMDS plots were constructed based on the Bray-Curtis dissimilarities at species level between the samples. Each point represents one sample taken at each of the three sampling zones: the coast (green), and transition (purple) and offshore (orange) zone. (NMDS with $k = 2$).

metabarcoding failed to identify *Platichthys flesus* as an indicator species (Data S1, Indicator Species; Figure S7). In the transition zone *Merlangius merlangius* was the only common indicator species and in the offshore zone *Echiichthys vipera* and *Mullus surmuletus* were detected as indicator species by both methods (Data S1, Indicator Species; Figure S7).

In the coastal zone, COI eDNA metabarcoding was able to detect all the indicator species identified with morphological determination of the invertebrate species in the beam trawl samples. These were *Chrysaora hysoscella*, *Crangon crangon* and *Abra alba* (Data S1, Indicator Species; Figure S8). However, COI eDNA metabarcoding also detected an additional 26 indicator species. For the transition and offshore zone both methods identified different invertebrates as indicator species (Data S1, Indicator Species). *Ophiura ophiura* was detected as the only indicator species for the transition zone by morphological determination, but was identified as an indicator species for the coastal zone by COI eDNA metabarcoding (Data S1, Indicator Species; Figure S8). Both invertebrate species, *Sepia officinalis* and *Liocarcinus marmoreus*, detected by morphological determination to be indicative for the offshore zone were not detected by COI eDNA metabarcoding (Figure 3; Data S1, Indicator Species). The seven offshore indicator species detected with COI eDNA metabarcoding were not caught in the beam trawl samples since they belong to the Cnidaria (*Amphinema dinema*, *Bougainvillia muscus*, *Clytia hemisphaerica*, *Lovenella assimilis* and *Tubularia indivisa*) or were zooplankton species (*Centropages hamatus* and *Ditrichocorycaeus anglicus*) (Figure 3; Data S1, Indicator Species).

3.5 | Fish and invertebrate community structures in and outside OWFs in the transition and offshore area

For the fish community structures a significant 'zone * impact' interaction effect was detected for the 12S eDNA metabarcoding dataset (two-way PERMANOVA: $p < 0.001$; two-way PERMANOVA Table S1.1). Pairwise tests indicated that the fish community structures differed between all four sampling areas: inside and outside the OWFs in the transition and offshore zone (pairwise tests $p < 0.01$; two-way PERMANOVA Table S1.2). Hence, the differences in the fish species community structures are dependent on the zone in which the samples were taken and the impact of the OWF. The nMDS ordination showed no clustering of samples by impact (inside vs. outside the OWF) while the two zones (transition vs. offshore) were only partially overlapping

(Figure 6). These observations are supported by the betadisper plot that shows a distinct separation of the clusters per zone outside the OWFs but not inside the OWFs (Figure S5.2). In addition, different levels of dispersion were found for the fish community data by zone and impact combined (betadisper: $p=0.01$) and by impact alone (betadisper: $p<0.001$), but not by zone (betadisper: $p=0.15$) (Tables S1.3–S1.5). The heterogenous dispersion and betadisper plot both indicate that the heterogeneity of the fish communities is greater outside the OWFs compared to inside the OWFs (Figure S5.2).

For the invertebrate communities, a significant interaction effect was detected for the COI eDNA metabarcoding dataset (two-way PERMANOVA: $p=0.01$; two-way PERMANOVA Table S2.1). However, the pairwise tests only detected a significant difference between inside and outside the OWF in the transition zone (two-way PERMANOVA: $p=0.02$; two-way PERMANOVA Table S2.2). The nMDS plot did not reveal clustering based on zones or impact (Figure 6). For the invertebrate community data we found different levels of dispersion by zone and impact combined (betadisper: $p=0.001$), but not for the zone (betadisper: $p=0.41$) and impact of the OWF (betadisper: $p=0.08$) alone (Tables S2.3–S2.5).

4 | DISCUSSION

Our findings demonstrate that eDNA metabarcoding detects significantly more fishes and invertebrates than beam trawl surveys and is an appropriate tool to describe patterns in marine fish and invertebrate community structures both at a wider (BPNS) and a smaller scale (OWF). However, only 12S eDNA metabarcoding is able to accurately represent fish data from conventional beam trawl surveys.

4.1 | 12S eDNA metabarcoding adequately detects fishes found in beam trawl surveys, while very different invertebrate communities were observed with the used COI assay

Previous studies have already demonstrated that eDNA metabarcoding and morphological determination based studies are compatible and that the combined use of both methods increases the number of identified species in various marine environments (Derycke et al., 2021; Sigsgaard et al., 2017; Stat et al., 2019; Valdivia-Carrillo

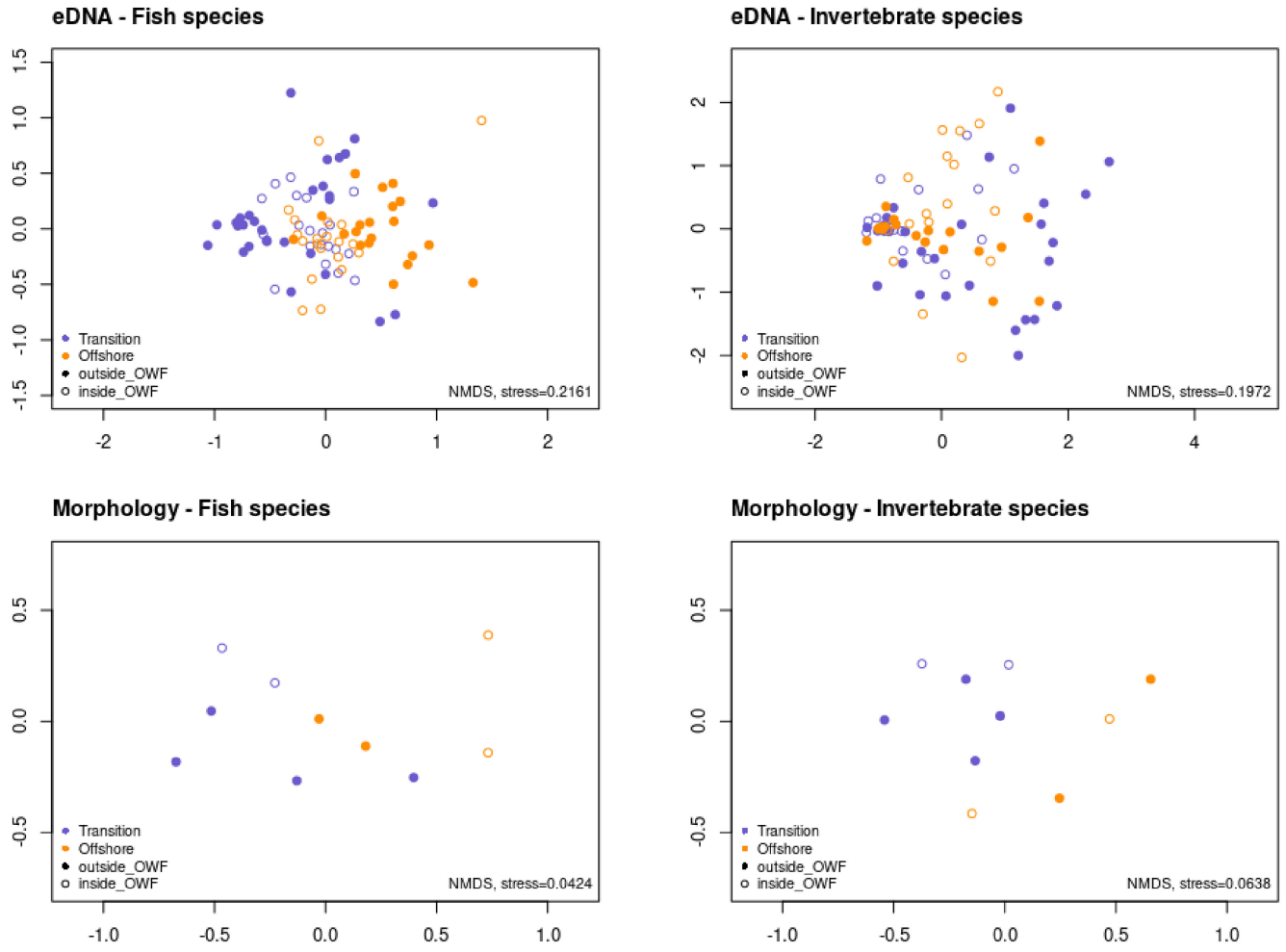

FIGURE 6 NMDS plot of the Bray-Curtis dissimilarities from eDNA metabarcoding and morphological determination of the offshore wind farm (OWF) and its reference areas. The NMDS plots were constructed based on the Bray-Curtis dissimilarities at species level between the samples. Each point represents one sample taken at each of the two sampling zones: the transition (purple) and offshore (orange) zone. The shape indicates if the sample was taken inside the OWF (open circle) or outside the OWF (full circle). (NMDS with $k=2$).

et al., 2021; van Bleijswijk et al., 2020). This is also true for the 12S and COI eDNA metabarcoding data collected in the BPNS.

Only six fish species were uniquely detected with morphological analyses of the beam trawls. These species were missed in the eDNA dataset due to the lack of taxonomic resolution of the 12S barcode, or because some species were very rare (for instance *Zeus faber*). In order to correctly report taxa, we recommend to group species with identical 12S barcodes to genus or family level.

COI eDNA metabarcoding failed to detect 35 out of 51 invertebrate species, only three of which (*Aequorea vitrina*, *Mulinia lateralis* and *Pirimela denticulata*) were absent from the reference database. Most invertebrates were likely missed due to the high amplification of species that do not belong to the kingdom Animalia and the detection of a high number of small pelagic animal species, such as cnidaria and zooplankton species which are not the target of beam trawling. Moreover, beam trawls target mainly benthic dwelling invertebrate species which are often characterized by the occurrence of an exoskeleton limiting an optimal release of DNA into the environment (Andruszkiewicz Allan et al., 2021; Crane et al., 2021). As such only the female egg carrying crustaceans are likely to shed the majority of the eDNA concentration of those species into the environment (Crane et al., 2021). Additional macrobenthic species may be detected by including eDNA from the sediment instead of the water column, but recent studies analyzing eDNA from sediment samples show that this number is likely to be limited in view of the low amount of MOTUs that were uniquely detected in the sediment samples compared to water samples (Tagliabue et al., 2023) and the low amount of invertebrate species that were simultaneously detected with morphological determination and COI eDNA metabarcoding (Willassen et al., 2022).

4.2 | eDNA metabarcoding captures community structure differences at wider BPNS scale

As already found in previous studies that used eDNA data from deeper and often calmer waters (Fraija-Fernández et al., 2020; Stat et al., 2019; Valdivia-Carrillo et al., 2021), our results confirm that eDNA is spatially organized in the marine environment. eDNA metabarcoding allowed for the distinction between fish and invertebrate communities between the coastal, transition and offshore area. This corroborated community results based on beam trawl surveys where the transition community consisted of a mixture of species from the coastal and offshore communities (De Backer et al., 2022). In the three zones, 12S eDNA metabarcoding was able to detect all but one of the indicator species identified by morphological determination. Of the 32 additional indicator species identified by 12S eDNA metabarcoding across all three zones, 15 were also reported in the long term monitoring study of De Backer et al. (2022). For 11 of them, the grouping of the indicator species into the three different zones matched with the abundance data that was visualized in the shade plot (figure 5 in De Backer et al., 2022).

Research based on morphological determined taxonomic classification detected different epibenthic communities related to the sediment type (De Backer et al., 2022). Our results show that morphological determination and COI eDNA metabarcoding detected strikingly different invertebrate community structures for all three zones. The three species (*Albra albra*, *Chrysaora hysoscella* and *Crangon crangon*), that were identified as indicator species for the coastal zone by both methods, have previously been described as coastal species by long term biomonitoring of the BPNS (De Backer et al., 2022; Kerckhof & Houziaux, 2003). *Ophiura ophiura* was identified as an indicator species for the transition zone by morphological determination, however, COI eDNA metabarcoding identified it as an indicator species for the coastal zone. This may be explained by the fact that *Ophiura ophiura* is known to be common across all epibenthic communities in the BPNS, but with higher abundances in the coast compared to the transition and offshore zone (De Backer et al., 2022). Morphological determination was also able to correctly identify two known offshore species, *Sepia officinalis* and *Liocarcinus marmoreus*, as indicator species for the offshore zone (De Backer et al., 2022). However, these invertebrates had either very low read abundances or remained undetected with the COI eDNA metabarcoding pipeline, respectively, which is most likely linked to the overwhelming amplification effect of species that do not belong to the kingdom Animalia.

4.3 | eDNA metabarcoding distinguishes between fish and invertebrate communities in and outside OWFs

With the eDNA metabarcoding analyses we were able to detect differences in fish and epibenthos community structures at a wider scale (minimum distance between coastal and offshore sampling sites was around 13 km), and at a narrower scale in areas that are located in closer proximity (distance between sites ranged from 3 to 10 km), as shown by the data collected inside and outside both OWFs. Although the residual currents in the BPNS are directed to the north-east, the eDNA metabarcoding method was able to capture spatial patterns inside and outside the OWFs. As reported in previous studies, this is most likely due to the rapid decay of eDNA in marine environments, which for marine temperate species eDNA detectability quickly decreases within the first 48 h following release (Holman et al., 2022). Trawl survey campaigns performed since 2005 have revealed small but significant differences in the fish community structures inside the OWF C-Power. An expansion of the reef-effect towards the sandy environment in between the turbines was observed through the increased occurrence of hard substrate-associated species like edible crab *Cancer pagurus*, common squid *Loligo vulgaris* and seabass *Dicentrarchus labrax* in the soft sediment trawls. Especially the significantly increased abundances of blue mussel *Mytilus edulis* and anemones *Anthozoa* sp., two species dominating the epifouling

communities on the turbines suggested the artificial reef expansion. In addition, slight but significant increased densities of some common soft sediment-associated fish species (*Buglossidium luteum*, *Callionymus lyra*, *Echiichthys vipera* and *Pleuronectes platessa*) indicated the first signs of a refugium effect, probably because of a combination of fisheries exclusion and increased food availability (De Backer et al., 2020). However, no such pattern could be detected through 12S eDNA metabarcoding (Figure S7). This may be explained due to the fact that eDNA metabarcoding is less suited to determine absolute species abundances (Kelly et al., 2019). In addition, the mentioned epifouling invertebrate species were not detected with the COI eDNA metabarcoding method. For the OWF Belwind, no significant differences in fish communities have yet been observed in the long term beam trawl monitoring data (De Backer et al., 2020), while eDNA metabarcoding was able to detect differences.

4.4 | The co-amplification of microbial species in eDNA metabarcoding reduces the detection power of epibenthos species

The advantage of the COI barcode compared to other barcodes is the extensive reference database, for which there are more public sequences available than for other marker genes (Leray et al., 2022). For half of the samples taken in this study, the read depth of the COI barcode was, however, too low to capture the full invertebrate diversity (Figure S9). This was most likely caused by the amplification of species that do not belong to the kingdom Animalia, and the large fraction of ASVs (95.0%) that did not receive a taxonomic assignment. In the past, the mlCOIintF and jgHCO2198 primers that target a 313 bp long COI-fragment, have shown good results in metabarcoding experiments using bulk DNA (i.e. DNA extracted from the solution obtained after blending or crushing all macrobenthos species sorted from a sample) (Derycke et al., 2021; Gleason et al., 2021). However, for eDNA samples, our data indicate that the taxonomic groups targeted by the degenerated primers are too broad, which might have caused an increased co-amplification of species, other than Animalia, that overwhelmed and outcompeted the focal species under study (Collins et al., 2019; Siddall et al., 2009). In addition, the 313 bp COI-fragment is relatively long, and as a result the primers may bind better to high quality cellular DNA compared to the more degenerated eDNA (Suter et al., 2021). Picoplankton and zooplankton species that are very abundant in nutrient-rich waters such as found in the BPNS might be collected on the 0.45-μm Sterivex filters (Suter et al., 2021). This may lead to a low number of reads for the invertebrate species of interest. Due to these drawbacks, COI is not recommended for the detection of epibenthos species through eDNA metabarcoding of samples with low eDNA concentrations of the species of interest and a high microbial and plankton diversity (Collins et al., 2019). However, good results were reported using COI eDNA metabarcoding on the slush water running down the fishing nets (Russo et al., 2021), which was most likely due to the higher abundance and resulting eDNA concentration of the species caught with the fishing nets (Russo et al., 2021).

The amplification of non-target sequences did also occur during the PCR-amplification of the 12S barcode with the degenerated MiFish_U/E primers. Compared to the COI barcode, this did not cause such a significant loss in the read depth for the fish species present in the BPNS, but it could, however, have affected the detection resolution of rare species (Figure S10). The majority of the ASVs (2648 ASVs, containing 19.2% of the reads) were on average 77 bp longer than the 12S barcode. Of these longer ASVs, 1212 ASVs received a taxonomic assignment that matched the 16S gene found in Bacteria (959 ASVs), Chromista (222 ASVs), Plants (26 ASVs) and Protists (5 ASVs). The co-amplification of non-target 16S sequences with the MiFish primers has also been described by other researchers, where the number of reads from the non-target amplification varied between 10% and 50% (Bylemans et al., 2018; Collins et al., 2019; Gold et al., 2020; Miya et al., 2020). This has been linked to the fact that mitochondria originated from microbial endosymbionts by ancient eukaryotes (Gold, Curd, et al., 2021; Roger et al., 2017). Moreover, the homology between the vertebrate 12S and prokaryotic 16S genes has been well described (Gold, Curd, et al., 2021; Minamoto et al., 2021). Since the non-target sequences are longer than the vertebrate 12S fragment a double size selection during the library preparation could reduce the wasted sequencing effort in the future (Di Muri et al., 2023; Guri et al., 2023; Larson et al., 2022).

4.5 | Quality control is needed to identify false positive and false negative detection with eDNA metabarcoding

The strengths and weaknesses of eDNA metabarcoding have already been well documented (Beng & Corlett, 2020; Derycke et al., 2023; Elbrecht et al., 2017; Hinz et al., 2022). During this study we also came across some of the weaknesses associated with eDNA metabarcoding. During data analysis, it became clear that all taxonomic assignments must be considered with care. Some ASVs received ambiguous hits that matched multiple species or were matched to species that are not present in the BPNS. The MiFish_U/E primers target conserved sequences that flank a highly variable region in the mitochondrial 12S rDNA (Gold, Sprague, et al., 2021; Miya et al., 2015; Zhang et al., 2020). For some closely related fish species the 12S barcode used in this study was highly conserved, resulting in false negative detections depending on the taxonomic assignment algorithm used (DADA2 vs. BLASTn). For seven families present in the BPNS (Ammodytidae, Clupeidae, Gadidae, Gobiidae, Mugilidae, Pleuronectidae and Triglidae) the p-distances between fish species were below 0.05, which is too low for an accurate assignment using the RDP classifier in DADA2 with a minimum bootstrapping support of 80. Subsequent identification at species level using the custom reference database

was possible by BLAST for an additional 96 ASVs (Data S1, 12S Taxonomic assignment). In addition, the taxonomic assignment by BLASTn against GenBank sometimes results in false positive detections due to the assignment of ASVs to species that are not native to the BPNS, such as the fish species *Chelidonichthys spinosus*, *Clupea pallasii*, *Myzopsetta ferruginea* and *Platichthys stellatus*, and the sponge *Clathria prolifera*. The identification of non-native species was also described in the study of Sigsgaard et al. (2017), when sequences assigned to *Clupea* matched with a 100% identity to both *Clupea harengus* and *Clupea pallasii*. During this study, we learned that when taxonomic assignment with DADA2 and BLASTn are used successively, most ASVs assigned to fish (92.6%) could be assigned to species level. For the other 7.4%, the assignment to species level was not unique for one single species. Thus, both methods are compatible but not exclusive and should be used together to maximize species identification.

The absence of *Zeus faber* from the eDNA 12S metabarcoding data is a false negative detection. A single specimen of this species was found in the beam trawl in two sites (ftWT2tris and ftWBB07) and COI eDNA metabarcoding detected the species in three samples (ftTrack2_2, ftTrack2_3, ftWBB03_1, in close proximity to the two mentioned sites) indicating that eDNA of the species was present in our extract. Here, PCR bias is the most likely cause of this false negative result, which can originate from PCR stochasticity, which is the random preference for individual DNA strands during PCR amplification, the preference of more abundant DNA templates and amplification bias due to sequence variation in the primer annealing sites (Schenekar et al., 2020).

4.6 | Negative controls and contamination of eDNA metabarcoding data

After amplification with the MiFish_U/E primers, most of our Niskin controls had high read counts (2383 to 130,855 reads per non-rarefied sample) that were comparable to the number of reads present in the biological samples (16,884 ± 14,961). Furthermore, nearly all of these reads (95.5%) assigned to fish species were present in the eDNA water sample taken just before the inclusion of that Niskin control (Appendix S1: Negative Control). A positive correlation was also observed between the read counts of each fish species in the Niskin controls and the read counts in the biological samples taken just before each of those controls (Figure S11), indicating that the most common or abundant species are also most likely to be detected in the Niskin controls. On the other hand, only 11 fish species caught in the beam trawl taken in the previous location prior to the Niskin control also occurred in the Niskin controls, suggesting that potential contamination via this pathway is unlikely. Similar observations in Nisikin controls have been reported in a freshwater study by Gehri et al. (2021). Importantly, in the eDNA 12S metabarcoding data, the sequence diversity in the Niskin controls was much lower than in the biological samples (42 ± 41.97 ASVs per non-rarefied sample, biological samples had an average of 581 ± 245.8 ASVs per sample), indicating that a high number of reads were assigned to a low number of ASVs (Figures S1, S2 and S12). These observations suggest that amplification in the Niskin and in the negative filter controls is linked to a relatively low number of eDNA molecules that remain in the Niskin bottle or the tube of the pump after rinsing with the source water and 10% bleach respectively, and since only little competition with other eDNA molecules in the PCR reaction are possible, these remaining copies get easily amplified. This idea is already accepted by the plant research community using High-throughput sequencing for the detection of pathogens and pests, where the use of positive and/or alien controls containing a DNA quantity similar to the analyzed samples instead of negative field controls has been put forward as guideline for proper interpretation of metabarcoding data (Massart et al., 2022). In contrast, the read count in all the Niskin controls was relatively low compared to the biological samples for COI eDNA metabarcoding (13–3518 reads). This suggests that overamplification of a few left-over sequences in the Niskin controls is possibly very much dependent on the marker gene used.

A recent study showed that DNA was completely degraded after 1 min exposure to 10% bleach (Stoufer et al., 2023). Spraying bleach in the Niskin bottles followed by submerging in the water column for several minutes at each sampling location may reduce the carry-over eDNA signal in the Niskin controls. In this study, the Niskin bottles were kept just below the surface for 3 min to calibrate the conductivity-temperature-depth instrument which flushed the Niskin bottle with local seawater. Therefore, we expect that the low number of "contaminating" eDNA copies that remain in the Niskin bottle will not interfere with the eDNA signal in the biological samples. A signal of contamination that would interfere with eDNA patterns in the biological samples would consist of both high ASV diversity and high read depth.

With respect to the negative filter controls, we have extended the bleaching step of the tubes from just flushing to keeping the bleach in the tubes for 10 min in our most recent sampling campaign. This removed all traces of eDNA as no sequencing reads were obtained in these samples. Good laboratory practices were further verified by the low contamination from the laboratory negative controls which indicates that cross-contamination between samples, plates and wells was kept to a minimum (Data S1, 12S_Origin of reads and COI_Origin of reads). These results also highlight the need for proper guidelines on the kind of negative controls required and on reporting the ASV and read abundance in all negative controls to allow correct interpretation of eDNA metabarcoding data.

5 | CONCLUSION

This study in the BPNS has demonstrated that eDNA metabarcoding of seawater samples is a powerful tool to capture spatial patterns in fish and invertebrate communities in shallow and well mixed marine waters, even at small spatial scales such as the OWF areas. Especially for the fishes, the detected species corresponded well

with beam trawl sampling and morphological determination. Due to the ease of sampling and non-destructiveness to the marine ecosystem, eDNA metabarcoding can greatly improve the temporal and spatial coverage of biomonitoring the marine environment. We foresee that this increased monitoring effort and the increased detection of fish species will improve our understanding on the spatial and temporal composition of marine ecosystems and how such systems are exposed and impacted by multiple pressures. For epibenthos, COI eDNA metabarcoding detected many other invertebrate species than the beam trawl surveys while similar spatial patterns were observed between both methods. For this group of animals, eDNA metabarcoding with other marker genes may increase the detection of epibenthic taxa found in the beam trawl.

AUTHOR CONTRIBUTIONS

IC acquired, analyzed and interpreted the data and wrote the manuscript; ADB designed the study, interpreted the data and critically reviewed earlier versions of the manuscript; SM helped processing samples in the laboratory; JV helped in bioinformatic processing of the data, RB and TR contributed to the conception of the study and critically revised earlier versions of the manuscript, KH organized the sampling cruise and sample collection, SD acquired funding, designed the study, conducted the field work, interpreted the data and helped in writing the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All sequencing data and sample metadata have been archived on NCBI under the BioProject number PRJNA1032405 (SUB14012338 for the 12S eDNA metabarcoding data and SUB14013523 for the COI eDNA metabarcoding data). A version of the inferred biodiversity data is available at GBIF.org: DOI 10.15468/nvabg3 (12S eDNA metabarcoding data) and DOI 10.15468/tvng39 (COI eDNA metabarcoding data). This version includes all the species detected in the unrarefied and concatenated data after decontamination with Decontam instead of microDecon. All scripts used for bioinformatics, taxonomic assignments, and statistical analyses are available on GitHub (https://github.com/icornelis90/eDNA_metabarcoding_NJ2021.git) and Zenodo (DOI 10.5281/zenodo.11280892).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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