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RESEARCH ARTICLE

Fish communities in Baltic Sea coastal bays; using eDNA metabarcoding to assess vertical profile and traditional method comparison

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Abstract. Fish communities in the coastal Baltic Sea are currently monitored using passive fishing gears, such as gillnets. In recent years, eDNA approaches have gained popularity for fish detection thanks to their non-invasive nature, faster processing, and more precise identification, although such methods have been underutilized in comprehensive fish community assessments in the Baltic Sea. This study reports fish diversity using eDNA metabarcoding within different depth profiles for the first time in temperature-stratified coastal bays in the Baltic Sea, while also offering some comparison with traditional net-based approaches. Comparing samples above and below the thermocline revealed exclusivity in fish species at both depths, emphasizing the importance of vertical sampling in capturing a comprehensive understanding of fish distribution patterns in such systems. Results indicated that eDNA captured more fish taxa per sample compared to gillnet sampling, with similar or higher fish diversity, although variations occurred between bays. This study highlights the importance of incorporating eDNA metabarcoding, alongside traditional survey methods, to aid assessment of fish communities in aquatic environments.

Keywords: marine, brackish, thermocline, shallow-water, deep-water, Sweden

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Introduction

Fish communities are part of dynamic ecosystems shaped by various environmental factors, including biotic (e.g., predation, competition, food availability) and abiotic aspects (e.g., nutrient levels, temperature, dissolved oxygen) (Jackson et al. 2001, Benoît and Swain 2008, Pecuchet et al. 2016). In vertically stratified waters, depth can also play a crucial role in influencing fish community composition and structure (Chouinard and Dutil 2011).

The Baltic Sea is a semi-enclosed, brackish-water basin where the average salinity in surface waters is 7–8 psu. This is due to the substantial freshwater influx from rivers in the north and limited exchange with the saltier waters of the North Sea through the narrow Danish straits in the south. It is relatively shallow (depth: average 55 m, max. 459 m) and exhibits large-scale gradients from temperate

marine to subarctic limnic conditions, which make the Baltic Sea and its mix of flora and fauna unique. Vertical stratification from a seasonal thermocline in summer, as well as a permanent halocline limits the vertical mixing of oxygenated and nutrient rich water, creating distinct environmental conditions in the water column. Coastal areas are additionally shaped by local variations in temperature, salinity, photic zone depth, bottom type, and water movement (Snoeijs-Leijonmalm and Andrén 2017).

Monitoring fish populations in the Baltic Sea is carried out for commercially important species (Atlantic herring, *Clupea harengus* (L.); Atlantic cod, *Gadus morhua* (L.); European plaice, *Pleuronectes platessa* (L.); Atlantic salmon, *Salmo salar* (L.); European sprat, *Sprattus sprattus* (L.)) in countries adhering to procedures and methods established by the International Council for the Exploration of the Sea (ICES) and the European Commission. In the Swedish Baltic Sea, fish survey monitoring for fish stock and environmental assessments use trawling and other traditional netting methods (e.g., gillnets), which result in the death of millions of fish each year (Nilsson et al. 2022). However, incorporating non-invasive and non-lethal environmental DNA (eDNA) methods can be one option to reduce this mortality and refine monitoring methods (e.g. Knudsen et al. 2019, Rourke et al. 2022) for more sustainable fish assessments in the future.

In recent years, eDNA based approaches have gained popularity for detecting fish species in aquatic ecosystems, including in rivers (Pont et al. 2018, Deng et al. 2024, Staveley et al. 2025), lakes (Jerde et al. 2011, Valdez-Moreno et al. 2019 He et al. 2024,), oceans (Gold et al. 2021, Thomsen et al. 2012a), and coastal zones (Sigsgaard et al. 2017, Yamamoto et al. 2017, Miya et al. 2022, Carvalho et al. 2024). The benefits of eDNA as a cost-effective, non-invasive sampling method with high detection probability, even at low densities, (Hering et al. 2018) can offer more precise and objective fish identification compared to more traditional based survey methods (Jerde et al. 2011, Sigsgaard et al. 2015). In the Baltic Sea, few studies have used eDNA tools to investigate fish presence and biomass, which have focused either on single species (i.e., *G. morhua*, (Kasmi et al. 2023), northern pike, *Esox lucius* (L.) (Ogonowski et al. 2023)) or exclusively on commercially valuable species (Knudsen et al. 2019, Urban et al. 2024). Nevertheless, exploring whole fish communities through metabarcoding in this region is somewhat lacking (although see Näslund et al. 2019).

This study reports fish community diversity using eDNA metabarcoding within different depth profiles for the first time in coastal bays in the Baltic Sea. In order to understand potential differences in fish communities in the shallower and deeper parts of the bays, sampling was conducted above and below the stratified boundary layer (thermocline). We further compared eDNA metabarcoding with traditional net-based approaches to assess their efficacy in identifying fish taxa and in relation to the number of sampling occasions. Finally, we identified and highlighted species of commercial value and those assigned specific Red List conservation statuses.

Materials and Methods

Location

This study was conducted in two coastal bays in the Baltic Sea between May and July 2022 (Figure 1). Kappelshamnsviken (area: 21.4 km^2; max. depth: 90 m), on the island of Gotland, is a relatively exposed bay and directly open to the sea to the north. The substrate is mostly composed of hard substrate as well as sandy and silty bottoms. Typical aquatic vegetation, such as filamentous algae and bladder wrack, *Fucus vesiculosus* (L.), is found in the shallower parts. Tvären, located on the eastern Swedish mainland coast, is smaller (area: 6.6 km^2; max. depth: 75 m) and more of an enclosed system, compared to Kappelshamnsviken. Soft substrates, e.g., sand and clay, dominate in deeper parts, while the shallower areas boast more hard, rocky substrates. Aquatic vegetation commonly found in this bay include filamentous algae and bladder wrack, alongside red algae varieties such as *Furcellaria lumbricalis*

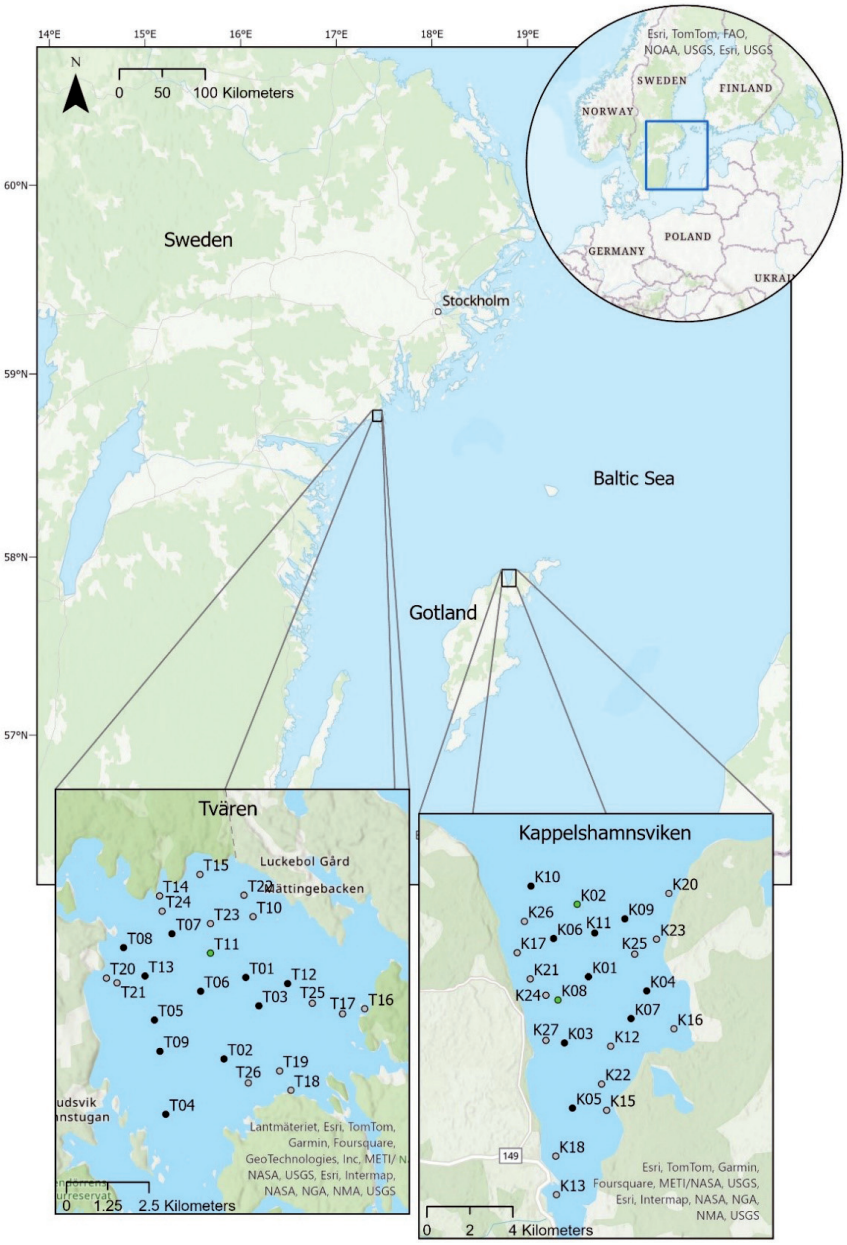

Figure 1. Location of the Kappelshamnsviken and Tvären bays in the Baltic Sea including eDNA and net sampling sites together with site names. Net sampling is indicated by the grey points, eDNA sampling is indicated by the green points, and black points mark locations where both nets and eDNA were used.

((Hudson) J. V. Lamouroux) and *Phyllophora crispa* ((Hudson) P. S. Dixon), which thrive particularly in the shallower parts.

Environmental variables

A vertical profile through the water column was conducted in Kappelshamnsviken prior to water sampling for eDNA on May 23, June 9, and additionally on July 3, 2022, and in Tvären prior to water sampling for eDNA on June 3, 2022, to collect temperature (°C) and salinity (psu) data. This was conducted in each bay at the deepest sampling site using a multiparameter probe (WiMo sonde NKE, temperature accuracy ± 0.02°C, conductivity ± 0.5% of the readings). Additionally, sonar (Deeper Smart Sonar

Chirp+ 2) was used at each site to check for the presence of a thermocline, which subsequently aided in classification between shallow (S) and deep (D) sites.

eDNA metabarcoding

Sampling procedure

Water samples for eDNA metabarcoding were taken at 11 sites in Kappelshamnsviken on May 23 and June 9, 2022, and at 12 sites in Tvären on June 3, 2022 (Figure 1; Supplementary Table S1). At shallow sites, that were classed by the absence of a thermocline, only one eDNA water sample was taken at 10 m. At deep sites between 35 and 85 m water depth, a thermocline was present and one sample was taken at 10 m and one between 35–55 m, depending on total water depth (Supplementary Table S2). This resulted in a total of 11 shallow and nine deep samples in Kappelshamnsviken, and 12 shallow and eight deep samples in Tvären. However, only six deep samples from Tvären yielded results. To the best of our ability, both eDNA and fishing sampling was conducted at the same sites. However, the number of eDNA samples was limited by resources, which is why deep gillnet sampling sites, which allow for vertical eDNA comparison as well as method comparison, were prioritized in the sampling design.

At all sites, five liters of seawater were collected from the boat using a two-liter Ruttner water sampler and transferred into one-time-use three-liter sterile bags (Fisherbrand™ Sterile Sampling Bags with Flat-Wire Closures, Fisher Scientific). The water was filtered immediately on site through a closed filter (5 µm GF top filter/0.8 µm PES bottom filter; 50 mm in diameter) supplied by Nature Metrics (UK). A preservative solution (modified Longmire's solution) was subsequently added to the filter, and sealed. Additionally, two positive controls and two negative controls were taken at the Ar Research Station, Gotland, Sweden (Supplementary Table S2). Positive controls were sampled in 15 m^3 tanks with recirculating seawater (17 psu, 10°C) containing *G. morhua*, which were fed *C. harengus* and *S. sprattus* daily. Negative controls used distilled water. The water sampler was cleaned between sites using bleach, and single-use sterile equipment was used for water filtration and DNA preservation to prevent contamination. The filters were stored frozen at -20°C for three weeks, before they were sent to the laboratory for analysis.

Laboratory protocol

All laboratory methods and analyses were conducted by Nature Metrics (UK). The following is a description based on information sent from Nature Metrics. A DNeasy blood and tissue kit (Qiagen) was used to increase DNA yields and extracted DNA from each filter (Supplementary Table S1) according to the protocol by Spens et al. (2017). Proteinase K was added directly to the filter housing to minimize the risk of contamination arising from handling the filter. Additionally, an extraction blank consisting of molecular grade water was processed for the extraction batch to monitor for exogenous DNA contamination. The extracted DNA was purified using a DNeasy Power Clean Pro Cleanup kit (Qiagen) to remove known PCR inhibitors, and the resulting yields were checked by measuring DNA concentration using a Qubit fluorometer with the Qubit dsDNA broad range assay kit (Thermo Fisher Scientific).

The purified DNA was amplified with a two-step PCR process for a hypervariable region of the 12S rRNA gene to target fish as part of the eDNA survey Fish pipeline. For this, tails were added to the 5' end of taxon specific primers to complement downstream adapter and index primer sequences. Amplification was performed with a commercially available high-fidelity DNA polymerase following manufacturers guidelines using MiFish-U-F 5'-[GCCGGTAAAACTCGTGCCAGC]-3' (forward) and MiFish-U-R 5'-[CATAGTGGGGTATCTAATCCCAGTTTG]-3' (reverse) primers (Alfaro--Cordova et al. 2022). The standard analysis involved 12 replicate PCRs per sample; each was performed in the presence of both a negative control (PCR-grade water) and a positive control sample (a mock community with a known composition). Amplification success was determined visually by observing gel electrophoresis. A total

of 4–12 successful PCR replicates were obtained for each sample submitted for sequencing, and no bands were observed on electrophoresis gels for the extraction blank or negative controls.

PCR replicates were pooled per sample, purified using MagBind TotalPure NGS magnetic beads (Omega Biotek), and sequencing adapters were added. Gel electrophoresis was used to confirm success, and all samples were successfully indexed with strong and expected size bands. No repeat reactions were necessary. A sequencing library was prepared from the purified amplicons using unique dual indexes, following Illumina's 16S metagenomic sequencing library preparation protocol. The resulting amplicons were subsequently purified, quantified, normalized, and pooled in equal volumes. The final pooled library was sequenced on an Illumina MiSeq system using a V3 600 cycle reagent kit (Illumina).

Bioinformatics

Sequence analysis was conducted by Nature Metrics. In order to process the sequence data, sequences were demultiplexed with bcl2fastq, and a custom bioinformatics pipeline was utilized. Paired-end FASTQ reads for each sample were merged with USEARCH (Edgar 2010). Forward and reverse primers were trimmed from the merged sequences using cutadapt (Martin 2011) with a length filter of 80–120bp. Sequences were quality filtered with USEARCH to retain only those with an expected error rate per base of 0.01 or below and dereplicated by sample, retaining singletons to obtain zero-radius operational taxonomic units (zOTUs). Unique sequences from all samples were denoised in a single analysis with UNOISE (Edgar 2016). Both negative and positive controls met the expectations. The data had minimal PCR and sequencing errors, resulting in very few discarded sequences prior to dereplication. The final dataset included a total of 3,227,505 high-quality sequences. Consensus taxonomic assignments were made for each zOTU by performing sequence similarity searches against the National Centre for Biotechnology Information nucleotide database (NCBI nt, GenBank) as a reference. Searches against databases were made using blastn (Altschul et al. 1990, Camacho et al. 2009) and required hits to have a minimum e-score of 1e-20 and cover at least 90% of the query sequence. The taxonomic identification associated with all hits was converted to match the GBIF taxonomic backbone.

Assignments were made to the lowest possible taxonomic level with consistent matches, and conflicts were manually flagged and resolved. Minimum similarity thresholds of 99%, 97%, and 95% for species-, genus-, and higher-level assignments were used, respectively. In cases where there were equally good matches to multiple species, public records from the Global Biodiversity Information Facility (GBIF) were consulted to determine the most likely species present in Sweden. If a taxonomic identification could not be resolved this way, higher-level taxonomic identifications or multiple potential identifications were reported.

zOTUs were clustered at 97% similarity with USEARCH to obtain OTUs. An OTU-by-sample table was generated by mapping all dereplicated reads for each sample to the OTU representative sequences with USEARCH at an identity threshold of 97%.

The OTU table was filtered to remove low abundance OTUs from each sample (< 0.025% or < 10 reads, whichever was the greater threshold for the sample). Finally, unidentified, non-target, and common contaminant sequences, such as human and livestock sequences, were removed.

Gillnet sampling procedure

Fishing was conducted on two occasions from June 15–17, 2022, in Tvären and on four occasions from July 2–4 and July 16–18, 2022, in Kappelshamnsviken. Occasions for net sampling were limited by weather conditions and logistical constraints. Great Lakes nets were used, which are modified coastal nets (comprised of nine different mesh sizes from 10–60 mm) with mesh panel sizes of 6.25 and 8 mm added. Each net was 55 m long and 1.8 m deep. Nets were deployed at a total of 22 and 25 locations

in Kappelshamnsviken and Tvären, respectively (Figure 1). The sampling locations varied in depth according to the bottom structure of the bays (Supplementary Table 1). Note that, due to the nature of net sampling, only one net was used at each site, in comparison to some eDNA sites where shallow and deep samples were taken. Two nets were destroyed during sampling, resulting in two sites without fishing data, but with eDNA data. The nets were set between 18:00–20:00 and taken up the following morning between 06:00–08:00. Fishes from each net were kept separately and treated as single units. All individuals were identified to the species level, where possible, and length was measured to the nearest mm and weight was recorded to the nearest g. If more than 30 fish within the same species showed little variation in length distribution (e.g., three-spined stickleback, *Gasterosteus aculeatus* (L.), of 50–60 mm length), the respective species was subsampled, meaning 30 individuals were measured for length, while the remainder were only counted. Identification of the gobies *Pomatoschistus microps* (Krøyer) and *Pomatoschistus minutus* (Pallas) were combined to *Pomatoschistus* sp. because of difficulties with species identification in the field.

Statistical analysis

The average difference in the number of taxa found between shallow and deep samples at one location including standard error was calculated for every site where a thermocline was present to investigate the importance of vertical sampling. To analyze fish community composition, non-metric multidimensional scaling (nMDS) was performed using the Jaccard dissimilarity matrix on presence-absence data across samples. NMDS was conducted using a four-dimensional solution, selected based on a stress value of 0.08, which provided the best fit. For visualization, the first two dimensions were plotted, with a stress value of 0.17. Permutational analysis of variance (PERMANOVA) was conducted to test the importance of temperature and salinity on fish communities from the different bays.

To examine the effectiveness of eDNA metabarcoding compared to net fishing to detect fish taxa, the average number of taxa detected per unit effort according to bay and sampling method was calculated and reported with standard error. To investigate potential differences in taxa detected per unit effort between eDNA metabarcoding and net fishing, a Mann-Whitney Rank Sum Test was conducted, as the data failed normality testing ($p \leq 0.05$, Shapiro-Wilk Test). To determine if fish communities in the bays were sufficiently sampled by the different sampling methods, taxa accumulation curves were generated based on randomized resampling of sites (1,000 permutations). Nonparametric bootstrap estimators were used to assess and extrapolate taxa richness in the surveyed bays (Gotelli and Colwell 2001). PERMANOVA was used to assess differences in fish community composition including bay and sampling method (shallow eDNA sampling, deep eDNA sampling and net fishing) as fixed effects. Taxa presence-absence data was used to compute a Jaccard dissimilarity matrix. These analyses were performed using the vegan package version 2.6-4 in R (Oksanen et al. 2022, Team R Core 2022).

Diversity descriptors

To further assess fish biodiversity between bays and differences in fish data collection methods, the Shannon-Wiener index (H), Pielou's Evenness (J), and Simpson's dominance index (C) were calculated as follows:

$$H' = -\sum_i (P_i - \ln(P_i))$$

$$J = -\frac{H'}{\ln(N)}$$

$$C = \sum_i (P_i^2)$$

where P_i is the proportion of reads or individuals represented by ith taxon and N is the total number of taxa for that method. For eDNA data, P_i was calculated as the number of reads for a given taxon relative to the total reads identified for that method. For net

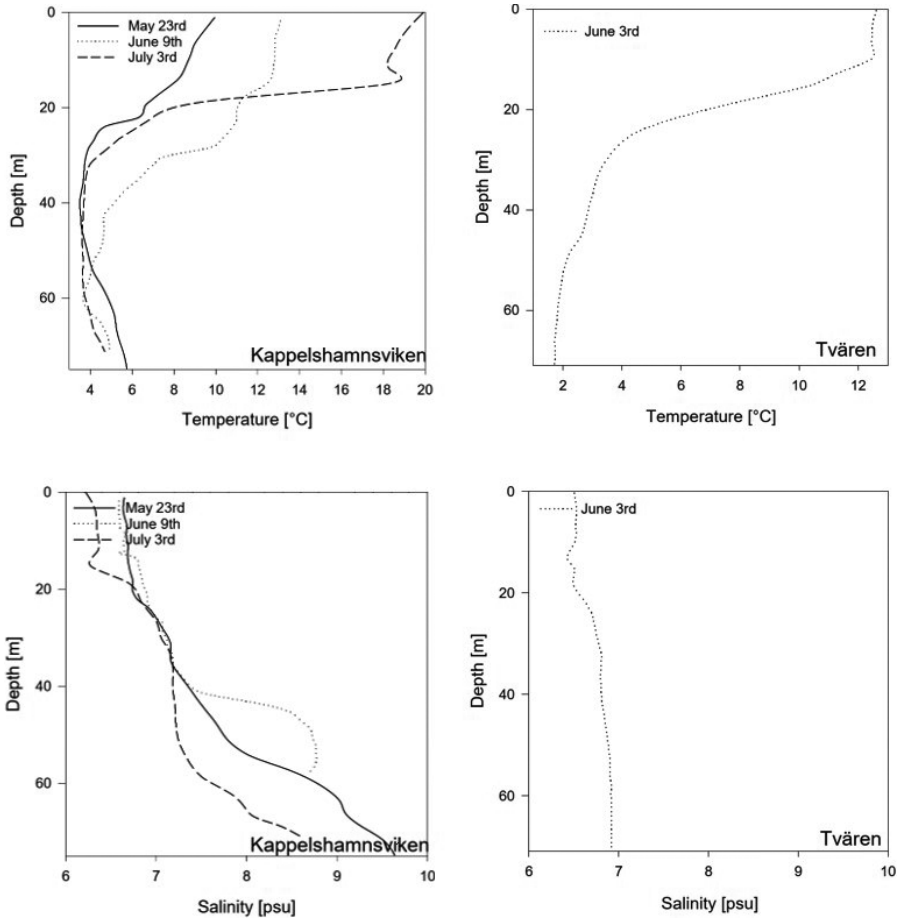

Figure 2. Vertical profiles of temperature (upper) and salinity (lower) in Kappelshamnsviken (left) and Tvären (right) bays during sampling occasions in 2022. Note different temperature scales.

data, P_i was calculated as a taxon's number of individuals caught relative to the total number of all taxa combined caught with that method. These indices were calculated based on total numbers of reads or individuals per taxon and bay.

Results

Environmental variables

From the vertical water profiles, water temperature in Kappelshamnsviken decreased from 10.0, 13.1, and 19.9°C at the surface, to 5.8, 5.0, and 4.7°C at the sea bottom in May, June, and July 2022, respectively (Figure 2). In Tvären, water temperature in June 2022 decreased from 12.6°C at the surface to 1.7°C at the sea bottom. A thermocline was present in both bays between approximately 12 and 30 m, separating the warmer, surface waters from the colder, deeper water layers. The salinity in Kappelshamnsviken was between 6.7 and 7.1 psu, which increased slightly to 9.7 psu below 60 m. In Tvären, the salinity was constant between 6.4 and 6.9 psu (Figure 2).

Fish richness from the eDNA

From all eDNA samples collected from both bays, 31 Operational Taxonomic Units (OTUs) were identified. Among these, 80% (25 OTUs) closely matched species in global reference databases and were assigned species names. The remaining OTUs were categorized at the lowest possible taxonomic levels:

16% at the genus level (5 taxa) and 3% at the family level (1 taxon). These taxa spanned 11 orders, 17 families, and 29 genera. The most prevalent species across both bays, based on sequence reads, were *C. harengus* and *G. aculeatus*, comprising 42% and 34% of the total sequence reads, respectively. These species were highly pervasive, detected in 95% and 97% of all samples, respectively.

In Kappelshamnsviken, a total of 25 taxa were identified, with *C. harengus*, *G. aculeatus*, and *G. morhua* being the most abundant, constituting 55%, 19%, and 11% of the total sequence reads, respectively. These species were detected in all samples from this bay. In Tvären, 19 taxa were found, where *G. aculeatus*, *C. harengus*, and *S. sprattus* dominated the sequence reads at 51%, 29%, and 13%, respectively. Similarly, these species were prevalent in ≥ 89% of the samples collected from this bay, indicating they are common and locally widespread species.

Comparison of eDNA from all shallow and deep-water samples

The majority of taxa captured at all sites by eDNA in Kappelshamnsviken, i.e., 96%, were found in shallow samples (compared to 64% in deep samples), while in Tvären most taxa, i.e., 84%, were detected in the deep-water samples (compared to 74% in shallow samples).

The following nine taxa were exclusively detected in shallow water samples in Kappelshamnsviken: *E. lucius*; black goby, *Gobius niger* (L.); *Thunnus* sp.*; Coregonus* sp.*; S. salar*; sea trout, *Salmo trutta* (L.); Arctic char, *Salvelinus alpinus* (L.), longspined bullhead, *Taurulus bubalis* (Euphrasen); and lumpfish, *Cyclopterus lumpus* (L.). In contrast, only the rock gunnel, *Pholis gunnellus* (L.), was identified solely in the deep samples in Kappelshamnsviken.

The straightnose pipefish, *Nerophis ophidion* (L.), was found solely in the shallow samples in Tvären, whereas four taxa - freshwater bream; *Abramis brama* (L.); *Carassius* sp.*;* saithe, *Pollachius virens* (L.); and common goby, *P. microps* - were exclusively detected in this bay's deep water samples.

When combining the eDNA samples from stratified sites in both bays (total samples: shallow = 15 and deep = 15), half (15 of 30 taxa) of the total fish taxa detected were present in both the shallow and deep-water samples. Nine taxa were found exclusively in the shallow samples while six taxa were found only in deep water samples (Figure 3). Demersal fish taxa, as well as commercially important taxa (see Table 1), were detected in both

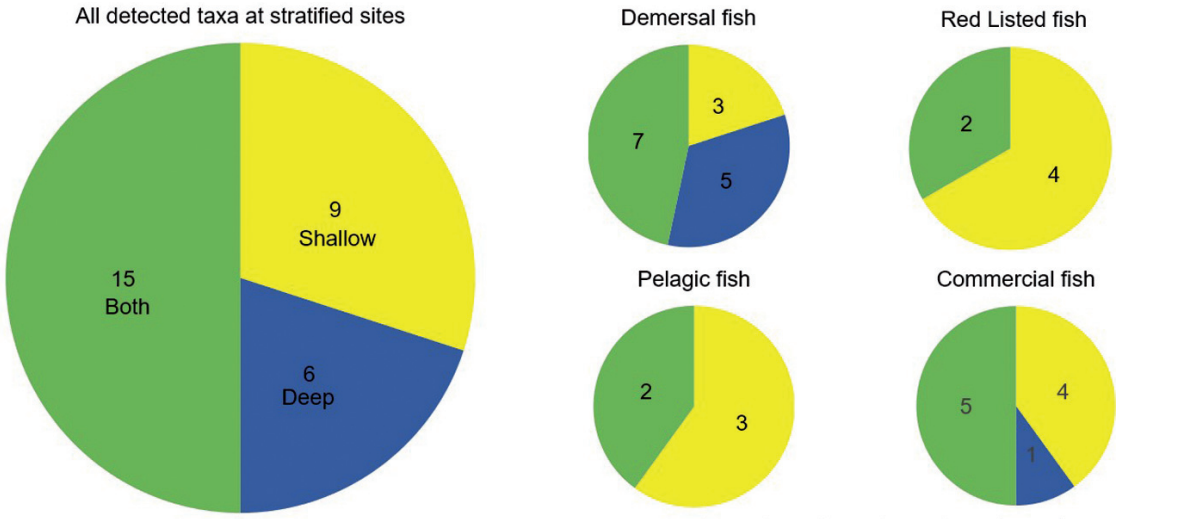

Figure 3. Number of taxa detected at stratified sites across both bays. Pie charts indicate taxa proportion from shallow samples (yellow), deep samples (blue), and from both sample depths (green). Separation into demersal or pelagic life history according to www.fishbase.org. Commercial value according to www.fiskbarometern.se. Red Listed fish are those categorized in either the IUCN or HELCOM Red List as near threatened, vulnerable, or endangered (see Table 1).

Table 1
Taxa found with specific sampling methods. * = recent historical and current commercial value in the Baltic Sea (source: Fiskbarometern, 2024), [a] = *Leuciscus idus* is the species likely to occur here and conservation status based upon this species, [b] = *Ammodytes tobianus* is the species likely to occur here and conservation status based upon this species, [c] = not known to occur in this region naturally, [d] = *Coregonus maraena* is the species likely to occur here and conservation status based upon this species. Conservation status according to HELCOM red list for fish and lamprey species (H) and the IUCN red list of threatened species (I); LC = least concern, NT = near threatened, VU = vulnerable, EN = endangered, DD = data deficient, NA = not applicable in the Baltic Sea.

			Kappelshamnsviken			Tvären		
Taxon	Common name	Conservation status	eDNA shallow	eDNA deep	net	eDNA shallow	eDNA deep	net
Belone belone	Garfish	H – NA, I - LC	X	X	X	X		
*Clupea harengus**	Atlantic herring	H – LC, I - LC	X	X	X	X	X	X
*Sprattus sprattus**	European sprat	H – NA, I - LC	X	X	X	X	X	X
Osmerus eperlanus	European smelt	H – NA, I - LC						X
*Abramis brama**	Common bream	H – NA, I - LC					X	X
Carassius sp.	Crucian carp	H – NA, I - LC					X	
Leuciscus sp.[a]	Eurasian daces	H – NA, I - LC	X	X				X
Rutilus rutilus	Common roach	H – NA, I - LC	X	X		X		X
Blicca bjoerkna	White bream	H – NA, I - LC						X
Alburnus alburnus	Common bleak	H – LC, I - LC						X
Tinca tinca	Tench	H – NA, I - LC			X			
*Esox lucius**	Northern pike	H – NA, I - LC	X					
*Gadus morhua**	Atlantic cod	H – VU, I - VU	X	X	X	X	X	X
Pollachius virens	Saithe	H – NA, I - LC					X	
Gasterosteus aculeatus	Three-spined stickleback	H – NA, I - LC	X	X	X	X	X	X
Pungitius pungitius	Nine-spined stickleback	H – NA, I - LC			X	X	X	
Ammodytes sp.[b]	Sandeel	H – LC, I - DD	X	X	X		X	X
Gobius niger	Black goby	H – NA, I - LC	X		X			X
Neogobius melanostomus	Round goby	H – NA, I - LC	X	X	X	X		X
Pomatoschistus sp.	Goby	H – NA, I - LC	X	X		X	X	X
Gymnocephalus cernua	Sand goby	H – NA, I - LC						X
*Perca fluviatilis**	European perch	H – NA, I - LC	X	X	X	X	X	X
*Sander lucioperca**	Pikeperch	H – NA, I - LC						X
Pholis gunnellus	Rock gunnel	H – NA, I - LC		X				
Thunnus sp.[c]	Tuna	H – NA, I - LC	X					
Zoarces viviparus	Viviparous eelpout	H – NT, I - LC	X	X	X	X	X	X
*Platichthys flesus**	European flounder	H – NA, I - LC	X	X	X	X	X	X
*Pleuronectes platessa**	European plaice	H – NA, I - LC			X			
*Scophthalmus maximus**	Turbot	H – NT, I - LC			X			
Coregonus sp.*[d]	Whitefish	H – EN, I - VU	X					
Salmo salar*	Atlantic salmon	H – VU, I - NT	X					
Salmo trutta*	Trout	H – VU, I - LC	X					
Salvelinus alpinus[c]	Arctic char	H – NA, I - LC	X					
Cottus gobio	European bullhead	H – LC, I - DD	X	X				
Myoxocephalus sp.	Sculpin	H – LC, I - LC	X	X	X	X	X	X
Taurulus bubalis	Longspined bullhead	H – LC, I - LC	X		X			
Cyclopterus lumpus	Lumpfish	H – NT, I - NT	X					
Nerophis ophidion	Straightnose pipefish	H – LC, I - LC				X		

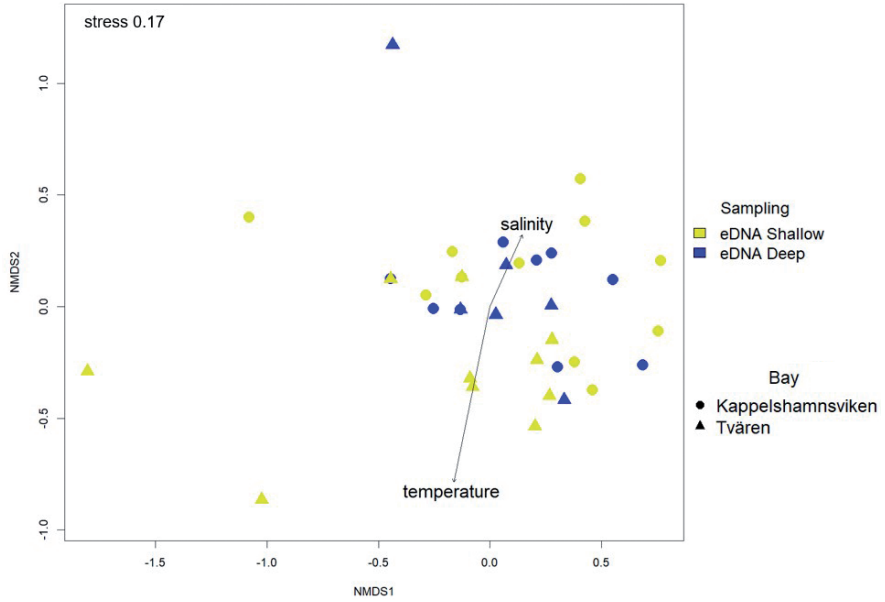

Figure 4. NMDS analysis of fish community composition based on eDNA samples showing the first two dimensions of the analysis. Each data point represents one sample.

sampling depths, with some exclusivity in shallow and deep samples. In contrast, pelagic and Red Listed fish were detected either solely in shallow samples or in both depths, but never only in deep samples.

Two-dimensional nMDS ordination showed differences between fish communities detected by eDNA from the different bays (Figure 4). Results of the PERMOVA indicated that the difference between bays was significant ($F = 2.81$, $R^2 = 0.07$, $p = 0.004$). Taxa composition also showed a significant association with water temperature ($F = 2.54$, $R^2 = 0.06$, $p = 0.007$), but not with salinity ($F = 1.05$, $p = 0.395$), likely due to low variation in the data.

Comparison of eDNA from specific shallow and deep-water samples

In those sites where a thermocline existed, eDNA sampling was conducted at the same geographic location but at two depths in the water column; shallow and deep, to enable comparison between the stratified water layers. Some distinct similarities and differences in fish community were observed between these samples, such as the dominance of *G. morhua* reads in K11.D and the switch in species dominance between *G. aculeatus* and *C. harengus* reads in the shallow and deep water samples at T03 and T11 (Figure 5). In Kappelshamnsviken, the average difference in specific taxa found exclusively in shallow or deep samples at one location was 5.8 ± 0.9, with a maximum difference of 11 taxa observed at site K10. Meanwhile, in Tvären, the average difference in the number of specific taxa exclusively in shallow or deep samples at one location was 4.7 ± 0.5, and the highest difference was 6 at sites T11 and T13.

Comparison of eDNA and net fishing

Generally, eDNA recovered more taxa per unit effort compared to the nets, i.e. average species per water sample was higher than average species per net ($U = 643.5$, $p < 0.05$). Using eDNA, 7.9 ± 0.7 and 6.6 ± 0.7 taxa were detected per water sample in Kappelshamnsviken and Tvären, respectively, while net sampling yielded 5.3 ± 0.7 and 5.9 ± 0.7 taxa per net, respectively. The comparison of taxon accumulation curves (Figure 6) suggests that in Kappelshamnsviken, net fishing reaches a plateau in taxon richness at approximately 15 taxa after five

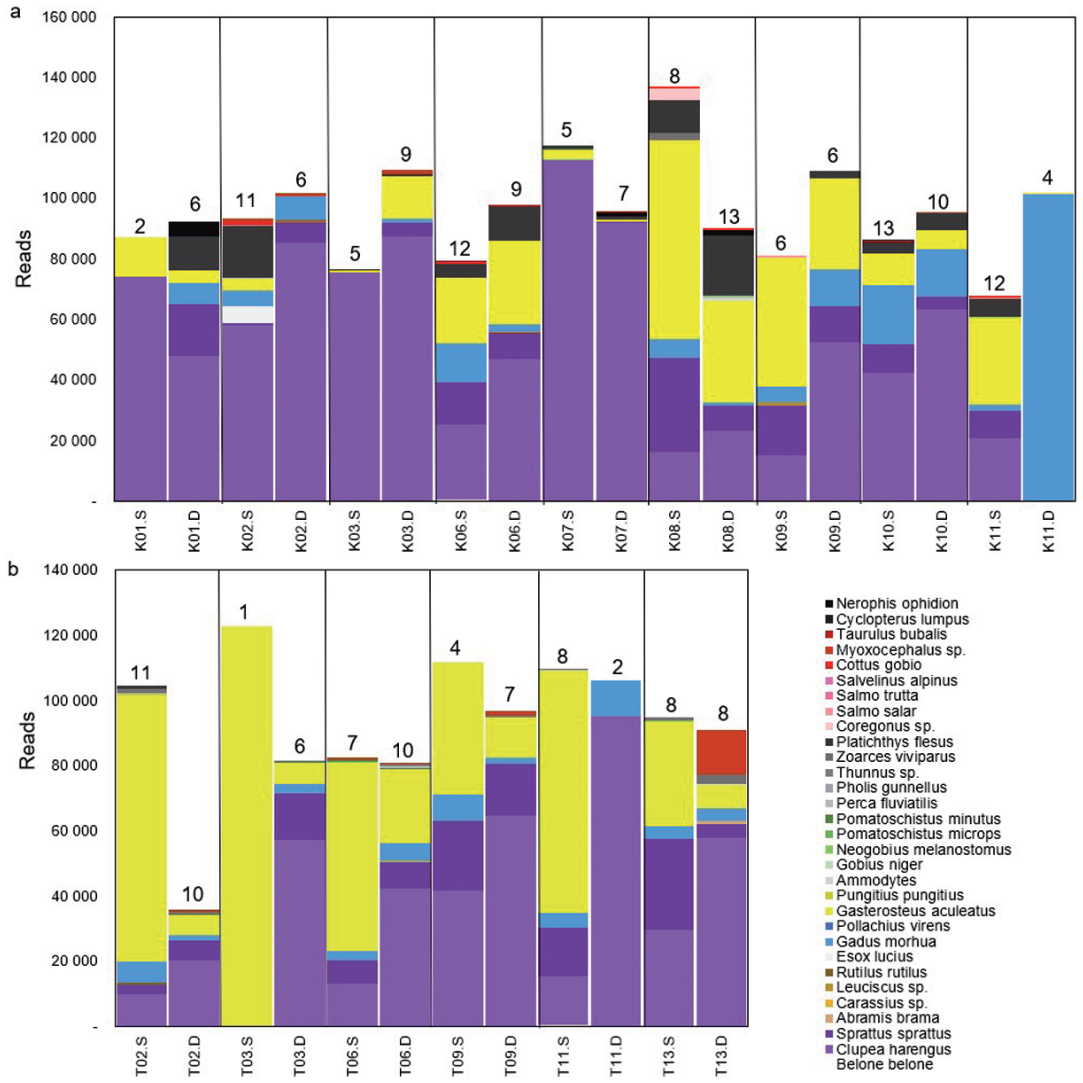

Figure 5: Reads from the shallow and deep samples coupled to the same location in the bays Kappelshamnsviken (a) and Tvären (b). The first sample (S) was sampled in the shallow layer, i.e., 10 m water depth and the second sample (D) was sampled in the deep layer, i.e., below the thermocline between 35-55 m. Numbers above the columns indicates total number of taxa found in each sample.

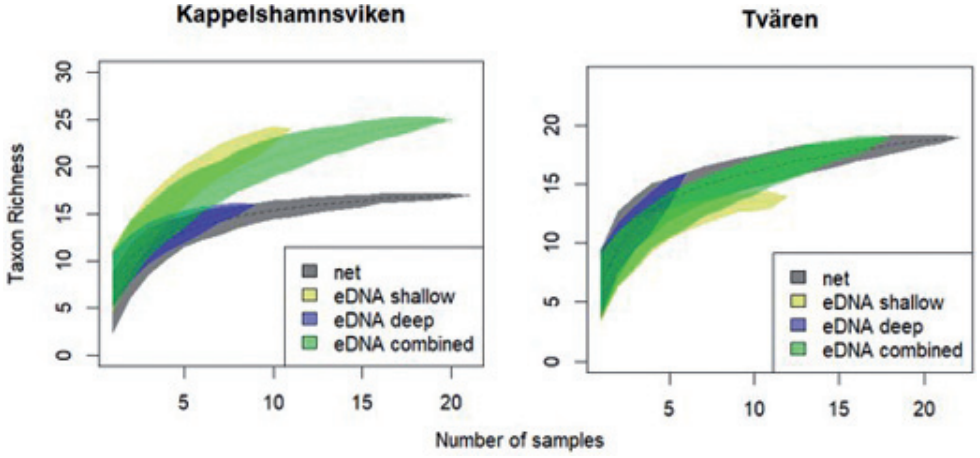

Figure 6: Taxa accumulation curves for net fishing and eDNA sampling in Kappelshamnsviken and Tvären based on all samples from the repective bay.

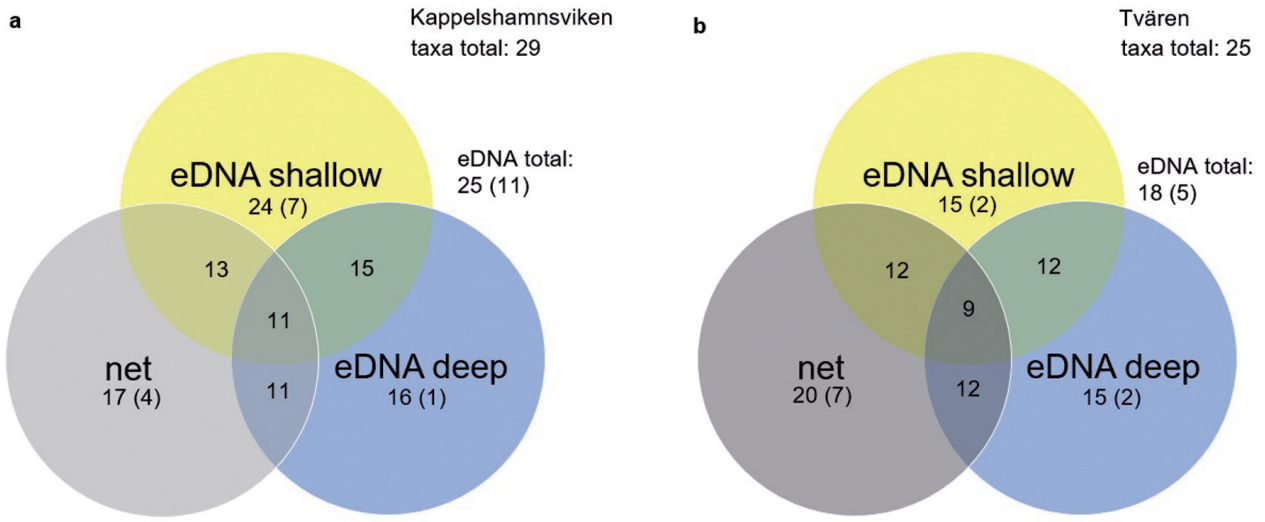

Figure 7. Venn diagrams displaying numbers of total (= present in the respective bay), shared, and unique (in brackets) taxa found by different methods in Kappelshamnsviken (a) and Tvären (b) bays.

samples. On the other hand, eDNA sampling in both shallow and deep waters results in a higher initial richness of around 25 taxa after 20 samples, and the richness continues to increase. In Tvären, both net fishing and eDNA sampling exhibit similar patterns, reaching less than 20 taxa after around 20 samples, and the taxon richness continues to increase with additional sampling.

The results of PERMANOVA indicated significant differences in fish community composition between bays ($F = 5.31$, $R^2 = 0.05$, $p \leq 0.001$), as well as between sampling methods ($F = 6.88$, $R^2 = 0.14$, $p \leq 0.001$). Overall, 29 fish taxa were found in Kappelshamnsviken, of which 25 (86%) were identified by eDNA and 17 (58%) by net (Figure 7). In Tvären, 25 fish taxa were found in total, of which 18 (72%) were identified by eDNA and 20 (76%) by net.

The most commonly found fish taxa, i.e., *G. aculeatus*, *C. harengus*, *S. sprattus*, and *G. morhua*, were captured by both methods (Table 1). Across all sites and methods, eight taxa were captured exclusively with nets, such as the flatfish *P. platessa*, turbot, *Scophthalmus maximus* (L.), and pikeperch, *Sander lucioperca* (L.), which were caught in several nets. Other taxa captured exclusively in the nets included white bream, *Blicca bjoerkna* (L.), and bleak, *Alburnus alburnus* (L.), although they each only appeared in one net.

From the eDNA, 11 taxa were detected using only this method, including the migratory *S. salar*, which was detected in several samples. Other taxa, e.g., other salmonids, *E. lucius, Thunnus* sp., *C. lumpus*, and *N. ophidion*, were detected in single samples, often with a low read count.

The Shannon index (H') was similar for net and eDNA sampling in Kappelshamnsviken (around 1.4), but significantly lower for net sampling compared to eDNA sampling in Tvären, with values of 0.3 and 1.3, respectively (Table 2). This shows that compared to net fishing, eDNA captures a similar or higher fish diversity. The evenness (J) and dominance (C) indexes were higher for net sampling compared to eDNA sampling in Kappelshamnsviken, which indicated a more even spread of taxa across the sites when using eDNA sampling. In contrast, net fishing yielded a lower evenness and higher dominance index compared to eDNA sampling in Tvären. This implies an uneven distribution of fish taxa and a high dominance of particular taxa when net fishing compared to eDNA sampling in this bay. Notably, these results represent only a snapshot in time and do not indicate general variation in the movement of fish taxa.

Table 2
Diversity index (H' = Shannon-Wiener index), evenness (J = Pielou), and dominance (C = Simpson's dominance index) for fish communities in the investigated bays based on different sampling methods

	Kappelshamnsviken				Tvären			
	net	eDNA shallow	eDNA deep	eDNA total	net	eDNA shallow	eDNA deep	eDNA total
Taxon Richness	17	24	16	25	20	15	15	18
H'	1.41	1.35	1.34	1.38	0.34	1.05	1.09	1.26
J	0.50	0.43	0.48	0.43	0.11	0.39	0.40	0.44
C	0.41	0.36	0.36	0.36	0.89	0.49	0.50	0.36

Discussion

Many fish species are known to occupy and move between certain depth ranges over varying time scales, whether they be daily, seasonal, or those associated with their life stages (e.g., Irisson et al. 2010, Freitas et al. 2016, Aspillaga et al. 2017). In this study, when comparing fish communities above and below the thermocline, some species were present throughout all sampling depths, yet certain species were found exclusively at specific depth intervals. Significantly, vertical sampling at varying depths in stratified waters is valuable for capturing differences in fish species that can help further understand, for instance, species distribution patterns (Aglen et al. 1999) and ontogenetic habitat shifts (Polte et al. 2017), while also facilitating detecting rare or non-native species (Stefanoudis et al. 2019). Using eDNA methods, such fish surveys can also be beneficial as complementary data for aquatic monitoring programs that use more traditional methods (Keck et al. 2022). As such, the results of this study showed that eDNA captured more fish taxa per sample compared to gillnet sampling and with similar or higher fish diversity in general, while important differences occurred at site and bay levels.

Summary of fish communities in the coastal Baltic Sea from eDNA metabarcoding

Even though the fish captured through the eDNA sampling gave a diverse range of marine and freshwater species that are most typical for this region, the dominance of *G. aculeatus* and *C. harengus* was widespread and occurred at most sites. *C. harengus* is one of the most common fishes in the Baltic Sea where it is often found in the pelagic zone, but it also uses shallower depths of 10-20 m, near coastal areas, for spawning (Aneer 1989, Svedäng et al. 2023). This likely explains the high levels of DNA detections during sampling (May-July), which coincides with the *C. harengus* spring spawning period in this region (Aneer 1989).

In the Baltic Sea, a regime shift towards *G. aculeatus* dominance has been observed of late (Eklöf et al. 2020). This small, adaptable, species has been estimated to constitute up to 10% of the total pelagic fish biomass in the open Baltic Sea (Olsson et al. 2019) and in some coastal areas, they even dominate the fish community by biomass (Ljunggren et al. 2010, Sieben et al. 2011, Staveley et al. 2020). Here, similar trends were observed as many sites, particularly in Tvären, where *G. aculeatus* detection numbers dominated, which indicate that this species is still very common in many parts of the Baltic Sea.

While spawning period has been shown to relate to peaks in DNA detection in other studies (e.g., Bracken et al. 2019, Hervé et al. 2022), and may be true for some species here (e.g., *C. harengus)*, it is unlikely that spawning was the main cause of higher DNA sequence reads for all species in this study. For example, the spawning grounds of *G. morhua* in the Baltic Sea are located much farther south from the investigated bays (HELCOM 2021), therefore this species may be utilizing these coastal bays for other reasons, such as prey availability or shelter (Staveley et al. 2019).

Importance of vertical profile sampling in relation to fish eDNA

Where a thermocline was present, the comparison of shallow and deep-water samples across bays indicated that the majority of taxa occurred throughout the sampling sites (52%), with approximately a third (32%) exclusive to shallow samples and the remaining 16% detected only in deep samples. Results here are in congruence with a study by Andruszkiewicz et al. (2017), which focused on marine vertebrates in Monterey Bay, California, USA, where they reported that both surface (0 m) and subsurface samples (20–40 m) resulted in variations in vertebrate communities at different depths, as well considerable shared taxa (64%) between the depths. In contrast, an eDNA metabarcoding study off the coast of New Jersey, USA found no difference between fish communities in surface and bottom samples (up to 30 m) in vertically stratified water (Stoeckle et al. 2021).

Interestingly, more taxa were detected at site K11 in the shallow sample, but one taxon, *G. morhua*, was dominant in the deep sample, yielding more sequence reads than all taxa combined in the shallow sample. Also, a switch in species dominance between *G. aculeatus* and *C. harengus* reads was noted in the shallow and deep water samples at sites T03 and T11. These results highlight the complexity and variability of fish communities in stratified waters not only at a larger bay-scale, but also at smaller, site-specific, scales.

In some temperate lakes, warm water fish species are detected in higher eDNA concentrations in the surface layer, while cold water species tend to concentrate below the thermocline (Klobucar et al. 2017, Littlefair et al. 2021). While in contrast, when stratification is broken down and the lake water is mixed, fish eDNA was more homogenous throughout the entire water column (Littlefair et al. 2021). While Baltic fish species reflect similar distribution patterns according to temperature preference for some taxa, i.e., the warm-water species *P. fluviatilis* was mostly detected in shallow samples and cold-water species such as *G. morhua* mainly in deep samples, these results are not consistent throughout, and can certainly differ depending on ecological and behavior traits for individual fish species. Surprisingly, for example, the more warm-water tolerant species *A. brama* was mostly detected in deep samples.

eDNA metabarcoding versus net fishing

In general, the sampling effort needed to observe taxon richness depended on the method and location. Comparisons between eDNA and net fishing revealed that eDNA achieved a higher taxa recovery per sample from both bays. Net fishing in Kappelshamnsviken reached a plateau in taxa richness in relation to sampling effort relatively early, suggesting that even a few nets captured all targeted catchable taxa. In contrast, eDNA sampling combining both shallow and deep samples in Kappelshamnsviken revealed more taxa than net fishing, but it required more extensive sampling at the bay level to capture and understand the full taxon richness. In Tvären, net fishing and eDNA sampling showed a more similar pattern of taxa richness recovery with increasing sampling effort. Interestingly, the ongoing increase in fish richness with additional eDNA samples suggests that taxon diversity may not have been fully characterized, and more effort may uncover additional taxa in both bays.

In assessing the methods, it is crucial to consider differences in sampling effort, as the number of sites sampled with nets in our study was twice that of sites sampled by eDNA. Moreover, bottom nets involve a more targeted approach, potentially biasing the data toward species with higher catchability close to the seafloor, yet taxon richness was similar or lower for net fishing than eDNA metabarcoding. Likewise, other studies have reported a greater number of species detected through eDNA in comparison to net fishing (Hänfling et al. 2016, Golpour et al. 2022, Li et al. 2023, Schreiber et al. 2023). This trend has also been observed when contrasting eDNA with various conventional survey methods, such as trawling, beach seining, fyke netting, angling, and snorkeling (Thomsen et al. 2012a, Golpour et al. 2022).

The spatial distribution of eDNA can be influenced by factors such as currents and boat activity, resulting in a more extensive spatial impact compared to traditional aquatic monitoring methods like net fishing (Pont et al. 2018, Harrison et al. 2019). Additionally, eDNA metabarcoding has the potential to capture a broader spectrum of species as it relies on genetic material released by organisms into the environment. However, it is essential to acknowledge the variability in DNA release among different fish species (Kirtane et al. 2021), their size and number (Takahara et al. 2012, Lacoursière-Roussel et al. 2016, Sassoubre et al. 2016), and their life stage (Maruyama et al. 2014). Furthermore, the degradation rates of eDNA in brackish waters remain unclear. Studies on eDNA degradation in seawater show that eDNA persists for only a few days above the detection threshold (Thomsen et al. 2012a, Sassoubre et al. 2016, Collins et al. 2018), while decay in freshwater happens on a scale of days or weeks (Dejean et al. 2011, Thomsen et al. 2012b), thus emphasizing the variability likely due to many physiochemical parameters.

Conducting both eDNA metabarcoding and net fishing has shown overlap but also some exclusivity in detecting/catching fish species both in this and previous studies (Gillet et al. 2018, Golpour et al. 2022). Here, the migratory *Thunnus* sp. and *S. alpinus* are not known to inhabit the Baltic Sea and were detected exclusively with eDNA in Kappelshamnsviken in single or few samples with low read count. eDNA has also previously been shown to be highly effective in detecting rare and non-native species (e.g., Jerde et al. 2011, Thomsen et al. 2012b), which highlights the importance of integrating eDNA metabarcoding in monitoring programs as these taxa can easily be missed by traditional fishing methods. On the other hand, the flatfishes *P. platessa* and *S. maximus* were caught only by net fishing, notably in several nets. Their demersal life history potentially limits DNA release into the water column and restricts it rather to the seafloor, which could explain the sole detection by net fishing, but this could also be due to the differences in method deployment since water collection for eDNA was a single time point compared to the nets that were left overnight. However, some pelagic taxa that are also common along the coastal Baltic Sea, such as *O. eperlanus* and *A. alburnus*, were only caught by net fishing. This indicates that the presumably more targeted approach of nets toward taxa with a higher catchability closer to the seafloor is not the only cause for differences in species detection compared to eDNA metabarcoding.

Impact on future monitoring

The analysis of fish communities in the Baltic Sea through eDNA metabarcoding has provided valuable insights into the diversity and distribution of species within the studied bays. The identification of 31 taxa, with 80% identified to the species level, highlights the value of eDNA as a powerful tool for monitoring fish in brackish-water ecosystems, particularly taking into account depth intervals and stratification in site selection.

While there are limitations in current monitoring methods of fish communities in the coastal Baltic Sea (Olsson et al. 2015), specifically that nets may exclude small-sized fish or less common species, this study highlighted that these taxa are more likely to be detected through eDNA metabarcoding. Although, in contrast, some species such as some commercially valuable flatfish, may not be detected by eDNA analyses. Other important commercial species in the Baltic Sea, such as *G. morhua* and *C. harengus*, have shown significant declines in population size recently (ICES 2023), therefore, non-invasive methods to accurately assess fish stocks are urgently needed. Although eDNA methods cannot currently offer direct biomass quantification, recent research demonstrates promising signs for incorporating genetic-based techniques for fish biomass assessment (Kasmi et al. 2023, Pont et al. 2023), which can hopefully be incorporated into more sustainable monitoring and assessment methodologies in the Baltic Sea and beyond.

Conflict of interest. The authors declare no conflicts of interest.

Data availability statement. All data used in this study is openly available in figshare at http://doi.org/10.6084/m9.figshare.27720507.

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