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ORIGINAL ARTICLE

Capturing drifting species and molecules—Lessons learned from integrated approaches to assess marine metazoan diversity in highly dynamic waters

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Abstract

Marine community diversity surveys require a reliable assessment to estimate ecosystem functions and their dynamics. For these, non-invasive environmental DNA (eDNA) metabarcoding is increasingly applied in zoological studies to complement or even replace traditional morphological identification methods. However, uncertainties remain about the accuracy of the diversity detected with eDNA to capture the actual diversity in the field. Here, we validate the reliability of eDNA metabarcoding in identifying metazoan biodiversity in highly dynamic marine waters of the North Sea. We analyzed biodiversity from water (eDNA) and zooplankton samples with cytochrome c oxidase subunit 1 (COI) and 18S rRNA (18S) metabarcoding at Helgoland Roads and validated the optimal molecular resolution by morphological and molecular zooplankton identification (metabarcoding) with the result of merely a few false-negative detections. eDNA and zooplankton metabarcoding resolved 354 species from all major and in total 16 metazoan phyla. This molecular genetic species inventory overlapped by 95.9% (COI) and 81.9% (18S) with published inventories of local, morphologically identified species, among them neozoa and rediscovered species. Even though half of all species were detected by both eDNA and zooplankton metabarcoding, the methods differed significantly in their detected diversity. eDNA metabarcoding performed very well in cnidarians and annelids, whereas zooplankton metabarcoding identified higher numbers of fish and malacostraca. Species assemblages significantly differed between the individual sampling events and the cumulative number of identified species increased steadily over the sampling period and did not reach saturation. About a third of the species were detected only once while a core community of 22 species was identified continuously. Our study confirms eDNA metabarcoding to be a powerful tool to identify and analyze North Sea fauna in highly dynamic waters and we recommend investing in high sampling efforts by repetitive sampling and replication using at least 0.45 μm filters to increase filtration volume.

KEYWORDS
biodiversity, environmental DNA, ground truthing, metabarcoding, metazoa, North Sea, zooplankton

1 | INTRODUCTION

Reliably and ideally comprehensively assessing biodiversity is fundamental to revealing marine biota and their changes as a consequence of environmental change. In the North Sea, the most severe stressors are eutrophication, climate anomalies, the occurrence of non-indigenous species, and a high impact of fisheries, contaminants, and noise (Andersen et al., 2020). For biodiversity research and conservation planning, having a reliable tool to assess species assemblages is essential. This remains challenging, specifically when the aim is to determine the overall marine metazoan pelagic and benthic diversity including organisms of different taxa, sizes, and life-history traits. Established marine metazoan sampling methods are numerous but limited to a certain subset of diversity (Walters & Scholes, 2017), such as different net types and mesh sizes capturing zooplankton (Berry et al., 2019), pelagic (Bleijswijk et al., 2020) and demersal fish (Thomsen et al., 2016), dredges and grabs for benthos, epi- and infauna (Aylagas et al., 2016), visual censuses for larger animals (Wilms et al., 2022) or hydro acoustics to detect mammals or fish producing distinctive sounds (Berger et al., 2020). Moreover, once the material is collected, traditional biodiversity identification primarily relies on morphological characteristics which can be impacted by sampling (e.g. fragile and gelatinous taxa) (Deagle et al., 2018; Greve et al., 2004) or are not distinctive enough to properly identify taxa down to species level (e.g. across e.g. sibling and cryptic species, life stages through complex life cycles) (Laakmann et al., 2020). In order to circumvent many of the described sampling and identification issues, molecular-based identification methods such as environmental DNA (eDNA) metabarcoding (MB) pose valuable tools to complement traditional identification methods. eDNA MB is regarded as a non-invasive sampling strategy for metazoa, which can easily be applied under difficult conditions in hardly or complicatedly accessible areas (Carvalho et al., 2019; Reinhardt et al., 2019) and is promoted to play a major role in marine conservation research.

Although desirable, no golden standard protocol exists for eDNA MB studies on marine metazoans. The methodology always needs to be adapted to the sampled ecosystem and to the specific research question (e.g., target taxa, targeted taxonomic resolution, timescale of changes). In order to contribute towards standardization, it is essential to investigate sampling both impact and efforts in terms of volume of filtered water, biological (filter) replication, and sampling repetition (Zinger et al., 2019). Especially in the context of identifying change or species turnover in marine environments, it is crucial to understand which differences are being caused by sampling and sample processing design and which differences factually occur in the community we are investigating. We need to validate if the taxa detected with eDNA MB are present in the local and regional community or if they are the result of false-positive or false-negative detection within a particular sample (Darling et al., 2020) or a result of the sampling design, PCR biases and/or sequence annotations (Zinger et al., 2019). Especially for the last point, plausibility checks on the reliability of the assignment, the reference entry, and the species' natural distribution ranges are needed. It has been found that investigators often lack expertise on the diversity at the investigated study site or do not consider weaknesses of the underlying reference database (Klunder et al., 2019) when analyzing and interpreting diversity based on eDNA studies. Furthermore, we need to consider false-negative and false-positive site occupations (Darling et al., 2020) which can be attributed to altered eDNA concentrations, for example, as a result of degradation or transport due to water dynamics. For instance, within calm waters, the transport of eDNA off its source is probably less relevant as eDNA is not detectable in temperate marine waters after a few days (Andruszkiewicz Allan et al., 2021; Collins et al., 2018; Thomsen et al., 2012). In contrast, in sampling areas where the sea is in perpetual motion such as systems where a strong tidal flow reverses itself in a shorter period than the time required for eDNA degradation, transport is an important factor to consider. One way to overcome poor coverage is to use highly temporal resolved sampling (i.e., sampling the same location several times over a short time window). The few studies that examined repetitive sampling and the effect of tides within shorter intervals were limited to periods of 4 days or even less (Bleijswijk et al., 2020; Ely et al., 2021; Jensen et al., 2022; Kelly et al., 2018) and found communities being largely steady across tides but in fact, changing with time among sampling intervals spanning from an hour up to about 12 h. It remains to be investigated if this finding is still valid in highly dynamic waters and how much species diversity patterns vary if frequent sampling is conducted over a longer period.

Hence, for each ecosystem, ground-truthing of eDNA MB is indispensable before implementing this method as a monitoring tool. With this study, we provide prerequisites for applying eDNA MB for future studies in the North Sea. We investigated an integrated approach to reliably identify marine metazoan biodiversity in highly dynamic waters and validate the ability of eDNA MB as a non-invasive approach. We conducted our study at Helgoland Roads, which is located between two offshore islands of Helgoland in the German Bight, North Sea. Helgoland Roads is characterized by strong diurnal tidal currents and the biodiversity, the dynamics and seasonal succession of both, the pelagic and benthic fauna is particularly well studied. As a result, we were able to study the efficiency of eDNA MB specifically in highly dynamic systems. One of the most important challenges for MB approaches is the use and dependency on reliable reference databases representing the biodiversity of the target groups in the investigated ecosystem. Therefore we make use of precise species lists that are available for the macrozoobenthos of the intertidal zone ("Felswatt") (Reichert & Buchholz, 2006) and

adjacent regions (Boos et al., 2004; Harms, 1993; Zettler et al., 2018) and for meso- and macrozooplankton, which has been monitored by traditional morphological identification in a high temporal resolution time series since the mid-70s (Greve et al., 2004). In addition, a comprehensive collection of open-access sequence reference entries is available for pelagic and benthic marine metazoan species from Helgoland waters, the German Bight, and the North Sea which complements knowledge of the species diversity and allows for an optimal framework for evaluating and validating eDNA MB studies in this region. This collection comprises validated sequence data for fish (Knebelsberger et al., 2014), crustaceans (Laakmann et al., 2013; Raupach et al., 2015; Rossel & Martínez Arbizu, 2019), echinoderms (Laakmann et al., 2017), cnidarian taxa (Holst et al., 2019; Holst & Laakmann, 2014; Laakmann & Holst, 2014) and molluscs (Barco et al., 2016).

Based on these sound scientific foundations, we investigated whether MB allows for capturing the metazoan fauna in Helgoland waters and how detected assemblages differed by sampling techniques and conditions (i.e., morphological identification of zooplankton (ZP) vs. ZP MB vs. eDNA MB, volumes of filtered water (eDNA), sampling frequency, replication, filter pore sizes to capture metazoan eDNA). In particular, we (i) validated the MB methodology to universally detect all marine metazoan taxa by comparing ZP MB to morphological species identification, (ii) validated discovered metazoan diversity to known species occurrences based on open-access databases and local/regional inventories, (iii) analyzed pelagic eDNA from highly temporally resolved and replicated sampling over several days using different filter pore sizes for filtration (0.2 and 0.45 µm) and single and pooled eDNA analyses, and (iv) evaluated the best choice of marker to identify species across metazoan taxa in this ecosystem.

Based on our results, we provide recommendations for identifying marine metazoan biodiversity from eDNA sampling and analysis in highly dynamic waters, especially regarding the implications of repetitive sampling and technical replication.

2 | MATERIALS AND METHODS

2.1 | eDNA and zooplankton sampling and sample processing

Sampling was conducted at Helgoland Roads, German Bight, North Sea on nine subsequent days between June 19 and 27, 2019 (Figure 1).

We sampled at two locations at Helgoland Roads, i.e., from the pier (54.185995 N, 7.888784 E) and at the long-term monitoring station "Kabeltonne" (54.186146N, 7.900457E) (Figure 1, Table S1). In a clean canister, we collected 20 L of seawater sampled with a bucket from ca. 1 m below the surface. From the pier, we sampled water throughout the entire sampling period twice a day at 9 a.m. and 1 p.m. At Kabeltonne, we sampled water and zooplankton (ZP) in the morning on board of the research motor boat *Aade* on weekdays. In order to collect both mero- and holoplanktonic ZP of different size spectra and developmental stages, we sampled ZP according to Greve et al. (2004). Briefly, a Calcofi net (ø 1 m, 500 µm mesh size) was towed horizontally and an Apstein net (ø 17 cm, 150 µm mesh size) vertically to sample about 14,500–17,000 L and 1000–3000 L of seawater, respectively. We fixed the samples in absolute ethanol after sieving over a sieve cascade (200 and 63 µm), replaced the ethanol after 24 h, and stored them at 4°C until further processing. For morphological identification and metabarcoding (MB) analysis, we split the samples by using a Motoda-splitter (Motoda, 1959) rotating 20 times back and forth before separating the equal halves.

eDNA was collected by vacuum filtration at 30 kPa (Rocker 400 oil-free pump) on sterile nitrocellulose membrane filters (Whatman, ø 47 mm) in technical replicates of 3 × 2 L per sampling assay (0.2 and 0.45 µm pore-sizes for pier station and 0.2 µm filters at Kabeltonne). After filtration, filters were stored in 2 mL low-DNA-bind Eppendorf tubes filled with absolute ethanol and frozen at −20°C. Contaminations were avoided and controlled (Supplementary data 2).

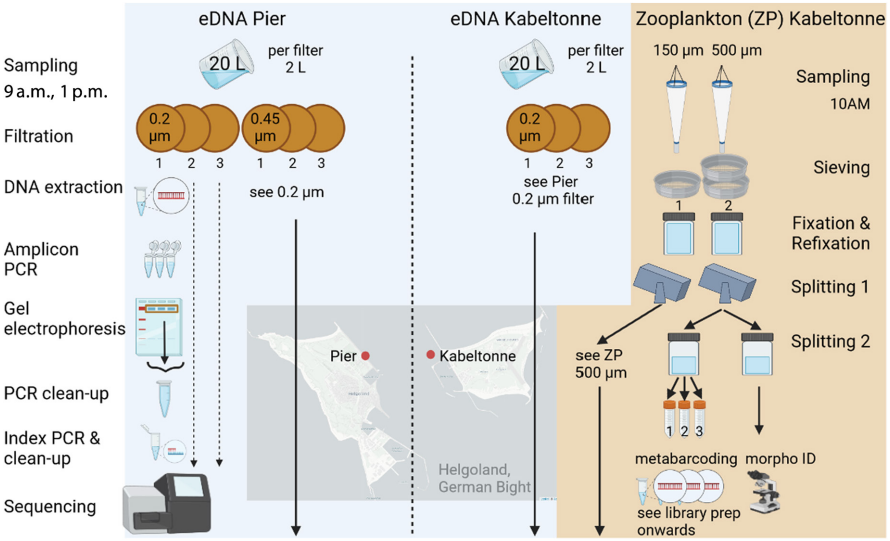

FIGURE 1 Schematic overview sampling design. Sampling was conducted at Helgoland Roads at the stations pier (eDNA; twice per day with 0.2 and 0.45 µm filters over 9 days) and Kabeltonne (eDNA with 0.2 µm filters and zooplankton (ZP)-net (150 and 500 µm net over 7 days); once on each working day; morphological analyses of ZP samples from 20th June in addition to metabarcoding). Maps were generated by R packages mapview (Appelhans et al., 2022) and leaflet (Cheng et al., 2022), and the figure was created with BioRender.com.

2.2 | DNA extraction, library preparation, and sequencing

DNA was extracted from eDNA and ZP samples by using a modified protocol of the DNeasy Blood and Tissue kit (Qiagen) (Appendix S3). Each filter technical replicate and each ZP-bulk extraction replicate was processed and sequenced individually. An exception was the "pooled samples" that were additionally sequenced to test whether replicate pooling would be an alternative to biological replication to save costs (subset pier filters 0.2 µm). For these, extracted DNA of filter replicates from the same sampling event (triplicates from morning or afternoon samples) or from the entire day (six replicates) were pooled before amplification with cytochrome c oxidase subunit 1 (COI). Libraries were prepared according to a modified protocol of the 16S Metagenomic Sequencing Library Preparation Guide from Illumina (Illumina, 2013). Modifications specifically implied the amplicon PCR with the 25 µL reaction containing 50 ng DNA template, 0.5 µL of each forward and reverse primer (20 µM), 0.25 µL HotMaster Taq DNA polymerase (QuantaBio), 2.5 µL HotMaster Taq Buffer, 0.25 µL BSA (0.1 g/L), 0.5 µL dNTP (10 mM) and 18.5 µL molecular grade water. A non-proof-reading polymerase was used since the suggested KAPA polymerase failed to amplify in combination with highly degenerated COI primers.

Two distinct markers were amplified with metazoan-specific universal primers tailed by Illumina overhang adapters: (a) COI with mlCOIintF-XT (Wangensteen et al., 2018) and jgHCO2198 (Geller et al., 2013) amplifying a region of 313 base pairs (bp) with improved taxonomic coverage and resolution in comparison to the standard Leray primer pair (Clarke et al., 2021; Wangensteen et al., 2018) and (b) 18S rRNA variable region 4 (V4) with Uni18S and Uni18SR (Zhan et al., 2013) targeted an approx. 430 bp region.

Amplification conditions for the COI amplification were modified from Lacoursière-Roussel et al. (2018), with an initial denaturation at 95°C for 15 min, followed by 30 cycles with 94°C for 30 s, 42°C for 90 s, 70°C for 60 s and final elongation at 70°C for 10 min. Cycling for 18S consisted of initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 30 s, 42°C for 30 s, 70°C for 30 s, and final elongation at 72°C for 10 min. Each sample replicate was amplified in PCR triplicates. Negative PCR control reactions with PCR-grade water were included. The entire PCR products were applied to 1.5% agarose gel stained with SYBRSafe (Invitrogen). PCR triplicate amplicons of the same sample replicate were united by cutting out from gel and cleaned with NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel). Indexing was performed with Nextera XT Index Kit v2 Set A. For index-PCR clean-up, CleanNGS magnetic beads (GC Biotech) were used. Library concentrations were quantified on a LabChip (PerkinElmer). After equimolar pooling of 96 libraries per run, the final concentration was confirmed on a BioAnalyzer (Agilent). As an internal control, 15% PhiX was spiked in. Sequencing was run on an Illumina MiSeq with MiSeq v3 reagent kit producing 2×300 paired-end reads, aiming for about 120,000 raw reads per sample.

2.3 | Sequence processing

Demultiplexing and FASTQ sequence file generation were carried out using the "Generate FASTQ" workflow of the MiSeq sequencer software. After prefiltering the sequences using the v 1.20 DADA2 R package (Callahan et al., 2016), detection and trimming of primers were performed with cutadapt (v. 3.4) (Martin, 2011) and sequence pairs in which at least one primer sequence could not be detected at 20% maximum mismatch rate, were discarded. In consideration of the read quality, the forward reads were truncated after 250–270 bp and the reverse reads after 220–250 bp. For each sequencing run, error rates were learned independently and sequences were denoised. Paired-end reads were merged with a minimum overlap of 40 bp allowing 0 mismatches and chimeras were predicted and removed. Taxonomy was assigned with the DADA2 implementation of the RDP Naive Bayesian Classifier (Wang et al., 2007) with bootstrap confidence minBoot = 95 for COI and 100 for 18S. As sequence reference library we used the MetaZooGene database (v. 2.2) (Bucklin et al., 2021) for the COI marker. For 18S, we assigned ASVs against metazoan sequences from a subset of SILVA (v. 138.1) and PR2 (v.4.14.0) both curated against WoRMS (WoRMS Editorial Board, 2022) to allow for annotation down to species level also for SILVA and merging of the resulting annotations. For curation, SILVA sequences were downloaded from SILVA's "Browser" function including all reads belonging to metazoa (on 08.10.2021). Species names were retrieved from the fasta file headers. Taxonomic ranks were queried from WoRMS with the package *worrms* (v. 0.4.2) (Chamberlain, 2020) for both PR2 and SILVA species names only allowing for entries where the species names' status were either specified as "accepted" or "alternate representation." This approach allowed for a compliance examination of the different assignments at each rank level. Moreover, we compared the assignment between SILVA and PR2 by comparing assigned ranks (phylum, class, order, family, genus, species) from these two databases for each corresponding ASV. A rank of an ASV was treated as "unassigned" if there was any disagreement of that rank assigned by SILVA and PR2 (as were the subsequent lower ranks of that ASV). In cases for which assignment to a lower rank was completed by one of the databases only, downstream rank information was accepted if at least the assignment was congruent at phylum level. In addition, species assignments were checked with 10 best hits in Genbank using the local BLAST algorithm (Altschul et al., 1990). ASVs annotated to non-metazoan according to BLAST (seven ASVs in the COI dataset) and singletons (reads of an ASV appearing only once in a sample) were removed from subsequent analyses. Analyses were run based on metazoan species level if not stated otherwise. Sequenced filtration blanks (four for COI and five for 18S) were used to screen for possible contaminants with the prevalence method provided by *decontam* package (Davis et al., 2018) in R (R Core Team, 2021).

To examine species assignments and identifications for plausibility (i.e. to identify potential false positives), we evaluated the likeliness of a species occurring in the sampling area based on their

distribution range documented in OBIS (obis.org), WoRMS, species inventory lists of Sylt (North Sea) (Armonies et al., 2018), as well as consultation of taxonomic experts (pers. communication). Based on this information, we scored a species' likeliness of occurrence as "very likely" (i.e. known to be present in the German Bight), "likely" (unknown from the German Bight but recorded distribution in adjacent waters), or "very unlikely" (species' known distribution outside the German Bight, North Sea and North East Atlantic). In addition, the invertebrate species list was compared with species inventory lists generated for the North Sea at Helgoland, "Tiefe Rinne" and "Steingrund" (Zettler et al., 2018), Helgoland Roads (Greve et al., 2004), and Helgoland rocky shores (Boos et al., 2004; Harms, 1993; Reichert & Buchholz, 2006) and Neozoa identified (Lackschewitz et al., 2015; Zettler et al., 2018) (Table S4).

2.4 | Validation of metabarcoding approach by zooplankton morphological identification

Specimens of the two net samples of June 20, 2019 (2nd sampling day) were determined by morphological identification to the lowest rank possible by using different identification guides (Castellani & Edwards, 2017; Conway, 2012a, 2012b, 2015; Hayward & Ryland, 2017; Larink & Westheide, 2011). The samples were divided into eight subsamples using the Motoda splitter as mentioned above. Species were counted in all subsamples until >50 individuals were detected and their numbers were extrapolated for the remaining subsamples. Ambiguous species identifications of seven specimens were validated by COI barcoding (Folmer primers LCO1490 and HCO2198) (Folmer et al., 1994) using Sanger sequencing according to the protocol in Laakmann et al. (2013) with the modification of an annealing temperature of 45°C. Amplification products were cleaned according to the NucleoSpin PCR clean-up manual and dispatched for sequencing to LGC Genomics, Berlin, Germany. Forward and reverse sequences were analyzed with Geneious (Biomatters Ltd.), aligned with the Geneious Alignment tool, curated, and blasted with the integrated tool against NCBI Genbank.

2.5 | Data analysis

The maps were created with the packages *mapview* (Appelhans et al., 2022) and leaflet (Cheng et al., 2022). All analyses were conducted with R (v. 4.1.2) and the packages *tidyverse* (Wickham et al., 2019) and *phyloseq* (McMurdie & Holmes, 2013) were used for data wrangling and most visualizations. Venn diagrams were created with *VennDiagram* package (Chen, 2021). Statistical analyses tested the difference of species assemblages induced by filter pore size (0.2 µm vs. 0.45 µm), station (Kabeltonne vs. pier), and sampling assay (eDNA vs. ZP MB) as well as the effect of sampling event (i.e., the 18 sample collections of eDNA at pier station) and tidal direction. A detailed description of our statistical analysis is found in S5.

3 | RESULTS

3.1 | Verification of the metabarcoding methodology

After bioinformatics processing with DADA2 and cutadapt, a total of 3,707,757 reads (12.0%) with 2333 ASVs for COI and 6,585,314 reads (19.4%) with 420 ASVs for 18S were assigned to metazoan species (Table S6). Rarefaction curves of reads assigned to metazoan species for both markers reached plateau indicating no further discovery of ASVs with increasing sequencing depth for both eDNA and ZP MB for metazoan at species level (Figure S7). We identified and removed 58 ASVs (COI) and 21 ASVs (18S) from the data set identified as contaminants.

In order to validate the molecular genetic coverage of the taxa, we cross-checked the detected diversity in ZP samples using MB and morphological identifications (Table S8) with additional confirmation of COI barcoding (Table S9).

Both methods revealed a high proportion of meroplankton on species level such as larvae of Decapoda (14), Thecostraca (4), Bryozoa (9), Echinodermata (3 Ophiuroidea, 1 Echinoidea *Echinocardium cordatum*), Polychaeta (22), Mollusca (8 Gastropoda, 4 Bivalvia), and ichthyoplankton such as eggs of Actinopteri (17) as well as holoplanktonic taxa such as calanoid and cyclopoid copepods (11) also represented by a large proportion of copepodites and nauplii, Branchiopoda (3), Ctenophora (3) and one appendicularian species (*Oikopleura (Vexillaria) dioica*). Confirmed by ZP morphological identification, all phyla, except for the phylum Phoronida, were identified with the MB methodology. For taxa identified morphologically at lower taxonomic rank levels such as families, genera, or species, representatives have been identified in the MB approach with the exception of Asteroidea (Echinodermata), and the species *Lagis koreni*, *Lanice conchilega*, *Euterpina acutifrons*, *Caligus elongatus* and *Lernaeenicus sprattae*. MB recovered 18 out of 24 genera and 9 out of 15 species which were identified by morphology. Rotifera and Nemertea were only identified by MB. On species level, MB clearly outperformed morphological identifications.

3.2 | Biodiversity recovered from metabarcoding approaches

3.2.1 | Comparison of genetic marker

A total of 354 species in 16 metazoan phyla were detected in the entire eDNA and ZP MB dataset (COI: 269 species, 18S: 127 species with 42 shared species, see S10–S12). Despite the overall success in recovering species, detection success of certain taxa was strongly determined by marker: COI identified more species within Arthropoda, Annelida, Mollusca, Cnidaria, Chordata, Bryozoa, Echinodermata, Ctenophora, Nemertea, and Rotifera and 18S complemented the dataset with phyla such as Entoprocta, Gastrotricha, Nematoda, Porifera and Xenacoelomorpha and performed much better in detecting Platyhelminthes.

3.2.2 | Validation of species identification and assignment

Our screening of false positives confirmed a high identification rate of species known to occur in the study area. Of the 269 species detected by COI, 95.9% were in agreement with known distribution records in the Helgoland area or adjacent waters (238 assigned as "very likely," 20 assigned as "likely") based on comparisons to OBIS, WoRMs and compiled species lists (Table S4). Of the total 127 species sequenced with 18S, this applied to 81.9% with 84 and 20 species, respectively. Only 11 species (4.1%) detected by COI and 23 species (18.1%) by 18S were classified as "unlikely" and are not expected in the sampling area (e.g., the fish *Gaidropsarus guttatus* and the decapod *Neocrangon sagamiensis*, both with high read abundances; see S10). Based on the compiled Helgoland fauna species list (S4), 207 (64.5%) of the 321 invertebrate species identified in this study are well-known representatives of the Helgoland fauna and we identified additional species not previously listed in the literature, most of them belonging to the classes Copepoda (30), Malacostraca (13) and Polychaeta (11). These are however among the most taxon-rich classes in this area of which we identified 25% (Copepoda), 14.5% (Malacostraca), and 18.3% (Polychaeta) in comparison to the literature-based records. However, in the literature, most of the copepods were identified down to genus level only (and according to this in the compiled list from the literature, for example (Greve et al., 2004)), and the occurrence of 23 of these is very likely and likely according to OBIS and WoRMS. For the other groups, we identified different proportions of the known fauna (e.g., Scyphozoa: 40%, Hydrozoa: 31%, Bivalvia: 14%, Gastropoda: 20%, Porifera: 3%, Echinoidea: 13% and Ophiuroidea: 38%). However, this study failed to detect all species within Asteroidea, Holothuroidea, both chaetognath and phoronid species, and all cephalopods; see S13).

Furthermore, we detected 15 macrozoobenthos and 2 planktonic species classified as neozoa according to Zettler et al. (2018) and Lackschewitz et al. (2015) such as polychaetes, anthozoa, crustacea, bryozoan, gastropods, and copepods. Moreover, we also recovered species which have not been registered since 1950 (e.g., the hydrozoa *Dicoryne conferta* and the Anthozoa *Sagartia (Cylista) viduata*) or were even registered last before 1900 (the polychaete *Megadrilus purpureus*) (Table S4).

3.2.3 | Diversity comparison from zooplankton and eDNA metabarcoding

To assess the overall effect of the sampling strategy on the resolved biodiversity from the field we compared the ZP and eDNA MB assays.

The molecular approaches recovered both, pelagic species such as ZP (holoplanktonic copepods and ctenophores; meroplankton, ichthyoplankton, and taxa with metagenetic life cycle such as Hydro- and Scyphozoa), nekton and typical benthic representatives of the epi-, in-, and meiofauna (e.g., Polychaeta, Malacostraca, Bryozoa, Ascidiacea, Anthozoans, Echinodermata, Entoprocta, Gastrotricha, Mollusca, Nematoda, Nemertea, Platyhelminthes, Porifera, and Xenacoelomorpha).

As metazoan assemblages recovered from eDNA 0.2 µm filter MB significantly differed between sampling stations pier and Kabeltonne (ANOSIM: COI: $R=0.40$, $p=0.001$; 18S: $R=0.24$, $p=0.001$), we only compared communities from Kabeltonne eDNA and ZP to reduce differences generated by spatial and temporal effects rather than by sampling assay. Biodiversity recovered from eDNA and ZP MB differed significantly for both markers (ANOSIM based on COI: $R=0.60$, $p=0.001$; based on 18S: $R=0.65$, $p=0.001$, Figure 2) with recovery of more species from the ZP samples (Figure S12). However, about half of the species were present in both eDNA and ZP (COI: 94 species; 43.5%; 18S: 36 species; 43.4%). Based on COI, three times more Malacostraca and Actinopteri species and twice more Copepoda, Gastropoda, and Hydrozoa species were identified in the ZP compared to eDNA while based on 18S, Malacostraca, Actinopteri, and Gastropoda were exclusively detected in the ZP but Anthozoa, Demospongiae, Myxozoa, Polyplacophora, and Stenolaemata in the eDNA only. Of the 216 species identified by COI, almost half (102; 47.2%) were uniquely detected by ZP MB, while the fraction of species detected only by eDNA MB was low (20; 9.3%). In the 18S dataset, the ratio was more balanced as uniquely sequenced species were 18 (21.7%) and 29 (34.9%) in eDNA and ZP samples, respectively.

We tested the detection of the same species in both the eDNA and ZP samples for every single day during the sampling period. Only about a quarter of the species were detected in both approaches during single days (25.1% COI, 26.4% 18S). Most species were only detected in the ZP MB approach (56.2% COI, 39.2% 18S), while numbers were lower for detections in eDNA only (18.7% COI, 34.4% 18S).

3.3 | Validation of eDNA methodology as biodiversity measure

As eDNA sampling locations Kabeltonne and pier for identifying marine metazoan diversity differed significantly from one another and thus influenced recovery of species and diversity patterns, we analyzed the effect of eDNA sampling strategies on biodiversity patterns on pier samples only.

3.3.1 | Effect of filter pore sizes

As clogging of filters and long filtration times (three times longer for 0.2 µm than for 0.45 µm filters) are a common problem for water filtration in turbid waters and high replication, we assessed whether a coarser pore size could be used to circumvent this issue. Thus, we tested if the filter pore size of 0.45 µm or 0.2 µm has an effect on the metazoan diversity detected. The recovery of species numbers and communities did not differ between 0.2 and 0.45 µm pore sizes (ANOSIM based on COI: $R=-0.03$, $p=1$; based on 18S: $R=-0.01$,

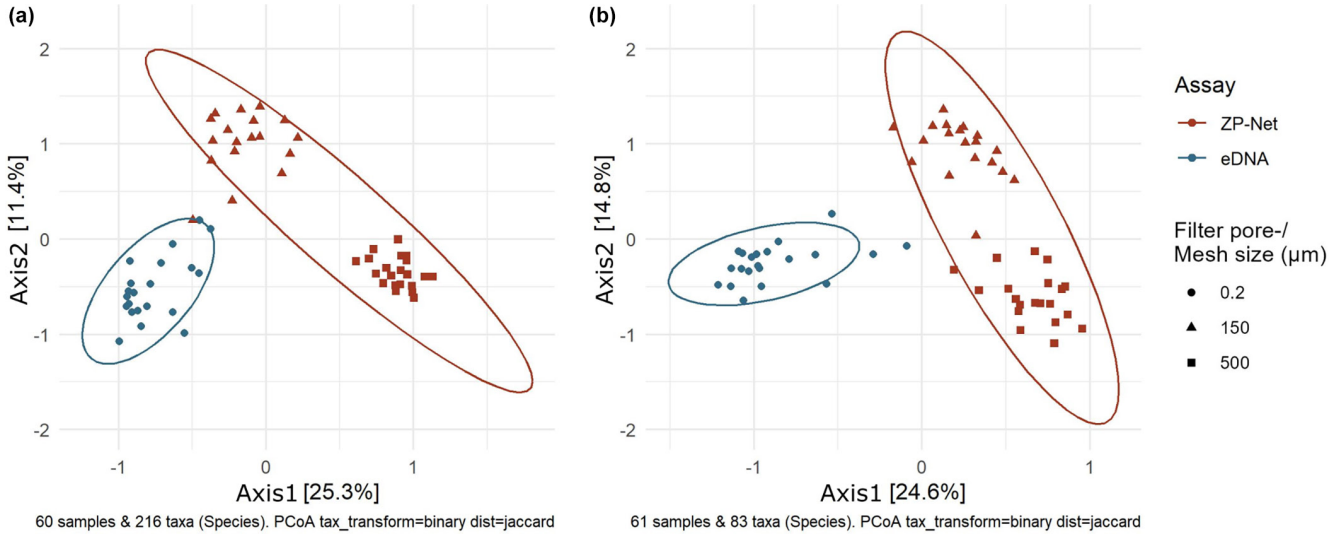

FIGURE 2 Principal Coordinate Analysis (PCoA) of zooplankton (ZP) and eDNA samples at Kabeltonne station based on Jaccard dissimilarity index on species-level. Ordination by sampling assay (ZP vs. eDNA metabarcoding). (a) COI; (b): 18S.

$p=0.9$) with more than 60% of the identified species which were found on both pore sizes for both markers (Figure S14). This was supported by PCoA plots (Figure S15) that did not show any clustering by pore size and Random Null Model analyses (Figure S16). The fraction of species uniquely found on one pore size was about the same, again for both markers without any evident trend by taxa (Table S10).

3.3.2 | Effect of sampling event and replication

As there was no significant difference in the recovery of the number of species nor species assemblages between the two filter pore sizes, the datasets of both filter pore sizes from the pier station were pooled for further analyses on the effect of sampling events.

To evaluate the relevance of sampling efforts for resolving biodiversity in highly dynamic waters from eDNA MB, we analyzed the cumulative number of species detected for both markers over the entire sampling period (Figure 3) as well as the influence of tidal currents. For all MB assays, cumulative numbers of species increased and did not plateau over the entire sampling period. COI MB resolved more species than 18S MB both for ZP and eDNA (Kabeltonne and pier) while at Kabeltonne, ZP MB identified more species than eDNA MB, both for COI and 18S. Most species were detected using COI with ZP net sampling.

Analyzing all filter replicates at the pier station identified a core community of 22 species (COI: 17 (8.9%), 18S: 7 (6.5%); two shared species) found on all 18 sampling events. In contrast to that, a third of the species were detected only once over the entire sampling period (COI: 60 species, 18S: 36 species) and species differed between the two markers. The species assemblages significantly differed between sampling events (Multiple Mantel test based on COI: $r=0.022$, $p=0.003$; based on 18S: $r=0.017$, $p=0.04$), and unique species detections were notified on all sampling events. In contrast, the tidal direction had no effect on the species assemblage (Multiple Mantel test, COI: estimate = −0.004, $p=0.2$, 18S: estimate = 0.0006, $p=0.9$). Still, species assemblages by sampling event shared overlapping species supported by PCoA plots (Figure S15) that did not show a clear-cut cluster pattern.

At Kabeltonne (with the exception of eDNA: 18S), more than a third of the species detected in both eDNA and ZP samples were identified only once in the seven sampling events (eDNA-COI: 39.5%, eDNA-18S: 24.1%, ZP-net-COI: 38.8%, ZP-net-18S: 38.5%). The ratios of the core communities were about the same throughout all assays (eDNA-COI: 16 species, 14.0%, eDNA-18S: 9 species, 16.7%, ZP-net-COI: 31 species; 15.8%, ZP-net-18S: 11 species, 16.9%).

Because we identified a strong variability in the six filter replicates analyzed from the pier station (Figure S17), we assessed whether the frequency of detecting a species on the different sampling events (max. 18) was correlated with the average number of detections on replicates per sampling event (max. six) in case of a positive detection. For both markers, we found a clear pattern that species that were detected overall more frequently during the 18 sampling events also were detected on more filter replicates on average (Figure 4). For instance, species of the core community were represented on 4.6 (COI) and 5.3 (18S) on average of the six replicates per sampling event. In contrast, for the large fraction of species that were detected on just a single sampling event, the majority (COI: 90.0%, 18S: 97.2%) was identified from a single filter replicate over the entire sampling period. An exception in the COI data were two malacostraca species *Campylaspis sulcata* and *Dexamine spinosa*, which have been identified on a single sampling event but on 4–5 filter replicates. The jellyfish *Cyanea lamarckii* in contrast was found on all but one sampling event where it was detected with an average of >5.4 filter replicates. Among the species amplified with 18S, the polychaete *Magelona mirabilis* was the one most outstanding, identified on four filter replicates during the same sampling event but not spotted on any other sampling event again.

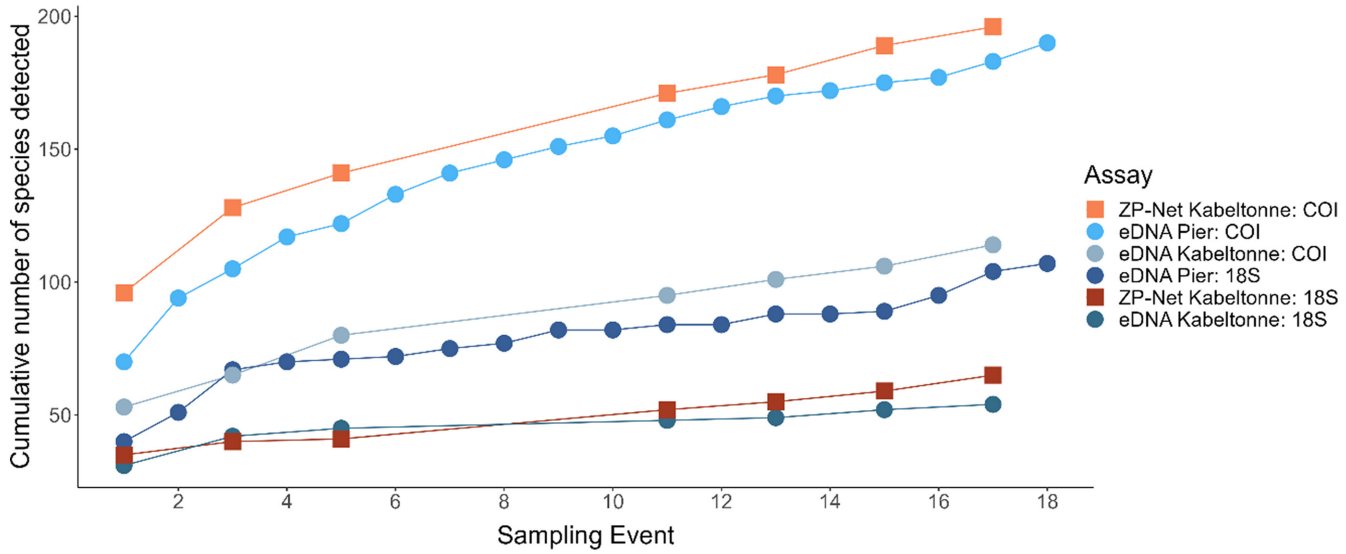

FIGURE 3 Increasing species accumulation curve from repetitive sampling at pier (eDNA) and Kabeltonne (eDNA, zooplankton (ZP)) metabarcoding over the entire sampling period. Pier station with 12 L filtered seawater per sampling event (3 × 2 L on 0.2 µm and 3 × 2 L on 0.45 µm filters) twice a day over nine consecutive days resulting in 18 sampling events. Kabeltonne station with 6 L filtered seawater per sampling event (3 × 2 L on 0.2 µm filters) and ZP 150 µm and 500 µm nets once per day on 7 days; eDNA (points), ZP (squares) MB. Markers were analyzed separately (COI: light; 18S: dark).

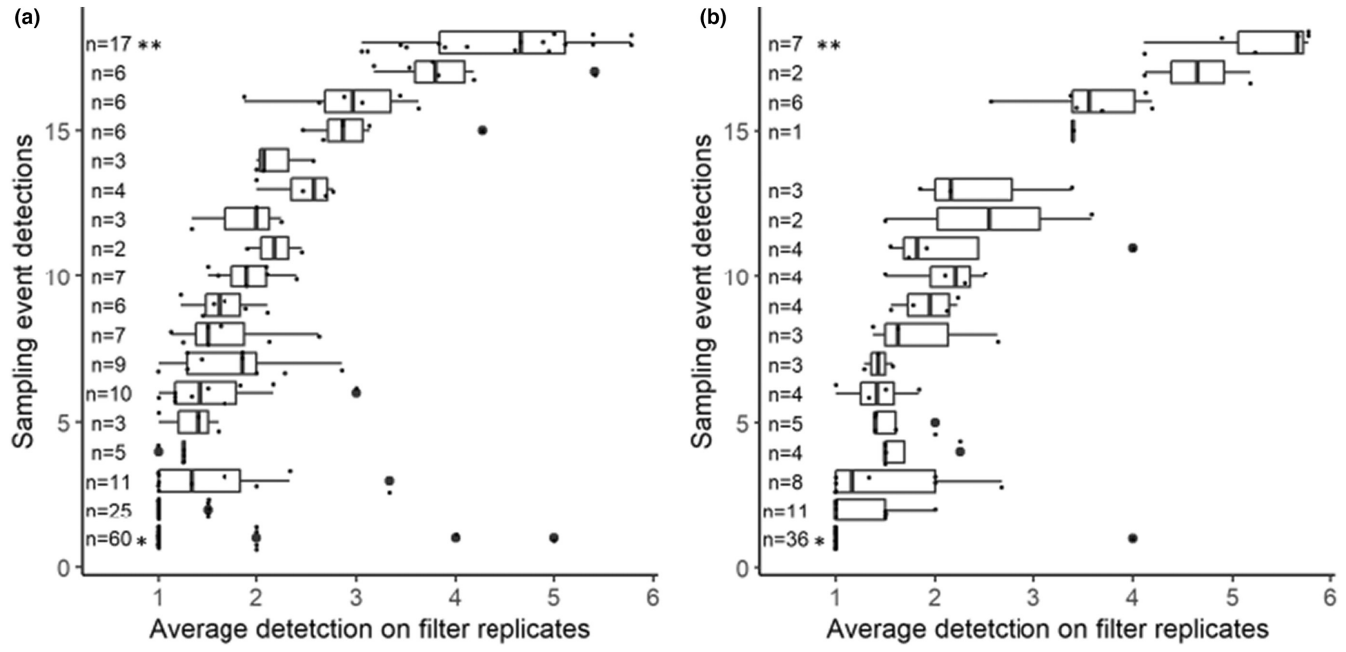

FIGURE 4 Average detection of each species on the six filter replicates (3 × 0.2 and 3 × 0.45 µm) per sampling event at the pier station, grouped by their frequency of detection during the 18 sampling events. Sampling event detections describe the frequency a species was detected over the entire time, with the core community of species detected throughout the entire sampling period at the top and single event detections at the bottom. Numbers indicate the number of species detected per sampling event detection frequency. (a) COI; (b) 18S. **: Core-community of species always detected, * species detected on one sampling event only.

As we confirmed that sequencing depth was sufficient to recover all metazoan ASVs (Figure S7) and we identified a high variability among filter replicates (Figure S17), we evaluated whether pooling of filter replicates (i.e., pooling of DNA extracts prior to PCR amplification) was as efficient in recovery of species numbers as sequencing replicates individually (Figure S18). Filter replicates of one sampling event differed not only in the number of species but also in species composition (Figure S17). Cumulative species numbers from individual filters resulted in higher species numbers compared to those analyzed from pooled DNA extracts of the individual replicates prior to amplification (Figure S18). The latter recovered about the same number of species as if an individual

filter was sequenced instead (Figure S18: single replicates), but only 42%–88% of the total species number when accumulating from individual filters. In many cases, a species sequenced with a low read number (usually <7 reads) on single replicates was not detected in the pooled sample and few species were sequenced uniquely from the pooled samples.

4 | DISCUSSION

With this study, we were able to ground truth and to confirm our integrated approach and specifically eDNA metabarcoding (MB) as a reliable tool to identify and to examine metazoan diversity and its potential changes in the highly dynamic waters of the German Bight, North Sea. We identified 354 species from 16 phyla whereof >90% are known to be distributed in the German Bight or adjacent waters with most species being typical representatives of the Helgoland marine fauna. We rediscovered species that have not been registered for decades, identified macrobenthos and neozoans as well as species that were not identified down to species level on the previous species list compiled for Helgoland waters. Among these, the largest fraction were Copepoda which were mainly listed to genus level only (e.g. *Acartia* spp., *Oithona* spp., and *Calanus* spp.) in the long-term monitoring (see Greve et al., 2004) (S4). All these results confirm the high sensitivity of the eDNA and ZP MB method as a reliable tool to monitor the pelagic and benthic metazoan communities in German Bight's highly dynamic waters. However, within Malacostraca, the new detection of *Polybius henslowii* is questionable as it was only represented by two reads and not distinguishable from *Liocarcinus holsatus* based on COI.

4.1 | Verification of diversity identified with metabarcoding

The morphological identification of the ZP sample acts as a proof of concept in order to cross-check the recovery of the different metazoan taxa in the MB approach and to identify potential issues related to primer specification, amplification biases, sequence processing, and species assignments. We verify the MB method to reliably identify North Sea marine metazoan communities as more than 90% of the genetically identified species are known to occur in this area. Species detected in ZP MB but not in eDNA MB, such as most fishes and decapods are likely the result of highly diluted or absent species-specific genetic material in the analyzed water volume due to patchiness in the highly dynamic waters at Helgoland Roads. As the rarefaction curves indicated that sequencing depth was sufficient for recovering metazoan ASVs and thus, species within the samples, we conclude that species-specific material was indeed missed by sampling effort, and not by the applied MB methodology. This might be circumvented by increasing the sampling volume of water as previously demonstrated (Bessey et al., 2020). Only a few taxa identified by morphology were not recovered by MB either as a result of misassignments to the wrong phylum (e.g. *Phoronis muelleri* with PR2 database) or lacking reference database entries (e.g. Asteroidea in MZGdb). Since there were only a few false-negative species detections (which were partly identified by MB on other sampling events within the 9 days), we assume a good sensitivity of primers and coverage of entries for North Sea metazoan species in sequence reference databases identifying the local community in highly dynamic waters of the German Bight. Thus, highly curated and complete reference databases are critical to properly identify metazoan assemblages when using molecular genetic methods.

The comparison against compiled Helgoland fauna species lists revealed only few missed or underrepresented taxa from both eDNA and ZP MB, such as sessile Anthozoa and Porifera and mobile Holothuroidea. Holothuroidea was not included in the COI MZGdb and for poriferan and anthozoan species COI is of poor applicability due to its conservative behavior in these two taxa (Bucklin et al., 2011). However, even the 18S marker did not identify these taxa and potentially other molecular markers may be needed and should be evaluated to identify these groups.

The COI primer performance exceeded our expectations as it even amplified taxa which caused problems in other studies, such as Cyclopoida, Cardiida, and Neogastropoda species (Zhang et al., 2018), analyzed from mock communities. On the other hand, the detection success of 18S, especially for copepods such as species of the genera *Acartia*, *Paracalanus*, *Temora*, *Tortanus*, and *Oithona* is in agreement with other studies (Zhang et al., 2018) with an additional sequencing success for *Centropages hamatus*. The two markers vary in their ability to amplify certain taxa. As demonstrated in other studies COI species identification was superior to 18S V4 (in other studies also V7, V8) (Clarke et al., 2017; Günther et al., 2018; Wangensteen et al., 2018) species identification which is reflected in the reliability of assigned species with <5% (COI) and >17% (18S) of annotated species which seem unlikely to be present in the North Sea and adjacent waters. Especially for 18S, these identifications are assumed to be false positive detections. *Gaidropsarus guttatus* and *Neocrangon sagamiensis* for instance were very likely *G. mediterraneus* and *Crangon crangon* as indicated by comparing the first top 10 matches from BLAST results, species well known from the sampling area. The same applies to *Calanus euxinus* (COI) which is identified as *Calanus helgolandicus* (morphology and 18S MB) and was already misassigned by COI elsewhere (Yebra et al., 2022). *Acartia (Acartiura) hudsonica* identified with both markers, might actually be correctly assigned as it is discussed to be present in the North Sea (pers. communication, see Acknowledgements). As read numbers are high for many of these species in question, we assume sequencing errors unlikely but rather an issue caused by false annotations due to closely related species. For 18S, the RDP classifier in combination with the integrated PR2 and SILVA assignment gave reasonable results for more than 80% of the species, and for the others, we assume unambiguous identification on species level, especially for closely related species. This issue was also apparent in other studies on various metazoan taxa (Brown et al., 2015, 2016; Clarke et al., 2017; Grey et al., 2018).

We used the 18S V4 marker as it is considered that more conserved markers allow a better coverage of a broad taxonomic range of metazoans and is thus, commonly applied in studies targeting marine communities (Brandt et al., 2021; Clarke et al., 2017; Di Capua et al., 2021; Loos & Nijland, 2021). Indeed, both markers complement each other and improve taxonomic coverage for metazoan species by recovering distinct community patterns which were also demonstrated in other studies (Grey et al., 2018; Wood et al., 2019). In our study, 18S particularly identified meiofauna taxa (Platyhelminthes, Nematoda, Gastrotricha, Xenacoelomorpha, and Entoprocta) while COI identified more holo- and meroplanktonic as well as epifauna taxa (e.g. Hydrozoa, Arthropoda, Annelida, Mollusca). As suggested earlier (Corell & Rodríguez-Ezpeleta, 2014), using a multimarker approach should be used whenever feasible or alternatively, investigating the taxa present with a universal 18S marker before applying primers with higher taxonomic resolution (West et al., 2020).

4.2 | Integration of zooplankton and eDNA metabarcoding to assess marine diversity

The combined eDNA and ZP MB approach successfully identified both the pelagic and benthic metazoan fauna. Our results clearly indicate that eDNA and ZP MB recover different species assemblages of the marine fauna which is in agreement with other studies (Djurhuus et al., 2018; Koziol et al., 2019; Leduc et al., 2019). ZP nets mainly catch holoplanktonic species, meroplanktonic larvae, and ichthyoplankton and are limited to certain size classes determined by the mesh size used (Djurhuus et al., 2018). This explains well why ZP MB was superior in detecting Actinopteri and Malacostraca as to both classes showed high proportions of eggs and larvae at the time of sampling confirmed by morphological analyses. ZP MB confirms a high proportion of typical North Sea meroplankton (Kirby et al., 2008). Moreover, it identifies the species-specific reproduction of benthic invertebrates and fish, gives insights into pelago-benthic coupling processes (upcoming species-specific larvae settlements), and highlights the reproductive turnover during that time of year. In particular, the trend of Arthropoda being represented by more species in ZP than eDNA samples was also observed in other studies (Djurhuus et al., 2018; Koziol et al., 2019; Leduc et al., 2019) as was the underrepresentation of larger and probably less evenly distributed taxa as Actinopteri in eDNA samples (Suter et al., 2021). On the contrary, eDNA MB additionally identified the marine fauna absent from the ZP at that time of year and gave a broader image of the pelagic and benthic fauna. This is congruent with our findings as to eDNA MB performed better in the number of species detected within many typically benthic-associated taxa, a pattern also seen in other studies (Djurhuus et al., 2018; Koziol et al., 2019; Leduc et al., 2019) and therefore more suitable for assessing the overall diversity. eDNA MB has the advantage of being not affected by missing specimens/species because of active net avoidance but is vulnerable to species' genetic material scarcely and patchily distributed. However, it is rather limited in the volume of water that can be processed and analyzed (i.e. in this study about 15,000 L by ZP net in contrast to 2 L per filter by eDNA). Thus, combining eDNA and ZP MB results not only gives the bigger picture of communities but is also an informative tool to estimate succession patterns and species-specific reproductive periods in more detail. For instance, two decapod species *Athanas nitescens* and *Hyas araneus* were detected with eDNA MB but not by net samples, potentially reflecting adult organisms present in the benthos which did not have a planktonic larval form during the time of sampling as it was off their reproductive phase. The same pattern was evident for 31 of the 53 Annelida species. Since both sampling strategies complement each other in the detected species community, providing complementary ecological information about recruitment and benthopelagic coupling processes, we recommend using both approaches in parallel for biodiversity assessments.

4.3 | Lessons learned from non-invasive biodiversity assessment

Non-invasive molecular genetic approaches such as eDNA MB are increasingly used to identify marine diversity and its change over time. Our evaluation of how the captured genetic material mirrors diversity in the field allows us to conclude and gives advice for future molecular assessments, especially for the implementation of these methods for monitoring biodiversity and inherent changes.

4.3.1 | Replication of sampling

In order to estimate a time- and cost-effective management of eDNA sampling and analysis to identify marine fauna in dynamic waters, we compared the recovery of species from individual filtration replicates to that of pooled DNA extracts from three replicates (S18). Our result of similar species numbers derived from a single replicate and from pooled replicates but high variability across the replicates clearly demonstrates replication as an important component to ensure high detection success. We identified some taxa uniquely in one of the two assays which are in agreement with other studies (Jensen et al., 2022; van den Bulcke et al., 2021). In our study, the shared species fraction among triplicates was about 32% which is only half as much as in other studies (>60%) (Jensen et al., 2022). However, the higher consistency in Jensen et al. (2022) was probably caused by allowing less variation in the species detected, as they specifically targeted fish and removed unique species detections within samples. Indeed, this variation in filter replication is caused by processes induced during sampling within turbid waters and patchy eDNA distribution rather than insufficient sequencing depth. Thus, pooling of DNA template of filter replicates prior to PCR cannot be recommended as an alternative to sequencing all filter replicates individually due to substantial variation among replicates which apparently cannot be circumvented by pooling.

4.3.2 | Choice of filter pore size

The recovery of species composition was similar for both filter pore sizes which is not in line with the detection of more operational taxonomic units (OTUs) on 0.45 μm filters than on 0.2 μm filters when analyzing specifically fish (Bessey et al., 2020). Hence, in our study, the only difference was the longer time used to filter the 2 L on the 0.2 μm filter (2/3 of the time used for filtration on 0.45 μm) because of clogging filters as a result of suspended particles in turbid water. We therefore recommend using coarser pore-size filters in order to reduce potential DNA degradation during filtration at room temperature, to reduce filtration time, and, more importantly, to increase the volume of filtrated water and thus, eDNA amount on the filters and in the samples. Especially the volume of processed water showed a strong positive correlation with the number of detected species in our study. In a few further studies, much coarser pore sizes up to 20 μm were successfully applied (Brandt et al., 2021; López-Escardó et al., 2018) and we therefore recommend using at least 0.45 μm filters in turbid waters.

4.3.3 | Sampling period and sample replication in highly dynamic waters

Specifically in regions with strong currents and continuous exchange of water bodies, it is important to evaluate to which extent eDNA sampling implied the potential to mirror the biodiversity in the field. In this context, different factors such as tidal flow with its translocation of eDNA (Hansen et al., 2018) as well as patchiness and dilution of genetic material in the water column are discussed to influence the recovery of species from eDNA. In our study, the tidal flow had no significant effect on the species assemblage recovered from the eDNA approach, although water movement is driven by the tide at Helgoland Roads. The same was found in other studies examining the tidal influence on species detection (Bleijswijk et al., 2020; Kelly et al., 2018; Lafferty et al., 2021; Larson et al., 2022). However, one study identified a significant effect of tides but this explained only 8% of the observed variation (Ely et al., 2021). Moreover, our results indicate high sampling effort is positively correlated with the detection of high numbers of species. Especially the low detection compliance of the same species at the same time in the ZP and eDNA reflects the patchiness and high dilution in the water column. Thus, increasing efforts by repetitive sampling and higher volumes of water pose drivers for the recovery of a comprehensive regional metazoan biodiversity based on molecular genetic approaches.

As tidal direction does not significantly shape the species community detected, we argue that transport of eDNA plays a minor role due to fast degradation and dilution and that most of the eDNA signals detected might be indeed shed from organisms present at the time of sampling. This does not only simplify sampling procedures as tide does not necessarily need to be considered but also provides valuable information concerning eDNA data congruency with locally appearing species. This is underpinned by our findings at Kabeltonne, where a quarter of all species detections at a specific time of sampling were consistent for eDNA and ZP MB. This ratio would presumably have been much higher if eDNA water sampling volume had been higher resulting in decreasing false negative detections. The concurring ZP and eDNA MB results suggest that ZP and eDNA particles are both subject to the same drift and transported in a similar manner, even in dynamic waters.

Only few studies addressed the effect of high-frequency water sampling to identify biodiversity by eDNA MB. Since filtration, processing, and sequencing more replicates is time-consuming and expensive, it still needs to be discussed whether it is worth increasing sampling efforts based on repetitive sampling to increase the amount of water and to analyze different water bodies and/ or to increase number of technical replicates. Our study revealed even narrow sampling events detect significantly different species assemblages which is in agreement with other studies. For example, the time point of sampling explained about a third of the variation in species communities of another tidal-shaped coastal marine environment in a 28 h sampling period (Kelly et al., 2018) as well as in a vertebrate-specific assay conducted in a protected bay over 96 h (Ely et al., 2021). This is supported by another study targeting marine vertebrates which found community composition was altered between three consecutive sampling days (Monuki et al., 2021). Another study conducting hourly sampling over 32 h found large variation in fish species richness and eukaryote classes as well as compositional dissimilarities changed with sampling time (Jensen et al., 2022).

Contrary to our findings, other studies reported very few samples being sufficient to recover habitat effects on fish assemblages (Larson et al., 2022) or present fish species diversity (Jensen et al., 2022). The latter study was however conducted in a harbor with marginal tidal effects as well as stable currents and to specifically detect fish, not universally the marine metazoan community. In contrast, we also found a core community of species present on all sampling events representing the well-known local biodiversity (with one exception). This is congruent with other studies which revealed even a small sampling size was sufficient to detect the most common taxa (Bessey et al., 2020). However, the highest proportion of detections were species detected only once out of the 18 sampling events. Hence, if a study's aim is to also capture species with low or patchy eDNA distribution, frequent sampling is essential. Concluding from our study, sampling 6 L of water per sampling event does not seem sufficient in order to capture rare genetic material in the water column as this presents itself as highly diluted, patchily distributed, or not detectable due to PCR stochasticity. Even sampling >200 L in a period of 9 days still tracked additional species without evidence of saturation not explainable by the succession patterns. Similar studies conducted in rather sheltered areas or in the vicinity of an Atoll also concluded marine metazoan diversity increased by the volume of water sampled between 5 and 30 L required for curves to plateau (Bessey et al., 2020; Ely et al., 2021; Grey et al., 2018; West et al., 2020, 2021). Models suggested even 45–95 samples per

site to detect rare species (Erickson et al., 2019) but even 6000 L were being discussed (Brandt et al., 2021).

Our results did not only show community patterns changing between sampling events but also a large variation between the technical filter replicates (these filter replicates are not considered as biological but as technical replicates as the filtered water came from 20 L well-mixed water in a canister). Analyses of marine vertebrate OTU variation in biological replicates revealed large variability with a maximum of only 13.7% of the OTUs shared among the triplicates (Andruszkiewicz et al., 2017). However, other studies demonstrated that increased sample volume per biological replicate could reduce variability to some extent (Andruszkiewicz et al., 2017; Bessey et al., 2020). In our study, the analysis of technical replicates leads to the assumption that in dynamic waters eDNA patchiness contributed to the variability between filter replicates and that the complex composition of genetic material varies largely even in a volume of 20 L when only analyzing subsamples of 2 L. Although rarefaction curves indicated sufficient sequencing depth and PCR triplicates were performed to reduce variability in amplification, we cannot exclude that sequencing DNA extract multiple times had a comparable effect in capturing additional species as sequencing multiple filter replicates separately as we did. Despite species composition, the eDNA concentration varied between technical replicates which was also demonstrated in other studies (Andruszkiewicz Allan et al., 2021).

In our study, the more frequently a species was identified over the entire sampling period, the higher the chance of it also being detected on multiple filter replicates of the same sampling event. This suggests the genetic material of the core community species is not only frequently present in the water column but also occurs in the surroundings to shed enough eDNA into the water to be detected frequently. If the aim had been to target the most common species, filtering once and sequencing only one or two replicates would likely have been sufficient. In contrast, this approach would have missed large parts of the diversity, as one-third of the species were only identified on one technical replicate over the whole sampling period. For these species, it can be assumed that the genetic material was very low concentrated/ highly diluted or patchy during the entire sampling period. There were however two arthropod species (*Dexamine spinosa* and *Campylaspis sulcata*) and the polychaete *Magelona mirabilis* which were found during only one sampling event but on most of the technical replicates of that very sampling event. This leads to the conclusion that the eDNA of these species was indeed much higher at this sampling event or even truly absent at other sampling events and that the timing of sampling determines if it can be detected. In contrast, for most other species found on one filter overall only, their detection was likely by chance and a stochastic effect due to low eDNA concentration and could theoretically be also found on other sampling events if more water had been filtered. For other species such as *Cyanea lamarckii* which have been missed on only one sampling event, we conclude that the eDNA of this species was indeed truly absent in the sampled water volume and q.e.d. not missed by chance. Thus, to increase the likeliness of detecting scarce species-specific genetic material, we recommend subsequent sampling, high replication of biological and technical replicates, and the increase of filtered water volume.

In conclusion, with this study, we verify both eDNA and ZP MB as reliable and sensitive tools to identify the marine fauna in the German Bight. The parallel eDNA and ZP MB analysis provides a comprehensive and concise picture of biodiversity and allows the identification of ongoing processes in pelagic and benthic communities. In using eDNA for marine metazoan community identification in highly dynamic waters we conclude that sampling a marine environment once, which could be referred to as "snapshot sampling", is robust in targeting the core community while this strategy is not sufficient to gain a comprehensive picture of the overall regional biodiversity.

AUTHOR CONTRIBUTIONS

Design of the study: AO and SL. Sampling: AO, ST, SL. Sample processing: AO, ST, UJ, SL. Bioinformatics/Data processing: AO, SN, SL. Statistics: AO, LK, SL. Manuscript: AO, SL. All authors discussed the results and contributed to the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

Sequence data for this study are available in the European Nucleatide Archive (ENA) at EMBL-EBI under accession number PRJEB59767 https://www.ebi.ac.uk/ena/data/view/PRJEB59767, using the data brokerage service of the German Federation for Biological Data (GFBio, Diepenbroek et al., 2014).

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SUPPORTING INFORMATION

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

[Correction added on 02 December 2023, after first online publication: The Supporting Information has been updated in this version.]

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