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Original Article

Towards the integration of environmental DNA analysis to profile the upper mesopelagic fish layer in the Northeast Atlantic Ocean

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Abstract

Interest in mesopelagic fish layers is on the rise due to the potential exploitability of their macrofauna; hence, profiling their fish community is crucial to enable the sustainability of future fishing practices. In this context, a dedicated survey was carried out in September 2022 along the Irish shelf break, where fishing (catch) and eDNA metabarcoding analysis using a portable high-throughput sequencer were performed to investigate the fish community of the upper mesopelagic layer. Catch data showed that the targeted layers consisted mainly of the young-of-the-year cohort of Mueller's pearlside (*Maurolicus muelleri*), a mesopelagic fish, with little bycatch. eDNA data reflected the high prevalence of *M. muelleri*'s eDNA (56%–97% of assigned reads), identified species undetected by fishing, and showed that the least represented species differed in water samples collected before or after fishing activities. While this reflects current limitations of each technique, it also shows that a multidisciplinary approach may provide an increased level of resolution for *M. muelleri* layer's ancillary fish community. Findings from the present study provided important insights to further refine sample acquisition and rapid processing of eDNA metabarcoding data, which beholds great potential to corroborate fishing methods when ground truthing acoustic approaches in mesopelagic fish layers assessments.

Keywords: environmental DNA; metabarcoding; mesopelagic fish; *Maurolicus muelleri*; Nanopore sequencing; trawling

Introduction

Environmental DNA (eDNA) is made up of the genetic material present in the environment that can be isolated and extracted directly from samples such as soil, water, and air (Taberlet et al. 2012). In the past two decades, there has been a substantial increase in the use of eDNA in monitoring and conservation efforts (Thomsen and Willerslev 2015). In particular, eDNA metabarcoding, a molecular technique that allows profiling of a community from an environmental sample using universal markers, has been widely used to study fish communities in both freshwater and marine environments (Rourke et al. 2022, Yao et al. 2022, Takahashi et al. 2023). eDNA has not been widely adopted as a standalone tool due to knowledge gaps in the understanding of its ecology (e.g. shedding rates, transport, decay) (Hinz et al. 2022, Coston-Guarini et al. 2023) and to the challenges described in Thomsen and Willerslev (2015) (e.g. contamination, inhibition, reference DNA databases). Instead, many studies have focused on the comparison and integration of eDNA with traditional survey methods. For instance, eDNA detected over 60% of the species identified by underwater visual census (Yamamoto et al. 2017), it equalled or outperformed multiple conventionally used survey methods (Thomsen et al. 2012, Valentini et al. 2016) and it detected more species compared to underwater videos from a cabled observatory (Mirimin et al. 2021). Therefore, eDNA has shown great potential to complement and corroborate direct methods in multidisciplinary approaches, as well as greatly reducing sampling efforts required by traditional methods (Valentini et al. 2016, Veron et al. 2023). The aforementioned studies based their metabarcoding analysis on high-throughput sequencing (HTS) technologies targeting short DNA fragments (e.g. Illumina), but recent technological advances have enabled the use and implementation of portable and affordable HTS instruments [i.e. Oxford Nanopore Technologies (ONT)], which have stood out as promising tools to scale up eDNA analysis at global levels (Thompson and Thielen 2023). Although few studies have used short-read ONT sequencing in the context of fish eDNA metabarcoding (Truelove et al. 2019, van der Reis et al. 2023, Munian et al. 2024), and fewer still have used long-read ONT sequencing (Doorenspleet et al. 2023), this technology is promising for its quick turnaround time (as little as 48 h in Truelove et al. 2019), reduced costs, and portability (Srivathsan et al. 2021). These characteristics allow sequencing of samples directly in the field with the potential to provide near-real-time results. In addition, ONT's ability to sequence

long fragments could provide better taxonomic resolution at the family and genus levels, which can still be challenging with short-fragment markers traditionally used with Illumina sequencing (Thomsen and Willerslev 2015).

In recent years, eDNA has found applications in fisheries science, with focus on both single-species and community approaches, with particular interest in supporting stock assessments (Rourke et al. 2022). Most studies have applied single-species approaches, based on real-time quantitative PCR (qPCR) of environmental samples, to show that eDNA can provide general quantitative information about fish stocks (e.g. Shelton et al. 2022). Instead, when applying metabarcoding, contrasting results have been shown, with some studies finding concordance between eDNA read abundance and fish biomass in catches (Afzali et al. 2021, Stoeckle et al. 2021), and others not observing consistency between results produced by the two methods (Fraija-Fernández et al. 2020, Veron et al. 2023). This indicates that, while eDNA metabarcoding is a reliable tool to describe the community and distribution of fish, further studies are needed to assess its application as a quantitative method.

The mesopelagic zone or twilight zone (200–1000 m of depth) is a largely understudied area of the ocean (St. John et al. 2016). Although the mesopelagic fish community has been virtually unexploited so far, estimates regarding the fish biomass of the mesopelagic ocean range from 1.8 to 16 gigatonnes (Proud et al. 2019), making it an attractive economic resource (e.g. as a source of protein and fatty acids, as well as biodiscovery in nutraceutical industry) (Gatto et al. 2023). Given their global distribution and large biomass, these organisms are likely to play a key role in the carbon cycle. Research gaps exist on the biodiversity of mesopelagic organisms, their life cycle, and their roles in the carbon flux and as food sources for key fisheries stocks. Thus, in order to hypothesize or build a sustainable mesopelagic extraction fishery, it is of utmost importance to address these research gaps (St. John et al. 2016, Martin et al. 2020, Standal and Grimaldo 2021). In the Northeast Atlantic Ocean, the shallow mesopelagic layer, between 100 and 250 m, is composed mainly by the Sternoptychidae *Maurolicus muelleri*, while the deeper layers, between 300 and 700 m, are usually more diverse and with a high biomass of the Myctophidae *Benthosema glaciale* (Grimaldo et al. 2020).

Current methods to study the mesopelagic zone focus on the use of echosounder acoustics, paired with direct catch, to detect the presence of fish and other taxa in the water column, associate a species to its acoustic signature, and provide fish biomass estimates (e.g. Gauthier et al. 2014, Dornan et al. 2022). Recently, studies have used eDNA sampling to target the mesopelagic zone, in conjunction with echosounder acoustic data. Particularly, Easson et al. (2020) and Govindarajan et al. (2021) collected samples at different depths of the water column, corresponding to acoustic layers, and used the 18S gene region to profile the taxonomic diversity. In addition, Govindarajan et al. (2022) expanded on their previous study by showing that filtering higher volumes of seawater led to improved biodiversity detection. Fewer studies have focused specifically on mesopelagic fish communities. Namely, Canals et al. (2021) used eDNA metabarcoding to investigate the vertical distribution of mesopelagic fish in relation with diel migratory behaviours, and Govindarajan et al. (2023) looked into the composition and vertical migrations of the twilight zone's fish layers in the Northwest Atlantic.

The overarching goal of the current study was to explore the integrability of eDNA metabarcoding analysis in the assessment of mesopelagic fish communities carried out in conjunction with acoustic analysis and fishing. Specific objectives were to (i) investigate whether water acquisition using the CTD rosette produced consistent results when collected in proximity to fish layers (as detected by acoustic methods) before versus after fishing hauls, (ii) adapt and apply an eDNA metabarcoding workflow using novel portable technologies, and (iii) integrate eDNA and fishing data to profile the upper mesopelagic fish layer.

Materials and methods

The Multidisciplinary Mesopelagic Scouting Survey (M_2S_2) was carried out from 31 August 2022 to 10 September 2022 on the Irish RV *Tom Crean*. Operations were conducted over 24 h and included echosounder acoustics, fishing, and eDNA sample collection. The survey area was the Irish shelf break in the Rockall Trough, between 54° and 55°N, and sampling efforts were concentrated on the upper mesopelagic zone (from 100 to 350 m depth). Four candidate sites were selected when echotraces of interest were detected and further investigated using two main techniques (i.e. fishing and eDNA, as further detailed below) (Fig. 1). When possible, seawater for eDNA analysis was collected by CTD casts before and after the deployment of the fishing gear, so to explore any variation in community detected due to spatial heterogeneity of target eDNA (as detailed in Fig. 1).

Acoustic detection and trawl sampling

Acoustic data were collected using a calibrated Simrad EK80 single beam scientific echosounder operating on four frequencies (18, 70, 38, and 120 kHz frequencies) for quantitative measurements. Directed trawling was carried out on echotraces of interest at each survey site using a graded four-panel single pelagic midwater trawl. The net had a vertical opening of 35 m and a fishing circle with a diameter of 422 m. The largest meshes measured 2.4 m in the mouth of the trawl tapering to a terminal mesh of 5 mm in the codend. During the survey, a total of eight trawls were conducted, of which seven on-target, with trawl duration between 36 min and 1 h 28 min, and one where the target aggregation was missed (trawl 4 > 2 h). Towing speed was between 2.2 and 2.6 knots to ensure the optimal geometry of the net was maintained. Table S1 in the supplementary materials provides metrics and catch information for each haul. All components of the trawl catch were sorted, weighted, and identified to the lowest taxonomical level possible aboard the RV by a fish taxonomist. The juvenile stage of one species (pearlfish, *Echiodon drummondii*), was identified at a later stage using the Cytochrome *c* Oxidase subunit I (COI) gene for molecular barcoding (Ward et al. 2005) (data not shown). The catch data are publicly available from: https://acoustic.ices.dk/submissions (survey code MEESO, country code IE).

Water sampling for eDNA analysis

All the procedures concerning eDNA processes detailed in the following methods sections were carried out in the CTD laboratory of the RV *Tom Crean*, or in dedicated eDNA laboratories on land in the Atlantic Technological University (ATU). On the RV *Tom Crean*, to avoid potential contamination, the

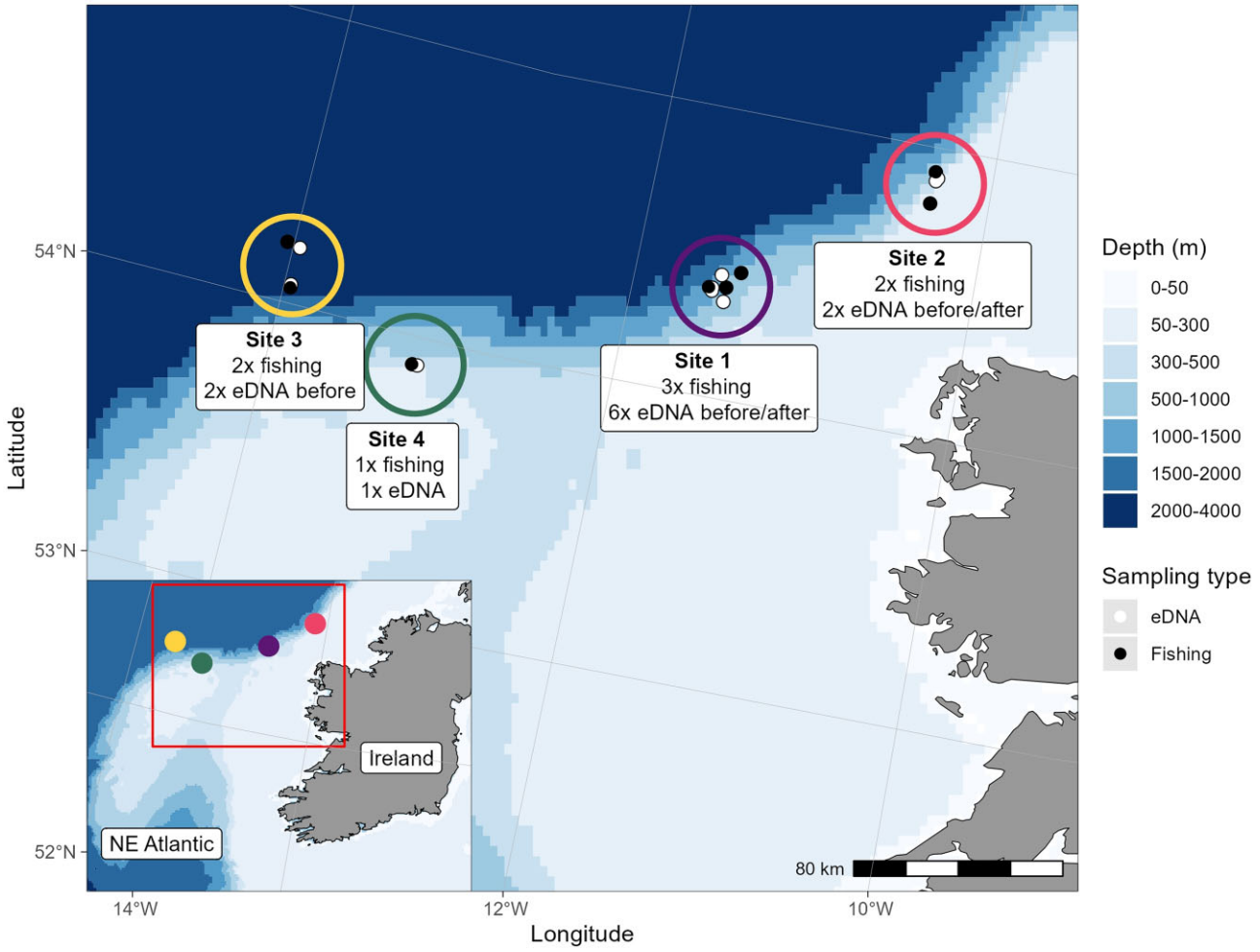

Figure 1. Sampling sites (1–4) investigated during M_2S_2. At all sites, eDNA sampling and fishing were carried out, with eDNA samples collected before and after relative fishing hauls in sites 1 and 2, only before fishing hauls in site 3 or independently from fishing activities in site 4.

CTD lab was dedicated solely to seawater work, and dedicated personnel collected/handled water samples for eDNA analysis. Before each step of the process, all relevant surfaces and reusable equipment were sterilized with 20% bleach and wiped with 70% ethanol to reduce the risk of contamination. In ATU, dedicated labs for eDNA extraction, pre-PCR, and post-PCR work were used.

eDNA sampling was conducted by collecting seawater at selected stations with a rosette of 10 l Niskin bottles mounted on the CTD frame. CTD stations were defined in each survey site based on the fishing activity. At sites 1 and 2, eDNA samples were collected before and after hauls 1, 2, 3, and 4. The location and depth of the 'before' sampling point were noted, and the vessel returned to the same location to collect the 'after' samples, closing the Niskin bottles at the same discreet depth. The sampling depth was selected by lowering the CTD as close to the fish layer as possible (Fig. 2) and firing two Niskin bottles. For haul 5, eDNA samples were not collected as adverse weather conditions did not allow the deployment of the CTD. At site 3, eDNA samples were collected only before hauls 6 and 7. At site 4, eDNA samples were collected to investigate a target layer without carrying out fishing activity. Table S2 in the supplementary materials includes metrics and further information about all CTD stations.

At least 15 min before deployment, the selected Niskin bottles were decontaminated by rinsing with 20% bleach. Only at CTD station 1, the bottles were rinsed with freshwater after the decontamination. At all other stations, the Niskin bottles were only decontaminated, as it was assumed that rinsing with seawater during the downcast would be enough to minimize the amount of residual bleach. From each Niskin bottle, four replicates of 2 l each were collected and filtered through a 0.45 μm Cellulose Nitrate membrane filter (Whatman™, UK) (47 mm diameter) using a GeoPump peristaltic pump (Geotech, USA) and a ramp that allowed independent filtering of up to three samples simultaneously. In total, eight samples were collected per station, i.e. four replicates from each of the two Niskin bottles fired. At CTD station 11, only 4 samples were collected as only 1 Niskin bottle was fired. The filters were folded in half and stored in sterile Ziplock bags at −20°C in a freezer dedicated to the CTD lab. Table S2 in the supplementary materials includes a summary of the samples collected.

Environmental DNA extraction

DNA was extracted from half of each filter using a modified version of the DNeasy Blood and Tissue Kit (QIAGEN, Germany) adapted for eDNA filter samples (Supplementary

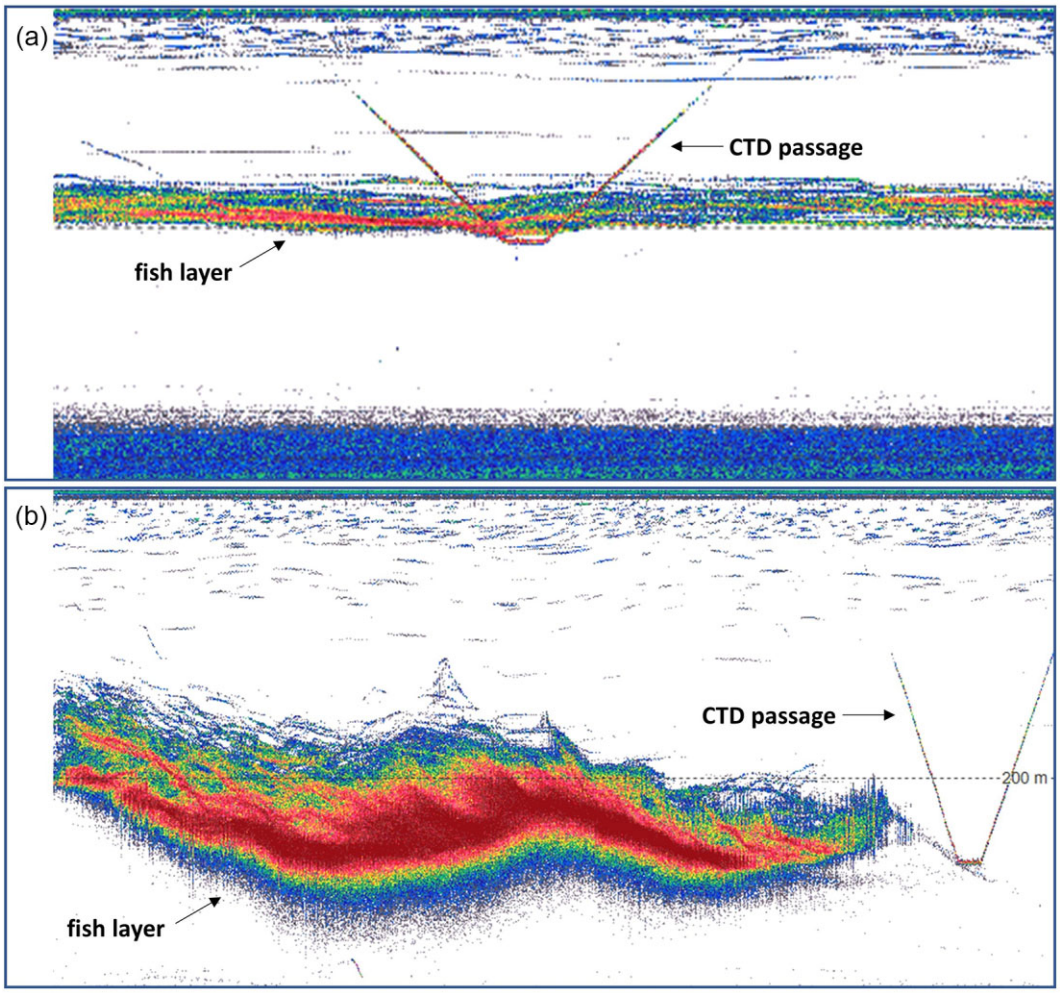

Figure 2. Deployment of the CTD before the haul either (a) in the fish layer or (b) as close to it as possible.

Appendix 1). Half of the extractions ($n = 44$) were processed on board the RV *Tom Crean*, and the remaining filters were extracted in an eDNA-dedicated laboratory in ATU. For all extractions, only filter tips were used to reduce the chance of cross contaminations. No extraction negatives were carried out during the extractions on board. Two extraction negatives were included during the extractions in the ATU lab, by performing the full extraction protocol on tubes that did not contain a filter.

Library preparation and HTS

Amplification was carried out through PCR in a MiniAmp™ Plus thermal cycler (Applied Biosystems), using universal primers targeting fish: a combination of the V05_898 forward primer (5′-AAACTCGTGCCAGCCACC-3′) and the teleoR reverse primer (5′-CTTCCGGTACACTTACCATG-3′) (Thomsen et al. 2016), producing a ≃650 bp long DNA fragment of the 12S mitochondrial gene. The PCR products were pooled for each CTD station (8 PCR products in each pool, 4 for CTD station 11).

Library preparation was carried out using the ligation sequencing kit (SQK-LSK110) with the barcoding expansion kit (EXP-PBC001), according to the manufacturer's protocol with minor modifications (see further details in Supplementary Appendix 1). For each CTD station ($n = 11$) amplicons were barcoded, and subsequently pooled in equimolar
quantities into a single library before loading onto an R9.4.1 flow cell with 500 active pores at the start of the run. Sequencing was carried out using the MinION Mk1C (ONT) for a total run time of 72 h, selecting active barcoding and fast basecalling in the run setup.

Bioinformatic processing

The Mk1C run produced demultiplexed sequences (one folder per sample), from which barcodes and ONT adaptors were removed with Guppy (v6.5.7+, Oxford Nanopore Technologies Ltd. 2023b) by selecting the EXP-PBC001 multiplexing kit. Quality of reads was checked by inspecting the results of the NanoPlot outputs (De Coster et al. 2018). The primers were removed using *Cutadapt* (v3.5; Martin 2011). The *decona* (v0.1.3; Doorenspleet et al. 2023) pipeline was used to process the trimmed reads. The reads were filtered based on length (min length = 550 bp, max length = 800 bp) and quality (q-score > 10). The clustering percentage was set at 90% and only clusters containing a minimum of five sequences were retained. The polished consensus from each cluster was retained in the final output and used for taxonomic assignment. Taxonomic assignment was carried out using the *blastn* function of the command-line version of Basic Local Alignment

Search Tool+ (BLAST+) (v2.12.0+; Camacho et al. 2009). The reference database used for this step was the MIDORI2 curated database (release 257; Leray et al. 2022) of metazoan 12S genes. The first 20 hits of the blast output were inspected, and consensus sequences were assigned to the top hit species (with a minimum alignment length of 500 bp and a minimum identity percentage of 97%). If a sequence was assigned with same alignment length, percentage of identity, and bit score to multiple species of the same genus, the species' global distribution was investigated, and the sequence was assigned to the endemic species. If reads were assigned to a species that was not expected to be found in that geographical area, all sequences were manually checked against the National Centre for Biotechnology Information (NCBI) nucleotide database using BLAST. Only sequences assigned to species level were retained for the scope of this study.

Data analysis

Statistical analysis and graphical outputs were obtained using R software (R Core Team 2021) and R Studio (Posit Team 2023). Packages *vegan* (Oksanen et al. 2022) and *tidyverse* (Wickham et al. 2019) were used for data analysis. Packages *ggplot2* (Wickham 2016), *VennDiagram* (Chen 2022), and *wesanderson* (Ram and Wickham 2018) were used for data visualization. Packages *ggOceanMapsData* and *ggOceanMaps* (Vihtakari 2022) were used to produce maps. A rarefaction analysis was run to evaluate if the sequencing depth was sufficient using the *iNEXT* package (Chao et al. 2014, Hsieh et al. 2016).

Principal coordinates analysis (PCoA) was performed to investigate similarities/differences between the eDNA samples collected before/after the hauls.

The full script for the bioinformatic processing and the R scripts for the data analysis are available in the following GitHub repository: https://github.com/maddalena-tibone/M2S2.git.

Results

Catch composition

A total of 17 fish taxa were identified, of which 16 on board, 14 to the species level, and 2 to the genus level (*Argentina* spp. and *Solea* spp.), and 1 additional taxon was identified later through post-survey analysis. After the survey, one pearlfish specimen was identified molecularly as *E. drummondii* to the species level. When considering only the fish species present in trawl catches, all hauls were dominated by *M. muelleri,* as it accounted between 85% and 99% of the fish biomass in the net (Fig. 3).

eDNA amplification and HTS

eDNA sampling was carried out at 11 CTD stations, collecting a total of 84 samples, that all amplified successfully. Both extraction negatives were also amplified using the V05_898/teleoR primer combination, and, when visualized on gel, one of them showed a faint band at the expected size. This product was sequenced and resulted in 46 reads assigned to metazoans (40 to *Homo sapiens* and 6 to *M. muelleri*). The relative read abundance of *M. muelleri* detected in this negative (0.03%) was subtracted from all samples extracted in the same batch (H1B, H1A, H2B, H2A, and H3B). All PCR negatives that were included in the amplification runs showed no bands on gel, indicating no evidence of contamination during the post-extraction steps, thus they were not processed further.

After initial quality filtering, a total of 1 370 361 reads were retained after primer removal and filtering based on length and sequence quality, for an average of 124 578 reads per sample (see further details in Table S3 in the supplementary materials). The rarefaction analysis showed curves that reached a plateau in the first 50 000 reads, confirming that the sequencing depth was sufficient to recover the diversity in all samples (Fig. S1 in the supplementary materials). Figure 4 represents the bar plots of the number of reads for all samples after each filtering step. Human DNA was detected in all samples (0.8%–63.4% of the clustered reads) and these reads were excluded from the dataset prior to further analysis.

Overall, eDNA allowed the detection of 19 fish species, of which ~40% belonged to mesopelagic fish families (Salvanes and Kristoffersen 2001) (Fig. 5). The target mesopelagic species (*M. muelleri*) was dominant (over 95% of the reads) in 5 out of 11 samples and overall had a relative read abundance ranging from 32.51% to 97.74%. In Fig. 4, 'Assigned to species' refers to reads that were assigned to a reference database of metazoan 12S sequences with a minimum alignment length of 500 bp and minimum identity of 97%. Across eDNA sampling points, 'unassigned' reads (i.e. unassigned to the reference database with a minimum identity threshold of 90%) were between 0.4% and 4.52% of the clustered reads and increased up to 23.97% for sampling point H2B and to 94.34% for H7B. Instead, considering the clustered reads across samples, between 1% and 7% were unassigned to species.

For all the fish species detected, the native range was checked manually by investigating the FishBase database (Froese and Pauly 2024) to detect potential false positives. All but two species, *Phycis chesteri* and *Helicolenus hilgendorfii*, were expected in the surveyed area.

The native range of *P. chesteri* is along the east coast of North America, but one species of the same genus, *P. blennoides*, is found in the Northeast Atlantic. The MIDORI2 reference database contained a partial 12S sequence for *P. blennoides* (415 bp) and a full 12S sequence for *P. chesteri* (953 bp). Given the geographical ranges of the species, all sequences assigned to *P. chesteri* were manually reassigned to *P. blennoides*, as the erroneous taxonomic assignment was due to an incomplete entry in the database.

Similarly, as the native range of *H. hilgendorfii* is the Northwest Pacific Ocean, all the sequences assigned to this species were manually checked against the NCBI nucleotide database using the online BLAST tool, which confirmed the assignment. As the 12S sequences of *H. hilgendorfii* and *H. dactylopterus* differ from only one nucleotide, the detection of *H. hilgendorfii* is most likely due to a sequencing error; therefore, these reads were manually reassigned to *H. dactylopterus*.

In Fig. 5, the first eight columns show the species detected in the eDNA samples before and after hauls 1–4. When comparing the species detected in the 'before' and 'after' samples, species with high relative read abundance were consistent, while species with low relative read abundance differed. The percentage of common species between 'before' and 'af-

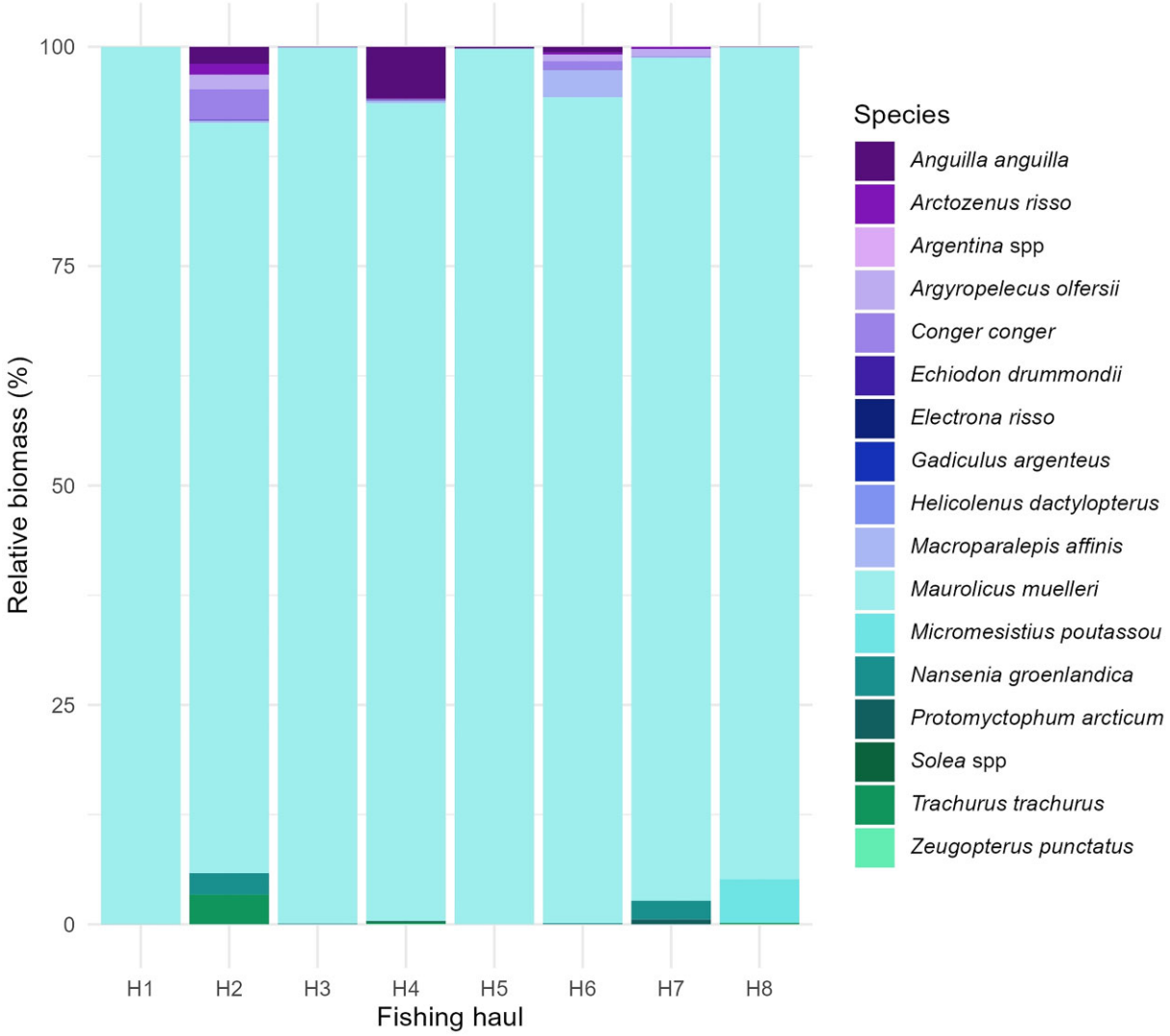

Figure 3. Stacked bar plots representing the species composition as relative biomass (%) for each haul. Large proportion of the catch in most hauls consisted of *M. muelleri*.

ter' samples varied from 37.5 to 83% (Fig. S2 in supplementary materials).

Comparison between eDNA and catch data

Species composition from hauls and eDNA samples were compared for individual sampling stations (Fig. S3 in the supplementary materials), showing that the species detected by both methods were 8%–25% of the total. Figure 6 shows the overlap between species/taxa detected across hauls 1, 2, 3, 4, 6, and 7 and relative eDNA sampling points. Each method detected 16 species/taxa, with 5 species (18.6%) being detected by both techniques.

The heatmap in Fig. 7 shows the percentages of relative read abundance (for eDNA) or biomass (for fishing) at each sampling point, including both 'before' and 'after' fishing eDNA samplings, where available. Overall, *M. muelleri* was the only species detected by both methodologies in all sampling occasions, with low overlap of the less present species between techniques. Only in some sampling points (H3B/H3A and H4B/H4A) consistency was noticed in the species detected by eDNA before and after fishing. This can also be observed in Fig. 8, which shows the output of the PCoA analysis for eDNA samples collected before and after hauls 1–4. In the plot, grouping is observed only for H3B/H3A and H4B/H4A, while for hauls 1 and 2, the communities detected by eDNA before and after fishing were different.

Discussion

In the current study, we present the use of eDNA analysis to describe the upper mesopelagic layer's fish community in the NE Atlantic. Different sample acquisition strategies were applied by sampling eDNA before and after fishing activities and results showed this had an effect on the communities profiled. In addition, we successfully showcased an eDNA metabarcoding workflow targeting a 650 bp metabarcode of the 12S gene using a portable MinION sequencer. Molecular data described the upper mesopelagic layer as dominated by *M. muelleri* and was in agreement with catch compositions, while also identifying species not found in the hauls. In the following sections,

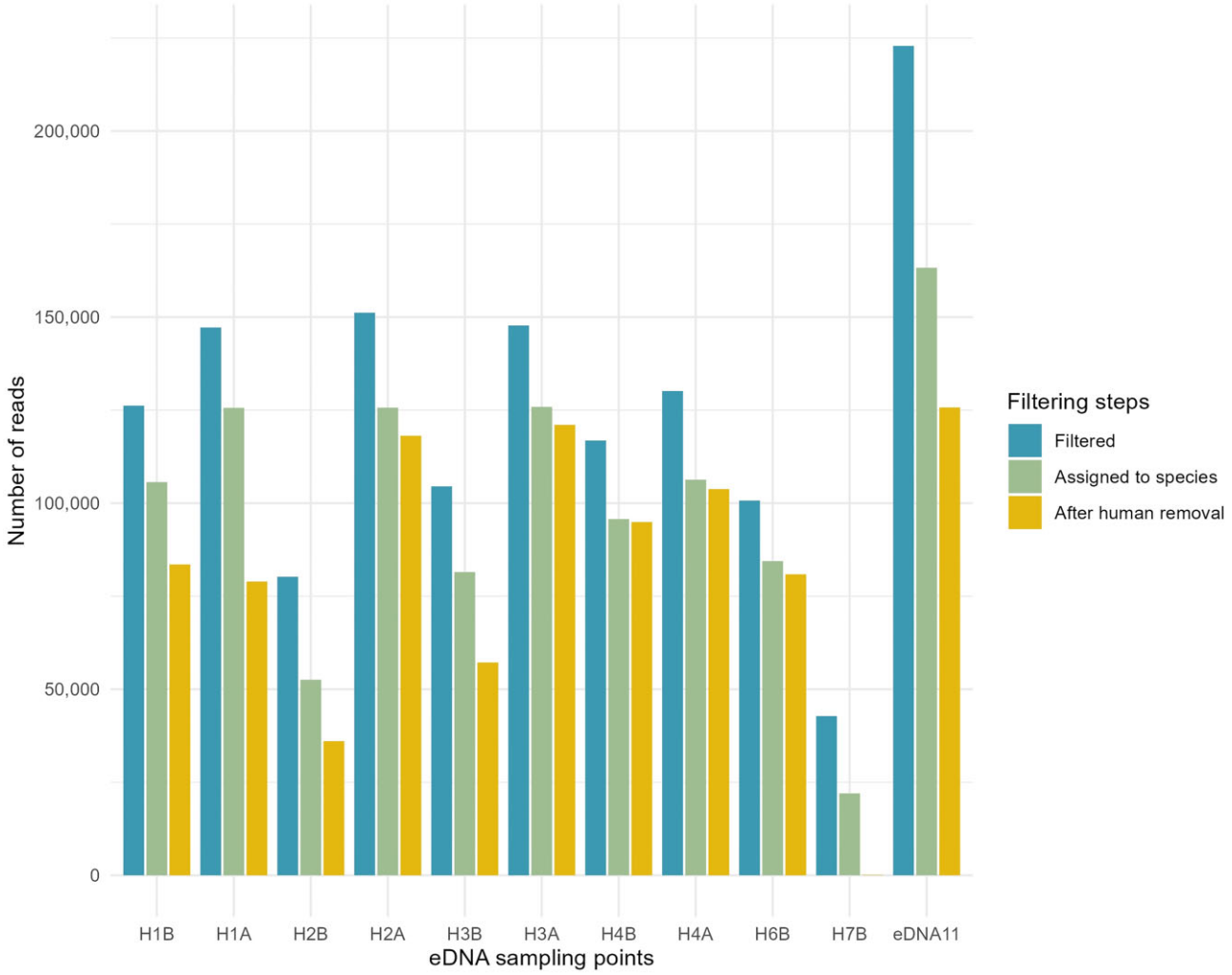

Figure 4. Bar plots of the number of reads retained in all samples after three steps of the bioinformatic processing.

we highlight advantages and drawbacks of the main study findings.

eDNA sampling and processing

At four out of six hauls, eDNA samples were collected with a CTD cast before and after fishing activities. Results show that species composition differed, with only the most abundant species being consistent before/after, whilst the least abundant species being more variable. Two of the four hauls (H3B/H3A and H4B/H4A) showed consistency before/after (Figs 7–8), while the other two did not. This suggests that the heterogeneous distribution of eDNA makes it more suitable to capture the local communities as opposed to large-scale communities, particularly when sampling low volumes. Although studies on the dispersal of eDNA in the mesopelagic ocean have suggested that eDNA can be found within tens of metres of where it was shed (Andruszkiewicz Allan et al. 2021), the time elapsed before the collection of the 'after' samples (from 1.5 to 4 h) could have had an impact on the species present in the area, thus on the less abundant eDNA retrieved.

The collection of eDNA samples is a process that requires a clean working environment and sterile equipment. Conducting such sampling on a research vessel added logistic difficulties, such as limited space and equipment, and potential contamination sources, as biological fish samples were processed in the neighbouring wet lab. These conditions highlight the importance of decontaminating, collecting field negatives, and checking extraction and PCR negatives to account for potential contamination during downstream protocols. Although we applied strict decontamination procedures during sampling and processing activities, the inclusion of additional field and lab negative controls would have enabled a more comprehensive assessment of potential sample contamination. Thus, any unaccounted/undetected contamination may have led to skewed species representation, especially when considering read counts. For future work in similar conditions, we recommend collection and processing of negative controls at all key steps (i.e. sample acquisition, eDNA extraction, PCR amplification, and HTS), as sequencing any negative that presents signs of contamination allows to account for contaminant DNA during the bioinformatic processing (Afzali et al. 2021).

High-throughput eDNA sequencing

This study used a primer set traditionally applied to barcode fish specimens (Thomsen et al. 2016) in a metabarcoding approach by taking advantage of Nanopore's long-read sequencing technology. The sequencing run resulted in an average

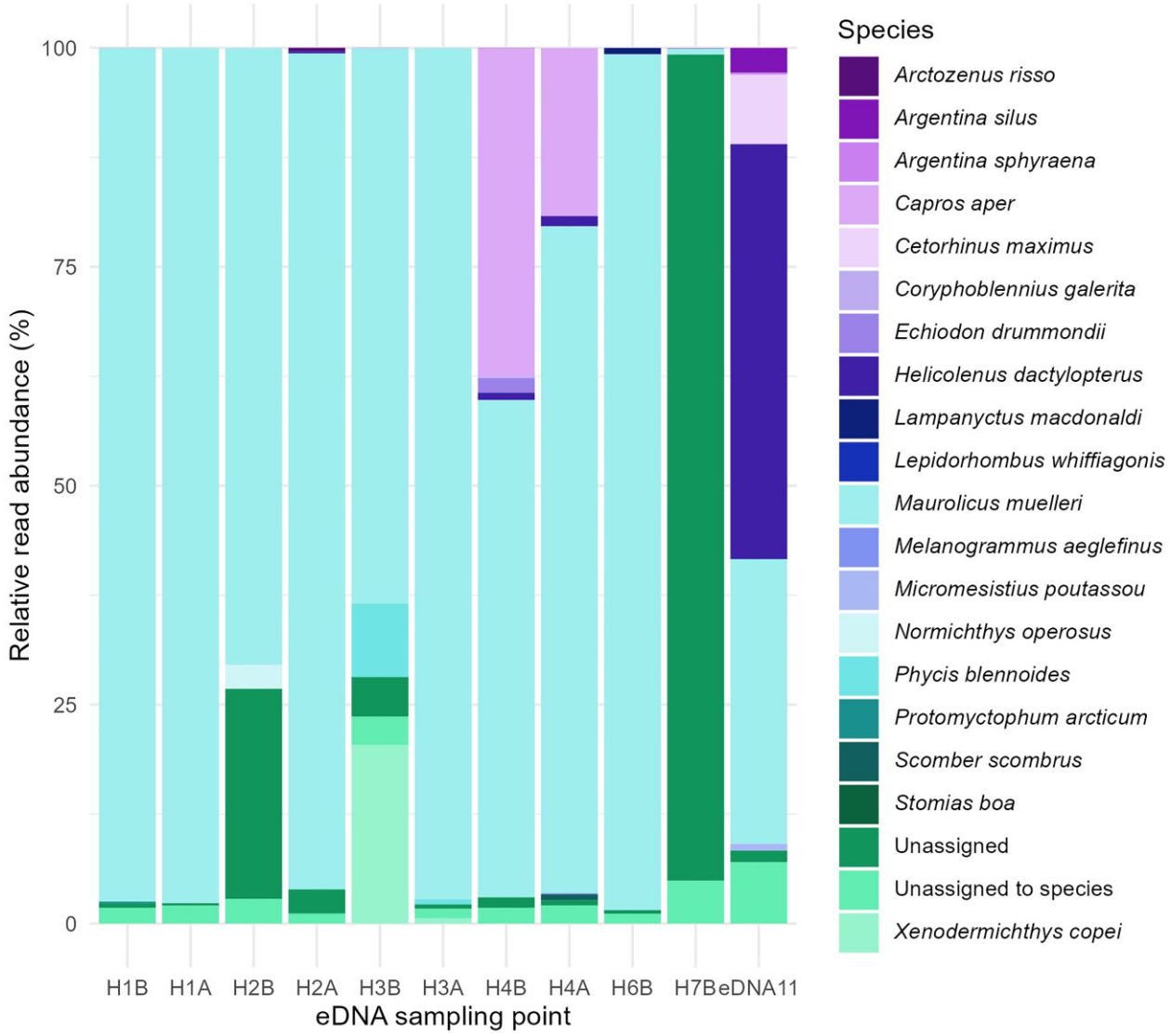

Figure 5. Stacked bar plots representing the species composition as relative read abundance (%) for each eDNA sampling point. For the hauls1, 2, 3, and 4, eDNA samples were collected before and after the fishing activities ('H1B' = before, 'H1A' = after); for hauls 6 and 7, eDNA samples were only collected before the fishing activities. The last column represents the CTD station 11 sampling point.

number of filtered reads per sample of 124 578, with an average of 52% of the raw reads retained after filtering. After rarefaction analysis, this sequencing depth was deemed sufficient to detect the local diversity (Fig. S1 in supplementary materials).

As most fish eDNA metabarcoding studies up to now have used Illumina sequencing technology, the amplicon length is usually limited. One of the issues associated with reduced amplicon length is the difficulty to resolve species from the same family or genus due to the low sequence coverage (Thomsen et al. 2016, Yamamoto et al. 2017, Afzali et al. 2021). Instead, ONT sequencing allows to target longer fragments, thus increasing the number of informative sites used for taxonomic assignment and the resolution of closely related species (Zhang et al. 2020, Doorenspleet et al. 2023). The length of the region targeted by the primers used in this study (~650 bp) provided good species resolution, with only 1%–7% of the clustered reads not assigned to species level. In comparison, other fish metabarcoding studies were not able to assign 6.5% (Zhang et al. 2020), 7.6% (Yamamoto et al. 2017), and 9.5% (Afzali et al. 2021) of the clustered reads to species level using MiFish primers, and 11.5% using teleo primers (Zhang et al. 2020). Unassigned reads can be a result of lack of resolution to species level but also incomplete reference databases. Whilst incomplete reference databases are a drawback for all sequencing platforms, Nanopore long-read sequencing could help increase resolution.

Despite the good specificity for fish of the V05_898/teleoR primers, they did also amplify human DNA, which is a known issue when targeting the 12S gene (Zhang et al. 2020, Afzali et al. 2021). Although strict decontamination protocols were followed, human contamination was inevitable, as sampling equipment (Niskin bottles, water drums) was routinely handled by both scientists and crew (including without gloves). To limit this non-target amplification, *H. sapiens* blocking primers have been used (e.g. Valentini et al. 2016). However, previous studies have shown that including a blocking primer for human DNA can result in a loss of species richness (Zhang

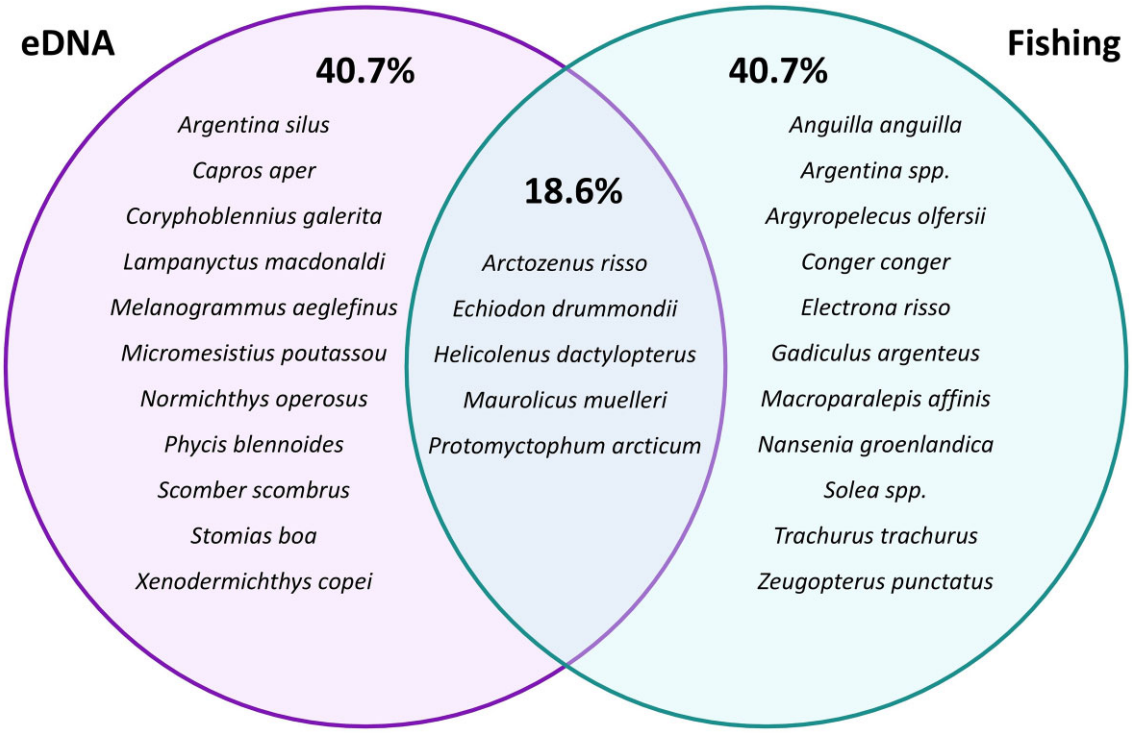

Figure 6. Venn diagram showing the species/taxa detected by eDNA and fishing, across hauls 1, 2, 3, 4, 6, and 7 and relative eDNA sampling points. The percentages represent the proportion of species detected by each method.

et al. 2020, Doorenspleet et al. 2021), particularly of taxa with low relative read abundance. Therefore, in the case of fish eDNA metabarcoding, an increase in sequencing depth could be the best solution to compensate for non-target amplification while avoiding a potential loss of species richness.

The erroneous detection of *H. hilgendorfii*, instead of *H. dactylopterus*, in one eDNA sampling point was due to sequencing errors. In fact, the main limitation of ONT sequencing is the higher error rates compared to Illumina sequencing (Menegon et al. 2017). However, ONT's technology and chemistries are advancing quickly (Srivathsan et al. 2021), leading to an improvement in sequencing accuracy that aims to align ONT with Illumina sequencing. The latest kits/chemistry, released in 2023, promise up to 99.5% raw read accuracy (Oxford Nanopore Technologies Ltd. UK 2023a).

On the other hand, the assignment of reads to *P. chesteri* instead of *P. blennoides* was due to an incomplete entry for the latter's 12S gene on NCBI (and thus MIDORI2). Although geographical range allowed a manual correction of the assignment, this issue underlines that the lack of complete reference databases is still a limitation of the application of eDNA metabarcoding (Miya et al. 2015, Thomsen and Willerslev 2015), and that manually checking the taxonomic assignment results is a necessary step.

Interpretation of fish communities obtained by eDNA analysis

In total, 27 fish species were detected, with both fishing hauls and eDNA identifying 16 species/taxa each across hauls 1, 2, 3, 4, 6, and 7 and corresponding eDNA sampling points. Only five species (18.6%) were detected by both methods: *Arctozenus risso*, *E. drummondii*, *H. dactylopterus*, *M. muelleri*, and *Protomychtophum arcticum* (Fig. 6). When investigating the hauls individually (Fig. S3 in supplementary materials), the overlap between the two techniques was as low as 8.33% (one species). Only *M. muelleri* was consistently detected by both methods and this reflected the nearly monospecific composition of catches (i.e. 85%–99% of the biomass) and eDNA (i.e. 56%–97% of relative read abundance, excluding H7B). The other four species were detected in small biomass percentages (0.097%–1.25%) and low relative read abundances (0.22%–1.68%). The dominance of *M. muelleri* confirms findings of a previous study located in the northeast Atlantic (Grimaldo et al. 2020), that described a high-biomass *M. muelleri* upper mesopelagic layer (100–250 m) as a potential source of protein and oil for human exploitation.

A total of 11 species were only detected by eDNA. Of these, five species (*A. silus*, *Coryphoblennius galerita*, *Melanogrammus aeglefinus*, *Micromesistius poutassou*, and *Stomias boa*) were detected with <200 reads (Table S4 in supplementary materials). Previous studies have observed positive relationships between eDNA concentration and species biomass (Rourke et al. 2022, Veron et al. 2023). Although further investigation is required to confirm if this could be applied to the present study, low biomass of these species in the environment could explain the lack of hauled individuals. In addition, *M. aeglefinus* is a demersal species, mostly associated with the seabed; therefore, it is expected to have low presence in the upper water column. *Capros aper* was detected in most eDNA sampling points and it is known to be present in this area (O'Donnell et al. 2022); therefore, its detection may indicate that schools of fish were present in the vicinity. Another 5 species (*Lampanyctus macdonaldi*, *Normichthys operosus*, *P. blennoides*, *Scomber scombrus*, and *Xenodermichthys copei*) were detected with higher number of

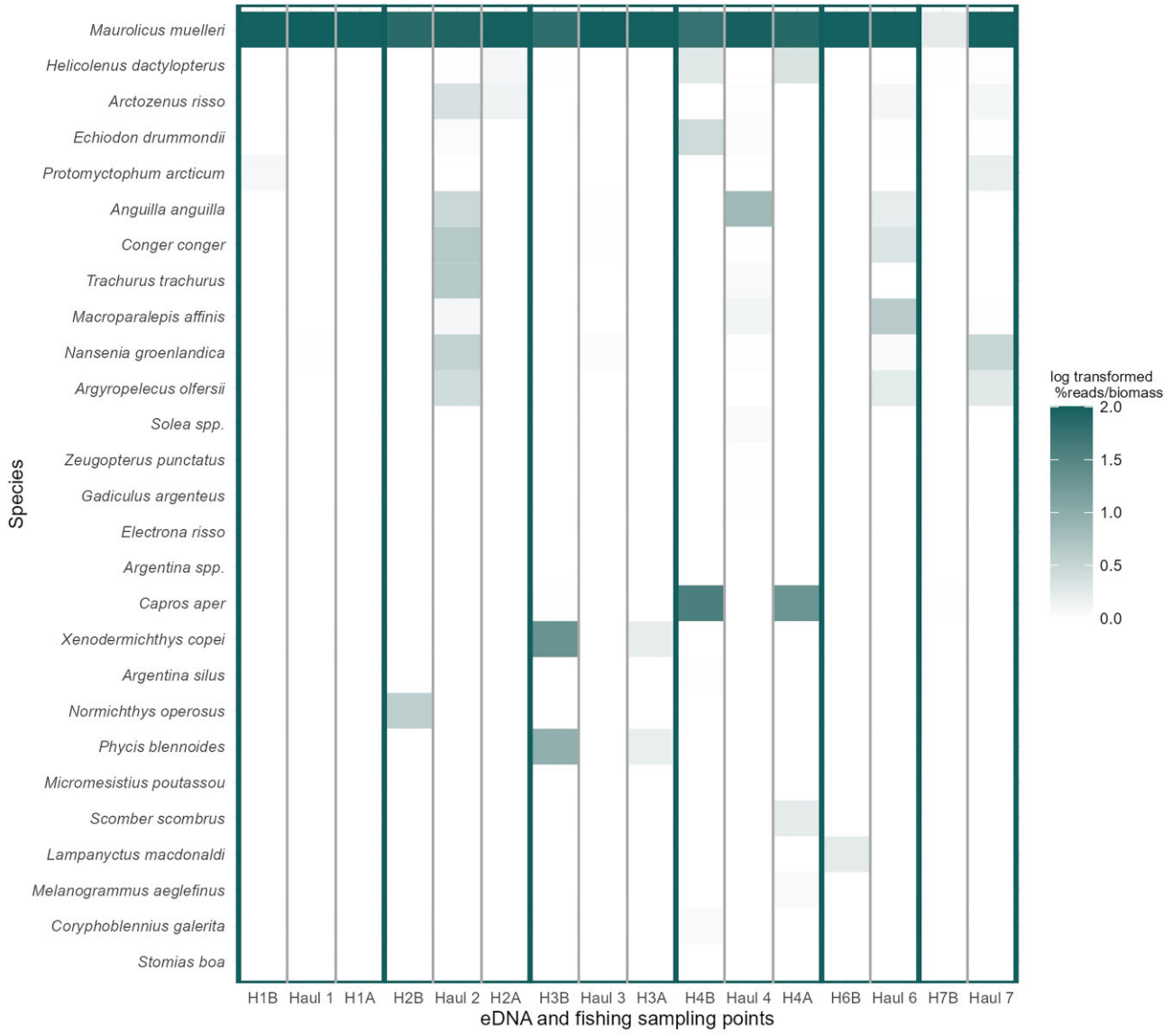

Figure 7. Heat map of the percentages of relative read abundance (for eDNA) and relative biomass (for fishing) of all species detected in the hauls and relative eDNA sampling points. The first four rectangles include species from hauls 1, 2, 3, and 4 and species from the 'before' and 'after' eDNA sampling. The last two rectangles include species from hauls 6–7 and only species from the 'before' eDNA sampling. All percentages were transformed with a logarithmic transformation: $x = \log(1 + x)$.

reads, from 500 to 12 700 (Table S4, supplementary materials). *Phycis blennoides* is a benthopelagic species that feeds in the pelagic environment, which explains its eDNA detection, but lives in muddy bottoms, which explains its absence in the catch. With regards to the other four species, their absence in the haul could be explained by active net avoidance (*S. scombrus*) aided by a low towing speed of 2.2–2.6 knots, behavioural traits such as diel vertical migration (*L. macodonaldi*, *N. operosus*, and *X. copei*) and/or stochasticity. Although the smallest individual caught by trawling was 11 mm in length and the mesh size at the cod end was very fine (5 mm), size could be an explanatory factor for absence in the hauls but detection by eDNA, as some species may have been present in larval or juvenile stages. In fact, *H. dactylopterus* in juvenile stages was detected only in 2 hauls, but its eDNA was found in 7 out of 10 eDNA sampling points in low relative read abundances. In addition, the benthopelagic *H. dactylopterus* made up 47% of the reads in eDNA11 sampling point, where Niskin bottles were closed at 400 m of depth, close to the seabed. As *H. dactylopterus* has been previously observed to spawn in the Rockall Trough and northern slope of the Porcupine bank, with juveniles being caught in shallow trawls (60 m) (Kelly et al. 1999), this difference reflects the sensitivity of eDNA to detect juvenile stages of fish that could be missed during pelagic trawling.

A total of 11 species were only detected by fishing. Of these only *Macroparalepis affinis* was missing from the reference database used for the taxonomic assignment of the eDNA data. *Argentina* spp. was detected only in haul 4, and it was not identified to the species level. In the corresponding eDNA sampling point, *A. silus* was detected, suggesting that this could be the correct taxonomic assignment. The remaining 10 species made up between 0.001 and 5.9% of the catch's biomass. Their low biomasses, partly due to species present in juvenile/larval stage (*A. anguilla*, *C. conger*, *Solea* spp., and *T. trachurus*), ranged from 0.2 to 112 g (excluding 192 g of

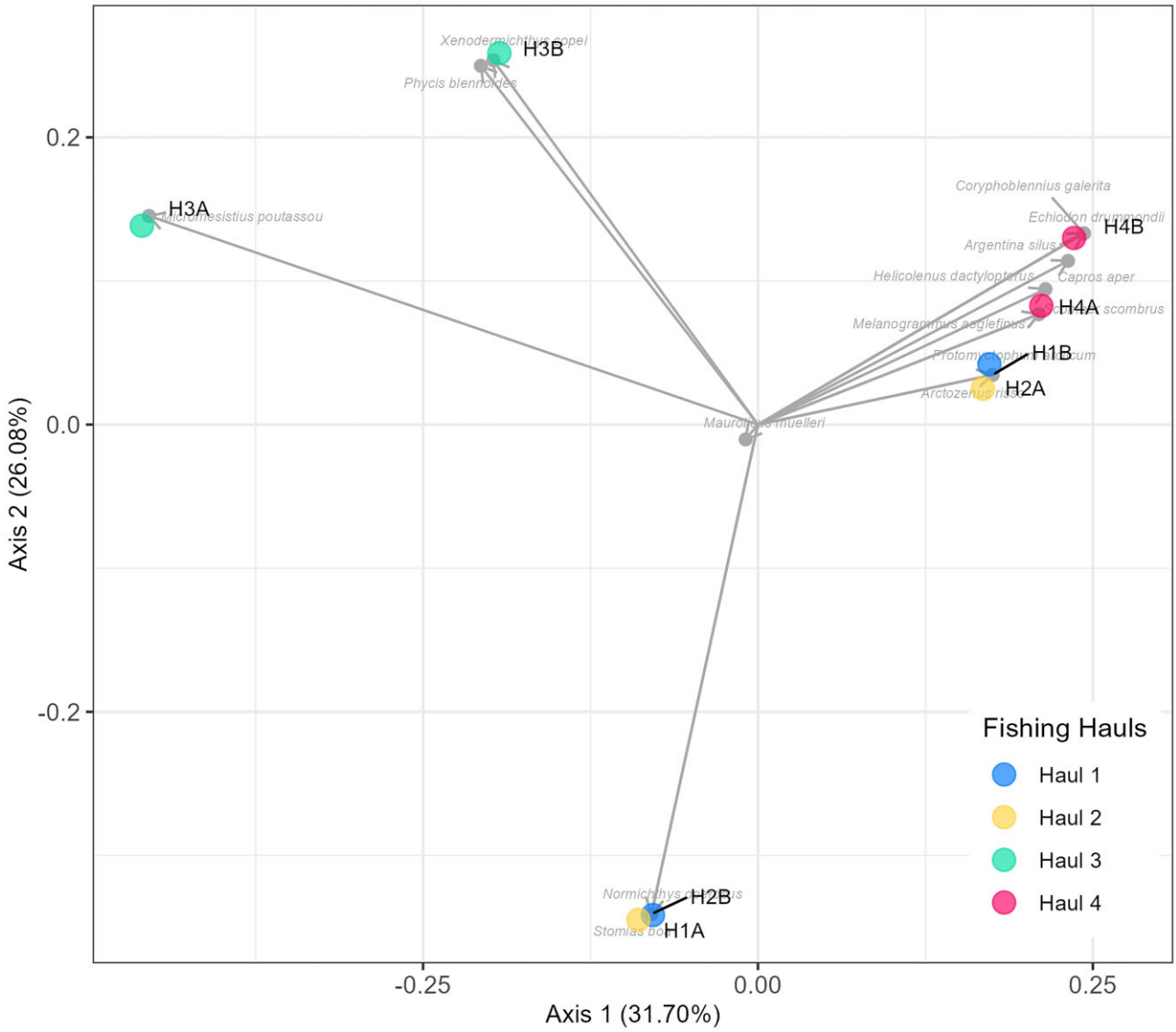

Figure 8. PCoA of the eDNA presence/absence data based on the Jaccard similarity index. The plot shows eight eDNA samples, collected before (B) and after (A) hauls 1, 2, 3, and 4.

M. affinis) and could explain the failed detection in the eDNA samples (Fraija-Fernández et al. 2020, Afzali et al. 2021). Additional explanations of the missed detection of the species found in the hauls include low primer selectivity and stochastic eDNA sampling. Primer selectivity is a known bias associated to 12S fish eDNA metabarcoding primers (e.g. Miya et al. 2020) and it could have influenced the eDNA detection rates. Further *in silico*/*in vitro* studies are recommended to understand this effect for the V05_898/teleoR primers, particularly for understudied communities such as mesopelagic fish.

Overall, the low overlap in species detected by the two methodologies can be explained by the differences in sampling strategy. In fact, while seawater is collected using Niskin bottles at a specific location and depth, during trawling the net covers a wider spatial and temporal area, from the surface to the target sampling depth and back up again. In addition, trawling is a targeted approach as the headline transducer allows to have a real-time sonar signal that facilitates dynamic targeting the fish schools. Instead, when collecting eDNA samples, the CTD cast is lowered to a specific depth based on the target echotraces seen on the echosounder, but there is no way to 'verify' if eDNA is present. In addition, seawater is collected in limited volumes (4 × 2 l in this study), compared to the volume of the oceanic environment, which determines, unavoidably, a stochastic sampling of the least abundant eDNA.

The differences between the two techniques applied reflect some of the advantages and limitations of both methods. With regards to eDNA, on top of well-documented drawbacks such as incomplete reference databases, an issue that emerged was the stochastic sampling of low abundance eDNA present in the environment. To mitigate this limitation, filtering higher volumes of water has been shown to increase the number of species detected (Govindarajan et al. 2022). On the other hand, eDNA was very sensitive in detecting rare species and was able to pick-up species from more than just the mesopelagic environment. With regards to fishing, while the net was able to cover a larger spatial area and

detect many species, including very small individuals, it did miss the detection of species that were present in eDNA samples.

Integrating multidisciplinary methods

Similarly to previous studies that have shown the advantages of integrating multidisciplinary methods (e.g. Thomsen et al. 2012, Valentini et al. 2016, Yamamoto et al. 2017, Mirimin et al. 2021), we show that integrating the data obtained from both techniques produces a more comprehensive description of the target layer. In fact, integrating both methods helped to ground truth target echograms as a nearly monospecific layer of *M. muelleri* while describing the low abundance/bycatch biodiversity that characterized the targeted mesopelagic fish layer.

Given the extremely reduced sampling effort of eDNA collection compared to trawling (Veron et al. 2023) and the ease of sampling standardization, eDNA samples could be routinely collected opportunistically on annual fisheries and oceanographic surveys worldwide (Stoeckle et al. 2021), with little disruption to core work programmes. Importantly, multiple taxonomic groups can be investigated from the same sampling effort, as shown by Govindarajan et al. (2023), that explored fish community compositions from the same sample set previously used in Govindarajan et al. (2021) to describe the invertebrate community of the mesopelagic layers.

We foresee that eDNA will be increasingly used in the near future to study fish communities, including the ones in the twilight zone. In this context, the use of ONT sequencing showcased here could find many applications as it is inexpensive, quick, portable, and can offer a high taxonomic resolution for fish species. Future recommendations include the filtration of high volumes, when possible, the inclusion of the metaprobe sampler (Maiello et al. 2022), as a simple and cost-effective way to obtain eDNA samples directly from the net, and the collection of water directly from catch tanks (Green et al. 2024, Maggini et al. 2024). Particularly, metaprobes and catch tank waters will increase the comparability of eDNA and fishing data. In addition, bulking up reference databases is always of utmost importance, particularly for less known areas of the ocean, like the mesopelagic layer and the deep seas (Govindarajan et al. 2023). As highlighted by Thomsen and Willerslev (2015), focus should also be targeted on mitogenome reference sequences, which would aid the application of ONT sequencing with longer metabarcoding fragments. Finally, the efforts presented here contribute to the implementation of omics sensors to be included on high-tech autonomous sampling platforms for the monitoring of deep-sea ecosystems (Aguzzi et al. 2019).

Conclusion

The present study showed the successful integration of eDNA and fishing in a multidisciplinary approach that described target echotraces as layers of *M. muelleri*. Considering the rising interest in the commercial exploitation of mesopelagic fish layers worldwide, further knowledge on their taxonomic composition is necessary. Integrating multidisciplinary techniques is recommended to maximize ship time efforts while providing direct and indirect data about these fish communities. Given the ease of eDNA sampling and the augmented species richness that it provides, future studies on the mesopelagic zone would benefit from the inclusion of this approach. In addition, the application of ONT sequencing with a long metabarcode was showcased as a promising tool for fish metabarcoding research, with advantages that include fast turnaround time, cost-effectiveness, and high taxonomic resolution.

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Author contributions

All authors conceptualized and designed the study. M.T., T.C., and C.O.D. collected samples and data. M.T. and L.M. conducted laboratory work. M.T. led bioinformatic and statistical analysis, with contribution from T.C. and help from L.M. and S.S. All authors discussed and interpreted the results. M.T. drafted the manuscript, with contributions from T.C. and L.M. All authors provided feedback and approved the final version of the manuscript. Ph.D. supervision to M.T. was provided by L.M., S.S., J.A., and B.O.N.

Supplementary data

Supplementary data is available at *ICES Journal of Marine Science* online.

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Data availability

The raw sequencing data are available on the NCBI SRA (BioProject PRJNA1112350).

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