Development and application of DNA based methods to quantify catch composition in Danish demersal and pelagic fisheries (DNAMIX)

Paulina Urban, Lars Magnus Wulf Jacobsen, Anders Nielsen, Brian Klitgaard Hansen, Sara Maggini, Dorte Bekkevold, Jos Kielgast and Einar Eg Nielsen

Development and application of DNA based methods to quantify catch composition in Danish demersal and pelagic fisheries (DNAMIX)

Paulina Urban, Lars Magnus Wulf Jacobsen, Anders Nielsen, Brian Klitgaard Hansen, Sara Maggini, Dorte Bekkevold, Jos Kielgast and Einar Eg Nielsen

Colophon

Preface

This report presents the results from the project "DNAMIX: Udvikling og afprøvning af DNA baserede metoder til kvantitativ bedømmelse af artsammensætning i mixede landinger", which has received financial support from the European Maritime and Fisheries Fund (EMFF) and the Danish Fisheries Agency (journal no. 33113-I-19-064). The project period was October 2019 to December 2022.

The project included collaboration between Danish fisheries producer organizations (DFPO and DPPO), MID (Marine Ingredients Denmark) and DTU. The organizations participated as consultants in relation to the design of the studies, by providing information on the fishery, vessel design and landing/catch processing procedures. They helped with the logistics in terms of getting access to fishing vessels and processing factories as well as providing fish for the experiments. Finally, they were asked to evaluate the results in relation to the feasibility of future method implementation from a practical fisheries perspective. They have not been involved in the data collection, data analysis or interpretation of the results.

We specifically thank our close collaborators from DPPO (Claus Reedtz Sparrevohn, Lise Laustsen), DFPO (Henrik Lund, Jakob Handrup) and MID (Anne-Mette Bæk). We also thank the many vessels, the fish processing factories, and their personnel for providing access to samples and catch information. Without their constructive and helpful attitude, we could not have completed the project. We also thank the many colleagues at DTU Aqua in Silkeborg, Hirtshals and Lyngby, who contributed to this project.
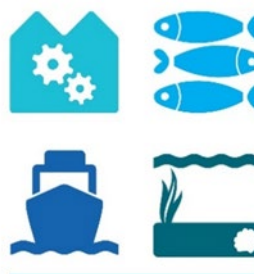
DTU Aqua, Silkeborg

Einar Eg Nielsen
Professor

Content

1. Summary

1.1 DNAMIX – Danish summary

Formål

At udvikle, afprøve og evaluere DNA baserede metoder til kvantitativ bedømmelse af artsammensætning i forbindelse med konsum og industrifiskeri. Herunder at vurdere både de nuværende og fremtidige anvendelsesmuligheder og implementeringspotentiale i relation til praktisk prøvetagning, tekniske og biologiske usikkerheder, sensitivitet samt omkostningseffektivitet.

Faktiske forløb

Projektet er forløbet som planlagt, med gennemførte aktiviteter, herunder 1) udvikling af molekylærbiologiske metoder, 2) eksperimentelle test til kalibrering af metoderne og 3) test implementering af metoderne i forhold til praktisk bifangstbestemmelse i både industri og konsumfiskeri.

Pelagisk fiskeri

I det pelagiske industrifiskeri fokuserede vi på bifangst af sild i brislingefiskeriet og i det pelagiske konsumfiskeri på bifangst af makrel i sildefiskeriet. Vi har udviklet og anvendt molekylære testmetoder, kvantitativ PCR (qPCR assays), der specifikt genkender og opformerer DNA fra henholdsvis brisling, sild og makrel og derfor kan bruges til at identificere og kvantificere mængden af DNA fra de enkelte arter i blandinger (på samme måde som en Covid test).

Efterfølgende er de specifikke qPCR assays anvendt til at kalibrere metoderne til DNA analyse af såkaldt "proces-vand". Princippet er, at DNA'et fra de forskellige arter i vandet der omgiver fiskene i processen fra fangst til forarbejdning er mere homogent fordelt end artsfordelingen i fangsterne. Man kan derfor få et mere præcist og integreret estimat af fangsternes sammensætning ved at tage DNA prøver af vandet end ved en traditionel visuel stikprøve, da arterne ofte er klumpet fordelt i fangsterne. Afgivelse af DNA til vandet per vægtenhed er forskellig fra art til art, så DNA koncentration giver ikke et 1:1 billede af vægtandelene i fangsterne, som jo er den efterstræbte værdi i fangstmonitering og kontrol. Vi gennemførte derfor en række eksperimenter med blandinger af arter med kendt vægtsammensætning, til at "oversætte" DNA andele til vægt andele. Ud over sammenhængen mellem DNA og vægt andele undersøgte vi effekten af fiskenes størrelse på mængden af afgivet DNA (små fisk forventes at afgive mere DNA par vægtenhed end store). Vi målte længden på brisling og sild i eksperimentelle blandinger og brugte størrelsen på fiskene, til at forsøge at optimere DNA baserede estimater af vægtandele.

De etablerede sammenhænge mellem DNA og vægt blev testet i forhold til praktisk fiskeri og landinger. Fra brislinge- og sildefiskeriet analyserede vi en lang række prøver af procesvand fra opbevaringstanke på fiskefartøjer ("blodvand") og ved landinger på fabrikkerne ("landingsvand"). På baggrund af DNA-andele af mål- og bifangstarter i prøver fra procesvand og statistiske modeller for DNA/vægt fra eksperimenterne, estimerede vi vægten af den totale bifangst i en række landinger. Estimaterne er sammenlignet med anvendte visuelle metoder til monitering og kontrol, såsom fiskernes logbog, 3.parts kontrol på fabrikkerne og fiskerikontrol. Disse estimater er baseret på den såkaldte "spande-metode", hvor et (statistisk repræsentativt) antal spande med fisk udtages fra fangsterne, sorteres og vejes manuelt, samt efterfølgende bruges til opskalering af artsandelene til hele fangsten. Sammenligningen af præcision, nøjagtighed og omkostningseffektivitet mellem de traditionelle metoder og DNA metoden dannede grundlag for en vurdering af potentialet og praktiske anvendelsesmuligheder for kvantitativ bedømmelse af artssammensætning i pelagisk fiskeri baseret på

DNA i samarbejde med fiskerne (DPPO), forarbejdningsindustrien (MID) og fiskeristyrelsen (se nedenfor).

Demersalt fiskeri

For det demersale konsum fiskeri har vi fokuseret på estimering af fangst og bifangst sammensætning i bundtrawl efter jomfruhummer og torsk (målarter). Dette fiskeri er mere komplekst i artssammensætning end det pelagiske fiskeri, så vi anvendte en anden molekylærbiologisk tilgang. I stedet for qPCR, anvendte og tilpassede vi en metode, der identificerer alt fiske-DNA (DNA metabarcoding) i prøverne. Derfor kan den anvendes bredt til at beskrive fangstsammensætningen. Ulempen ved metoden er, at den er mindre præcis i forhold til kvantificering. Disse analyser blev foretaget med en transportabel 3. generations DNA sekventerings maskine ("MinION"), som derfor potentielt kan anvendes på fartøjer og fabrikker. Som for det pelagiske fiskeri, testede vi først på en blanding med kendt DNA sammensætning (12 forskellige arter). Dernæst indsamlede vi prøver af procesvand, her på observatørture fra praktisk torsk/jomfruhummer fiskeri. Prøverne var fra opbevaringstanke med den totale fangst (både ønsket og uønsket fangst), samt delprøver af uønsket fangst alene og prøver med maksimal arts diversitet inklusiv PETS (protected, endangered and threatened species). DNA data blev sammenlignet for forekomst og kvantitet af arter i forhold til visuel vurdering af artsantal og vægt af den totale fangst (logbog), samt manuel optælling og vejning af delprøver af uønsket fangst (undermåls og ikke kvoterede arter) og prøver med maksimal artsdiversitet. Resultaterne for det demersale fiskeri, i forhold til implementeringspotentiale, blev diskuteret med erhvervet (DFPO) og fiskeristyrelsen.

Opnåede resultater i projektet

<u>Pelagisk fiskeri</u>

Vi har med succes udviklet molekylærbiologiske assays, der helt specifikt genkender og opformerer DNA fra mål- og bifangstarter, hvilket er en forudsætning for anvendelse af DNA og PCR tests til kvantificering af fangstsammensætning.

DNA analyse af eksperimentelle blandinger med kendte artsandele for de pelagiske fisk (brisling, sild og makrel) viste en meget stærk sammenhæng mellem DNA og vægtandele. Det betyder, at man med høj præcision og nøjagtighed kan bestemme vægtandele i en blanding af fisk, hvis man kender DNA andelene i det omgivende vand. Eksperimenterne tillod udvikling af statistiske modeller til omregning fra DNA andele til vægtandele i det praktiske fiskeri og på fabrikkerne. Eksempelvis svarer en estimeret silde DNA fraktion på 0.4 i sild/brisling blandinger, til en silde vægtandel på 0.54±0.07. Ved at tage højde for biologiske usikkerheder, såsom arternes relative størrelse, forbedredes præcisionen yderligere (0.47 ± 0.01 for silde DNA fraktion på 0.4). Arternes DNA-andele i vandet var konstante indenfor en i fiskeri og forarbejdningsmæssig relevant tidsramme. Det vil sige, at DNA analysens resultat ikke påvirkes af fiskenes opholdstid i vandet og vandet opbevaringstid inden DNA analyse.

Ved analyser af procesvand fra opbevaringstanke på fartøjer (blodvand) og på fabrikkerne (landingsvand), var de DNA baserede bifangst-estimater konsistent i samme størrelsesorden, som de visuelt baserede estimater (spande-metoden). De DNA baserede metoder havde dog langt større præcision (lille varians) end de visuelle metoder. Selv hvor andelen af bifangst var nul eller få promiller af den totale fangst havde DNA metoden høj præcision. Typiske landinger er på omkring 1000 tons, så der findes ikke totalt opgjorte fangster (alle fisk talt og vejet). Men baseret på den lave variation og store robusthed af de DNA analyserne, er det sandsynligt, at de også har en større nøjagtighed end spande-metoden.

<u>Demersalt fiskeri</u>

Vi har med succes udviklet en pipeline til DNA analyse af vandprøver fra det demersale fiskeri til bestemmelse og kvantificering af artssammensætning. Pipelinen inkluderer procedurer fra indsamling af vand ombord på fiskefartøjer over DNA metabarcoding på MinION, bioinformatik og yderligere kvalitative og kvantitative analyser af artssammensætning. DNA analyserne af kontrolprøverne viste at alle 12 arter kunne identificeres i sekvensanalysen, mens det kvantitative signal var svagt. DNA analysen fra subsamples af uønsket fangst viste en meget god kvalitativ korrespondance mellem antallet af arter detekteret visuelt (46-100%) og på DNA niveau. Der var også en klar positiv og konsistent sammenhæng mellem de hyppigt forekommende arter og deres DNA signal, uden det dog tillader en præcis omregning fra DNA andele til vægtandele. Lignende resultater blev opnået for høj-diversitets prøverne, hvor en meget høj andel (42-73%) af arterne, inklusiv PETS blev detekteret. For analyserne af prøverne fra det totale fiskeri blev 67-100% af de visuelt observerede arter (logbog eller observatør) i jomfruhummerfiskeriet detekteret med DNA, mens det var 62-92% for torskefiskeriet. En lang række arter blev detekteret med DNA, som ikke blev visuelt registreret (30-58%). DNA analyserne detekterede 7 forskellige arter af hajer og rokker (PETS). Der var generelt en relativt svag sammenhæng mellem vægt (logbog) og DNA andele. Ved anvendelse af overordnede mål for biodiversitet baseret på artssammensætningen af prøverne, kunne der detekteres klare forskelle i biodiversitet mellem DNA analyserne fra jomfruhummer og torskefiskeriet.

Opsummering og samlet konklusion

Projektet har vist at det er muligt at etablere DNA baserede metoder til kvalitativ og kvantitativ bestemmelse af fangstsammensætning i konsum og industrifiskeri. DNA analyserne af de komplekse prøver fra de demersale fiskerier (jomfruhummer og torsk) viste et højt potentiale for beskrivelse af arts-sammensætning og biodiversitet i prøverne, med specifikt potentiale ift. bifangst af PETS. Den sekventerings baserede (metabarcoding) metode anvendt, sammen med prøvernes kompleksitet, gør det dog vanskeligt at opnå mere end et semi-kvantitativt estimat af fangstsammensætningen, herunder af den uønskede fangst. For de pelagiske fangster til industri og konsum viste den anvendte metode (qPCR) meget stort potentiale for en præcis og nøjagtig bestemmelse af bifangst i de undersøgte fiskerier. Ud over at metoden er en klar forbedring i forhold til de nuværende visuelle metoder, er den også mere tids og omkostningseffektiv. Der er således et stort potentiale for umiddelbar implementering, som en generel moniterings- og kontrolmetode i det pelagiske fiskeri.

1.2   DNAMIX – English summary

The project objective was to develop, test and evaluate DNA-based methods for quantitative assessment of species composition for consumption and industrial fishing (for fishmeal and fish-oil production). This included assessing current and future applications and implementation potential related to practical sampling, technical and biological uncertainties, sensitivity and cost-effectiveness. Overall, the project progressed as planned, with completed activities: 1) development of molecular-biological methods, 2) experimental tests to calibrate the methods for DNA/weight relationships and 3) test implementation of the methods for practical bycatch determination for both industrial and consumption fisheries. The overarching rationale was that fish excretes DNA to the surrounding "process water" throughout the production chain. Since the water is sourronding the whole catch at all times, the DNA contained in it is better mixed than the catch itself, where the species can be very unevenly distributed.Therefore, the DNA content provides a precise and accurate integrated signal of the catch composition as a whole.

For the pelagic fishery for consumption and industrial purposes,the case studies were herring bycatch in the (industrial) sprat fishery and mackerel bycatch in the (consumption) herring fishery. We devel-

oped species-specific quantitative PCR assays for target species identification and DNA quantification. First, assays were applied to DNA derived from experimental samples, that consisted of species mixtures kept under fisheries-like scenarios to assess the DNA-to-weight relationship. Overall, the experiments showed a strong linear relationship between input fish weight proportions and DNA proportions, allowing the establishment of statistical models for converting DNA proportions to fish weight. Combining statistical models for converting DNA to biomass with DNA analysis of production water from actual fish catches at the fishing vessels ("blood water") and factories ("discharge water") allowed quantitative bycatch estimation in large pelagic catches (~1000 t). Results from the DNA analysis were in the same range as visually determined bycatch estimates from the fishers' logbooks, third party control at the factory and the fisheries control agency. More importantly the precision of the DNA-based estimates was higher than of the other catch assessment methods tested, even at very low bycatch rates.

For the demersal fishery, we focused on aqueous samples of DNA from Norway lobster and cod fisheries. Due to high species complexity we developed a Next Generation Sequencing analytical pipeline (DNA metabarcoding) using a MinION portable sequencing device. This method potentially allows identification of all catch species simultaneously, but is less precise for species quantification. The method was tested on a mock DNA mixture from 12 species and identified all species. However, the quantitative signal was weak. Subsequently the method was applied on real fishery samples, collected during a fisheries observer trip. Three types of water samples were analysed, originating from 1) the whole catch, 2) the unwanted catch alone (undersized and not quotated specimens) and 3) mock samples with maximal biodiversity including PETS (protected, endangered and threatened species). The results were compared to the fishers' logbook and visual observer records. In general, there was a high overlap for species identification between DNA based and visual data. Overall, the DNA based method identified more species than the visual approach. Moreover, the DNA based method identified seven sharks and rays species and identified differences in biodiversity indices between cod and Norway lobster fisheries. However, DNA/weight relationships were weak, suggesting further method development is required before implementation for quantification.

Overall, the DNA methods have potential for species monitoring (including PETS) and development of biodiversity indices for the complex demersal fishery. For large and less complex pelagic catches the methods has a large and immediate potential for implementation and will provide a significant improvement regarding, precision, speed, and cost efficiency. Some of the tools described here were developed in parallel with the Horizon 2020 project PANDORA for mesopelagic fishes.

2. Scaling from eDNA to biomass: controlling allometric relationships improves precision in bycatch estimation

Authors
Paulina Urban[1]*, Dorte Bekkevold[1], Henrik Degel[2], Brian Klitgaard Hansen[1], Magnus Wulff Jacobsen[1], Anders Nielsen[3] & Einar Eg Nielsen[1]

Affiliations
[1]Section for Marine Living Resources, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark (DTU), Silkeborg, Denmark
[2]Section for Monitoring and Data, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark (DTU), Lyngby, Denmark
[3]Section for Marine Living Resources, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark (DTU), Lyngby, Denmark

2.1 Abstract

Environmental DNA (eDNA) has attracted interest in relation to fisheries, with its possibilities for species identification and promises for species quantification. In the context of fisheries catches, eDNA can be most useful for the estimation of bycatch proportions. The assessment of species mixtures in large catches (>1000 t) is challenging, especially when morphologically similar species are to be differentiated. We used an experimental set-up to simulate industrial sprat fishery catches, and tested two types of water (blood- and discharge water) derived from this simulated fishery for their suitability in reliable species quantification. We analysed nine mixtures of sprat and herring – the main bycatch species. Species-specific quantitative PCR was used for species identification and quantification. Species-to-species weight fractions and eDNA fractions in mixtures showed a strong correlation. Accounting for size-based differences in DNA abundance (allometrically-scaled weight) reduced the estimated standard erroron weight fraction prediction from 0.064 to 0.054 in blood water, and from 0.080 to 0.075 in discharge water when comparing the weight-based model with the allometrically-scaled weight model respectively. Accounting for allometric scaling in genetic analyses of fisheries process water can serve as a more precise method for the assessment of bycatch, thus in a wider sense improve the quality of fisheries-dependent data.

2.2 Introduction

Genetic and genomic tools are implemented in a large number of research areas including fisheries (Segelbacher *et al.*, 2010; Jacobsen *et al.*, 2018; Nielsen *et al.*, 2018; Beng and Corlett, 2020). Environmental DNA (eDNA) based detection has received particular attention for the application to aquatic biodiversity and resource monitoring (Rees *et al.*, 2014; Ruppert *et al.*, 2019; Beng and Corlett, 2020). The concept exploits the inherent DNA release from macro-organisms into their environment through DNA shedding via e.g. feces, urine, skin cells, and mucus (Taberlet *et al.*, 2012; Rodríguez-Ezpeleta *et al.*, 2021). eDNA can subsequently be retrieved from an ambient environmental sample, most often

water, and used to identify the species present in a given area (Thomsen *et al.*, 2012; Boussarie *et al.*, 2018; Salter *et al.*, 2019; Hongo *et al.*, 2021; Knudsen *et al.*, 2022; Shelton *et al.*, 2022a). Analysis of eDNA is generally effective for high resolution species detection, and is at the same time fast, cheap, non-invasive and does not require taxonomic expertise (Thomsen *et al.*, 2012; Stoeckle *et al.*, 2021; Knudsen *et al.*, 2022). Recently, application of eDNA has been applied to fisheries science (Mauvisseau *et al.*, 2017; Jacobsen *et al.*, 2019; Salter *et al.*, 2019; Hansen *et al.*, 2020; Russo *et al.*, 2021; Stoeckle *et al.*, 2021). Here, primary research focus has been to estimate fish abundance or biomass in the wild from species-specific DNA copy numbers in environmental samples (Thomsen *et al.*, 2016; Knudsen *et al.*, 2019; Salter *et al.*, 2019; Russo *et al.*, 2021; Stoeckle *et al.*, 2021; Yates *et al.*, 2021; Shelton *et al.*, 2022a). However, correlations between eDNA and fish abundance can be relatively weak (Perez *et al.*, 2017; Deutschmann *et al.*, 2019; Knudsen *et al.*, 2019). For one thing due to the technical challenges associated with the amplification process (i.e. PCR) used in many studies, were varying primer efficiency per species (Kelly *et al.*, 2019) directly influence the read abundance recovered. For another thing, as eDNA captured at a location at a particular time and space is the result of a complex interplay between DNA production, degradation and transport, including dilution, all of which vary with biotic and abiotic conditions (Hansen *et al.*, 2018).

In contrast, the confined "environment" onboard a fishing vessel provides a unique opportunity for a more general evaluation of the eDNA approach's potential for quantitative analysis of species abundance and biomass. The DNA-abundance measured in a fisheries catch reflects a more controlled environment because of the lack of variation in abiotic factors such as current, temperature, sunlight etc., that typically hamper interpretation of eDNA estimates naturally occurring at low quantities if detected at all in ambient sea samples. Biotic factors like metabolism and health are also suggested to impact eDNA shedding rate in nature (Hansen *et al.*, 2018), but are likely of little importance in fisheries samples, especially in cases where the fish are already dead and not severely damaged due to the fishing process itself. As such, fisheries sampling allows for improved insights into the specific relationship between eDNA and biomass by focusing on the interplay between DNA-shedding (production) and DNA-decay (degradation) on standing eDNA concentrations, which has been notoriously difficult to predict (Thomsen *et al.*, 2012; Sassoubre *et al.*, 2016; Collins *et al.*, 2018). DNA-production rates can vary between species due to the type of material being shed (i.e. mucus, scales, skin-cells) (Sassoubre *et al.*, 2016) and the DNA-state (free DNA versus cell-bond (intracellular) DNA) (Zhao *et al.*, 2021; Mauvisseau *et al.*, 2022). Further, size based differences, following the general assumption that small fish are expected to shed more DNA per unit weight, due to the larger relative surface area, are expected (Maruyama *et al.*, 2014; Yates *et al.*, 2021). Also, species-specific differences in the release of DNA or variability in DNA-target (i.e. mtDNA) copy number in tissue can cause higher DNA abundance of some species in solutions, which do not reflect higher abundance or biomass *per se* (Hansen *et al.*, 2020). DNA-decay can further contribute to variation in DNA-abundance, due to variation in the speed of DNA degradation of different states of DNA (Jo and Minamoto, 2021). For example, free DNA is an easy target for microbes and thus quickly disappears in solutions with high microbial load (Salter, 2018), whereas the degradation of intracellular or even intraorganellar DNA can take longer, as it is protected by a cell or organelle double membrane (Mauvisseau *et al.*, 2022). Knowledge of both DNA-shedding and decay rates is thus of utmost importance when attempting to translate DNA abundance estimates into quantitative metrics, such as species abundance, biomass and density.

Pelagic fisheries catches commonly exceed 1000 tons total catch brought to shore (Nøttestad *et al.*, 2016), rendering visual estimation of species compositions and proportions virtually impossible. Nonetheless, it is often a formal requirement to report (non-targeted) bycatch proportions under control and enforcement schemes (Council Regulation (EC) No 1224/2009 (European Union, 2009)). Here, catch

mixture can be estimated through catch subsampling, in which all specimens are visually identified, weighed and the proportions scaled to the total catch (Fiskeristyrelsen, 2021). The complexity of the unwanted catch and its distribution, e.g. between individual hauls of the total catch, determine the amount of necessary subsamples needed to accurately represent the total catch (Fiskeristyrelsen, 2021). It can be hypothesized that instead, eDNA-based analysis of samples from the water surrounding the catch, which is expected to be better mixed than the fish themselves, could be an effective alternative (Urban *et al.*, 2022). One opportunity is to sample and analyse so-called blood water, which is the product of a commonly used onboard procedure, where cooled natural seawater is circulated around the catch for up to 15 days before landing, in order to maintain fish quality (del Valle & Aguilera, 1991). Another opportunity is to sample the discharge water, which is freshwater used when pumping the catch from the boat to the processing factory during landing, after the blood water has been drained. In contrast to blood water, discharge water is in contact with the catch for a relative short time (2-10 h). Compared to visual inspection of catch sub-samples, eDNA-based blood and discharge water analysis may provide a precise and cost-efficient opportunity to comply with legal framework, i.e. the EU landing obligation (European Union, 2009; STECF, 2013; Uhlmann *et al.*, 2019).

The industrial sprat fishery in the Baltic Sea has a relatively simple catch-bycatch mixture and therefore is a good case study to test quantitative eDNA-based catch assessment from fisheries production water. In the Baltic Sea there are only two commercially exploited pelagic fishes (ICES, 2020), European sprat (*Sprattus sprattus*, L.) and Atlantic herring (*Clupea harengus*, L.) that feed in mixed-species assemblages. Sprat is the targeted species and constitutes the largest part of the catches (quota statistics for Denmark 2021: sprat 21 993t, herring 2 367t, European Commission, 2020), although population sizes, and thus quota, fluctuate. Due to the natural ecology and distribution of the two species, the bycatch of herring in sprat fisheries is inevitable and variable. High morphological similarity of the two species (especially when damaged under storage), and high variability in proportions among hauls challenge the currently applied visual bycatch assessment methods. Yet, with dwindling spawning stock biomass (SSB) of herring in the Baltic Sea it becomes paramount to provide a robust assessment of the total landings, including bycatch, to facilitate population recovery (European Commission, 2020). In this study, we test the applicability and precision of a blood and discharge water eDNA-based method for the assessment of fish mixture fractions in experimental "mock" samples with known weight fractions of sprat and herring, mimicking catch fractions in the Baltic Sea sprat fishery. We hypothesize that, due to better mixing, water samples will be more homogeneous in eDNA content and thereby better represent species mixing, than the mixture of fish itself. We specifically test: *i*) whether DNA-based fractions estimated in blood and discharge water are accurate proxies for weight-based fractions of sprat and herring in mixed samples, *ii*) whether fish size variation (allometrically scaled weight, i.e. surface area) can improve the biomass estimations, and *iii*) if there is a differential shedding/decay of DNA for the two species over time, affecting the fractions estimated from blood and discharge samples at different time points.

2.3 Material and Methods

Experimental "mock" fishery samples were prepared by mixing sprat and herring to mimic fractions typically encountered in the Baltic Sea sprat fishery. Sprat and herring specimens were caught during the Baltic International Trawl Survey (BITS), by the research vessel DANA on the 8th of March 2021 using standardized survey fishing gear (type TV3, mesh size 20 mm) (Table 2.1).

Table 2.1. Overview of fish and seawater collected for the experiment.

	Date	Longitude	Latitude	Time	Gear
Fish	08/03/2021	015°35'111 E	54°36'775 N	19:30	TV3*
seawater	09/03/2021	015°19'367 E	54°48'730 N	11:00	CTD**

*TV3 is a standard fishing gear type used for fisheries monitoring programs in the Baltic Sea (ICES, 2014).
*CTD is an electronic instrument that collects information about the ambient water **c**onductivity, **t**emperature, **d**epth and can collect seawater at specific depths when coupled with Niskin bottles.

After visual species identification and sorting, herring and sprat were rinsed with seawater to remove excess mucous from the catch and kept cool (3-4°C) until the start of the experiment on March 9. While at sea, nine different sprat-herring mixtures were established ranging 10-90% sprat as a target,

Table 2.2. Overview data for the fish used in each experimental unit. Provided are: Targeted biomass mixture (whole fish) for the two species (S=sprat, H=herring) as well as numbers, weight, weight fraction and mean length of each species in each experimental unit.

N	Targeted Sprat/herring mixture	Species	Weight (g)	Weight fraction	Mean weight (g)	Mean length (cm)
362	90/10[a]	S	4401.6	0.887	12.13	12.63
16		H	560.7	0.113	35.04	17.79
360	90/10[b]	S	4235.7	0.877	11.77	12.57
22		H	594	0.123	27	16.27
374	90/10[c]	S	4069.1	0.846	10.88	12.24
28		H	740.1	0.154	26.43	16.07
343	80/20	S	4037.4	0.79	11.77	12.46
26		H	1038.4	0.203	39.94	18.7
287	70/30	S	3167.4	0.661	11.04	12.32
46		H	1624.7	0.339	35.32	17.92
251	60/40	S	2971.7	0.599	11.84	12.55
51		H	1989.7	0.401	39.01	18.31
205	50/50	S	2250.1	0.472	10.98	12.35
68		H	2520.3	0.528	37.06	18.53
182	40/60	S	2066.7	0.402	11.36	12.17
69		H	3081.1	0.599	44.65	19.34
124	30/70	S	1412	0.285	11.39	12.28
84		H	3548.7	0.715	42.25	18.99
85	20/80	S	1035.6	0.205	12.18	12.46
98		H	4010.1	0.795	40.92	19.05
46	10/90	S	545.5	0.104	11.86	12.38
109		H	4703.8	0.896	43.15	18.73

The target mixture 90/10 (percent sprat and herring, respectively) was replicated three times, resulting in a total of eleven mixtures (hereafter experimental units) (Figure 2.1). For each unit, fish were drained, and placed into separate 12L containers double wrapped with plastic bags (Cater Line,

Freezer bags, 40L). 2 -2.5 L of fresh seawater (Table 2.1) were prepared for each unit. Before the water was poured into the unit to start the experiment, 45 ml were sampled into a sterile falcon tube (Sarstedt, 50 ml), using a sterile syringe (Codan™, 60 ml) and served as blank samples for estimating levels of natural contamination for each unit. The plastic bags were closed to prevent possible cross-contamination, and the units were kept in a cold room (3-4°C) for the following nine days, being stirred energetically at least once per day.

Targeted sprat/herring mixtures in experimental units
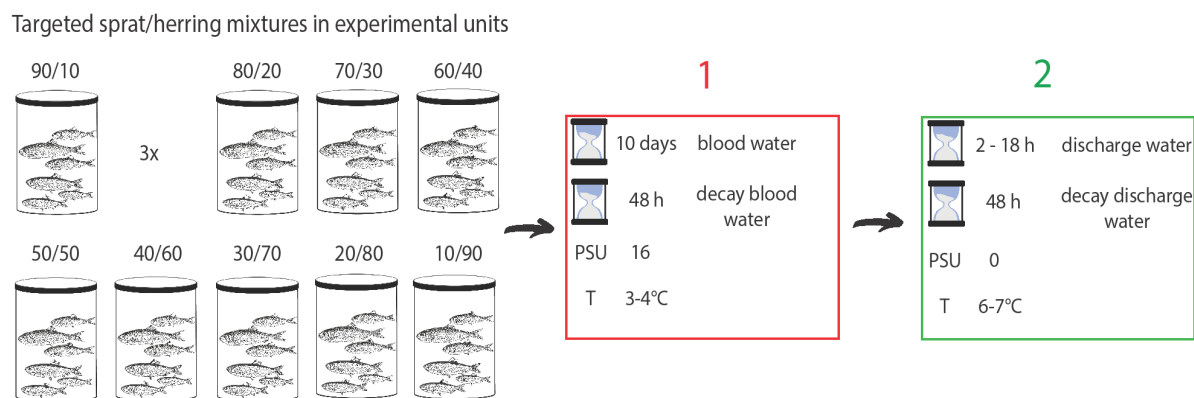
Figure 2.1. Overview of the experimental set up of the study. Eleven experimental units with nine different sprat-to-herring mixtures were prepared to simulate the sprat fisheries from the Baltic Sea. 1) The experimental units were covered with natural seawater (16 PSU) from the Baltic Sea and kept at low ambient temperature (3-4°C) for a duration of 10 days. The resulting blood water was separated from the fish and sampled. Then the blood water was kept (without fish) for another 48h and sampled at regular intervals to estimate the decay. 2) The fish were subsequently transferred to freshwater (0 PSU, 6-7°C) to simulate discharge (unloading process of the catch from the ship to factories). The discharge water was sampled at regular intervals for 18h, after which the fish were again separated and the water kept for additional 48h at the same conditions to monitor the decay.

To simulate the normal procedures associated with holding the sprat/herring catch onboard industrial fishing vessels and the processes associated with discharge of the fish to the processing factory, each experimental unit was subjected to four different sample treatments (see Table 2.3 and Figure 2.1 for details): 1) blood water, 2) blood water decay, 3) discharge water, 4) discharge water decay. For each treatment sampling was performed at different time points to assess possible changes in DNA fractions, resulting in varying numbers of observations (N) per treatment (Table 2.3).

Table 2.3. Overview of types of treatment, time intervals and experimental conditions tested for each aimed mixture (experimental unit).

Treatment	Type	N	Time intervals sampled	Experimental conditions	
				SAL (PSU)	T (°C)
Blood water	DNA-shedding	1	9 d	~ 9	4
Blood water decay	DNA-decay	6	2h, 4h, 6h, 12h, 24h, 48h	~ 9	4
Discharge water	DNA-shedding	7	2h, 4h, 6h, 8h, 10h, 12h, 18h	~ 0	5-6
Discharge water decay	DNA-decay	6	2h, 4h, 6h, 12h, 24h, 48h	~ 0	5-7

2.3.1 Blood water and the decay of blood water

The blood water experiment was set up while at sea, and blood water was sampled upon arrival at the harbour after nine days, which is in agreement with typical time intervals from catch to discharge in the industrial sprat fishery. Upon landing, experimental units were briefly transported from the ship to the lab (~ 1 km) and blood water was drained through a mosquito net (mesh size: 1.4 x 1.6 mm; to avoid larger tissue particles) into sterile plastic bags (Whirl-Pak® Stand Up, 2,041 ml). From here, 45 ml blood water was sampled into sterile falcon tubes (Sarstedt, 50 ml), using a sterile syringe (Codan™, 60 ml). The falcon tubes were immediately frozen at -20 °C and kept until DNA-extraction, and used to represent the 'blood water' treatment. The remaining blood water bags were sealed and placed in a cooling facility at 4 °C for the subsequent 48 h for the 'blood water decay' treatment. During that period, the decaying blood water was sampled at six different time points: 2h, 4h, 6h, 12h, 24h and 48h. Each time, 3 ml of the blood water decay samples were collected using a sterile syringe (Injekt®, 20 ml) into small collection tubes (Sarstedt, 57x15.3 mm).

2.3.2 Discharge water and the decay of discharge water

After draining (i.e. separating the fish from the blood water), the fish were returned to the experimental containers double wrapped with new plastic bags (Cater Line, Freezer bags, 40 L). To simulate the catch discharge, 2 L of freshwater (tap water) was added to each of experimental containers and stirred manually to ensure mixing. Plastic bags were closed to prevent possible cross-contamination and the containers were stored at 6-7 °C for the subsequent 18h to simulate the natural discharge process from ship to factory. The discharge water was sampled at seven different time points: 2h, 4h, 6h, 8h, 10h, 12h and 18h (= 'discharge' treatment). Subsequently, the fish were drained through a mosquito net and the discharge water was collected in sterile bags (Whirl-Pak® Stand Up, 2,041 ml) and left for a 48h decay period. These 'discharge decay' samples were taken at six time point: 2h, 4h, 6h, 12h, 24h and 48h. All discharge samples were collected in 3 ml tubes (Sarstedt, 57x15.3 mm) using a sterile syringe (Injekt®, 20 ml). All units were stirred at regular intervals and before sampling, to ensure a fully mixed samples. After collection, samples were frozen at -20 °C until DNA-extraction.

2.3.3 DNA-extraction and qPCR

Before extraction, samples were centrifuged at 3700 rpm for 30s to minimize the chance of extracting tissue particles present in water. 1 ml of water was used for the extraction of DNA with the Omega Bio-tek E.Z.N.A. Tissue DNA kit (Omega Bio-tek, USA) following an adjusted version of their standard "tissue DNA protocol", i.e., using a 2.5x volume of buffers and solutions to adjust for the large sample volume. Samples were eluted in 50 μl pre-heated elution buffer, and stored at -20°C.

DNA quantification was based on species-specific sprat and herring qPCR assays targeting the cytochrome *b* sequence of the mitochondrial DNA (mtDNA). The sprat assay was designed and validated for this study (Table 2.4). Both primer design and *in silico* validation was performed using Geneious (Version 2021.2.2) with the integrated Primer3 (version 1.1.1) software on sequences downloaded from National Center for Biotechnology Information (NCBI) GenBank database (Clark *et al.*, 2016) To target herring DNA we used the assay CluharCYB_02 assay (Knudsen *et al.*, 2019). Both assays were tested and validated *in vitro* in relation to assay optimization (primer and probe concentration adjustment), specificity (testing assay performance on closely related, co-occurring species) tested on tissue derived DNA extracts and sensitivity with determination of LOD (Limit Of Detection) and LOQ (Limit Of Quantification) (Merkes *et al.*, 2019). All samples were analysed in duplicates on the StepOne Real-Time PCR System (Life Technologies, USA) with triplicate negative controls and triplicated standard curves generated from a dilution series of diluted gBlocks ranging from 3 x 10^6 to 3 x 10^0 copies/reaction in each run. Total volume of each reaction was 10 μl with 3 μl of sample, 4 μl TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher Scientific), assay-specific volumes of primers and probes to obtain optimal reaction conditions (Table 2.4) and 1.2 μl TaqMan™ Exogenous Internal

Positive Control Reagents (Thermo Fisher Scientific) to monitor potential inhibition. The qPCR consisted of 5°C for 5 min and 95°C for 10 min followed by 50 cycles at 95°C for 30 s and 60°C for 1 min. Species-specific estimates of DNA copy numbers were then used to calculate herring and sprat fractions; i.e. herring DNA copy number to total DNA copy number (sum of herring and sprat DNA copy) ('DNA-based fractions').

Table 2.4. Overview of the assay elements (primer and probe) used for the species-specific quantitative PCR approach. Assay elements correspond to F: forward primer, R: reverse primer, P: probe, T: target sequence (gBlock). To increase delta-fluorescence assays used double-quencher probes (5'FAM/ZEN/3'IB®FQ (Integrated DNA Technologies, USA).

Target species	Assay element	Primer and probe sequence(direction 5'->3'), with FAM and BHQ1 modifications	Optimal concentration (nmol) per individual reaction	Target sequence length	Target mtDNA fragment	Avg. Eff. % (MIN) (MAX)	Avg. R2 (MIN) (MAX)	LOQ (following Merkes et al. 2019)	Ref.
Clupea ha-rengus	F	CCCATTTGTGATTGCAGGGG	200			89.454	0.997		Knudsen et al. 2019
	R	CTGAGTTAAGTCCTGCCGGG	600			88.19	0.991		
	P	TACTATTCTCCACCTTCTGTTCCTC	300	86	Cytb	91.7	0.999	43	
	T	CCCATTTGTGATTGCAGGGGC-TACTATTCTCCACCTTCTGTTCCTCCAC-GAAAC-GGGGTCAAACAACCCGGCAGGACTTAACTCAG							
Sprattus sprattus	F	CTCGTATAAGGACGCCCTAG	400			93.53	0.997		
	R	CGAAGAGGGCTAGAGATGTAA	400		Cytb	91.059	0.993	248	
	P	GCTTTGCGG/ZEN/TCATGCTGTTGGCTC	200	65		100.286	0.999		
	T	CTCGTATAAGGACGCCCTAGGCTTT-GCGGTCATGCTGTT-GGCTCTTACATCTCTAGCCCTCTTCG							

2.3.4 Bacterial quantification

To determine if the activity and quantity of microbes could potentially influence DNA degradation we measured the microbial quantity in 1 ml of blood water samples using Bactiquant®), which is a widely applied method for measuring bacterial load in the aquaculture industry (Reeslev *et al*., 2011). This method was applied on blood water samples only, as we hypothesized that the longest incubation time of fish in seawater would lead to the highest bacterial load.

2.3.5 Fish

All individuals used in the study originated from the same fishing haul (i.e. a single catch). Their average weight and size (length) varied slightly among the different experimental units (Table 2.2). Only whole fish were used leading to slight deviations in the actual gram-to-gram proportion in each experi-

mental unit and thus deviations between the targeted mix proportion and actual weight based fractions (Table 2.2). In general, herring were heavier and larger than sprat (Table 2.2). In total, 3239 individuals were morphologically identified to species, and their size weight, length measured at the end of the experiment.

The size of the fish, and ultimately its surface area is expected to influence the magnitude of the DNA-shedding of the individuals. Even though the surface area of the fish was not measured directly, we estimated it empirically, following the assumption that it will explain all the variation in DNA release. We determined the allometrically scaled weight (hereafter ASW) for each individual sprat/herring as follows:

$$AW \sim W^y,$$

where W is the individual fish's weight and y is the allometric scaling coefficient modified after (O'Shea et al., 2006). Due to the difference in degradation of the fish as a result of different treatments, the allometric scaling coefficient y is assumed to change and thus is estimated per treatment. The estimation of a suitable coefficient per treatment was done though visual inspection of model fit to observations.

2.3.6   Data analysis

To investigate the DNA to weight or allometrically scalled weight relationship the data (DNA abundance, weight, and allometrically scalled weight) was first converted into sprat-to-herring fractions, second we build a beta-distributed generalized linear model (GLM) with logit transformation on fractions using the package glmmTMB 1.0.2.9 (Brooks et al., 2017) in R 4.1.0 (R Core Team, 2016) and RStudio (version 1.4.1106).

$$O_i \sim Beta\,(\mu_i, \phi\,),$$
$$logit\,(\mu_i) = \alpha + \beta\,logit(X_i),$$

with O_i is the observed DNA based fraction. O_i is assumed to follow a beta distribution with mean value (μ_i) and a variance parameter ϕ. The logit transformed mean value (μ_i) is assumed to be a linear function of the logit transformed true fraction (X_i). The true fraction X_i is either weight-based fraction (kg) or allometrically scaled weight-based fraction (mm^2).

Figures were created using R 4.1.0 (R Core Team, 2016) and RStudio (version 1.4.1106) using the packages $tidyverse$ (version 1.3.1, Wickham et $al.$ 2019) and $dplyr$ (1.0.6, Wickham 2015).

2.4   Results

2.4.1   Assay performance

The developed sprat assay did not cross-amplify with herring DNA for the concentrations tested (0.01-1 ng/µl). The herring assay was tested on the same concentrations of sprat DNA and showed no cross-amplification for concentrations up to 0.1 ng/µl and weak, below LOQ cross-amplification for the highest concentration (1 ng/µl). However, even under cross-amplification, the target species amplification was six orders of magnitude higher than the non-target species amplification, pointing to negligible influence on results. The total DNA-concentration across the different treatments was similar (mean: 0.95 ng/µl, min: 0.20 ng/µl, max: 12.00 ng/µl) across all samples, except for two blood water decay samples that had the highest DNA concentration measured (~ 12.00 ng/µl). The amplification efficiency was comparable for both species through replicated qPCR runs. On average, the herring assay amplified slightly less efficiently than the sprat assay (herring average efficiency 89.5 %,

min=89.1 %, max=91.7 %; sprat average eff. 93.5 %, min=91.0 %, max=100.3 %), which is considered just within borderline acceptable for qPCR assay performance as defined in MIQE guidelines (Applied Biosystems, 2015; Bustin *et al.*, 2009).

2.4.2 Relationship between qPCR and weight input fractions

The estimated herring eDNA fraction showed a strong linear relationship with the weight input fraction across all experimental mixtures for both blood water (Figure 2.2A) and discharge water (Figure 2.2B). In both treatments, herring eDNA fractions were underrepresented in relation to expectations from weight fractions across mixtures. Because of the logit scaling on both variables, a single estimate of the change between eDNA fractions and weight fractions is not feasible. Instead, we visualize the relationship in Figure 2.1 and describe the relationship based on an example. As exemplified in Figure 2.2A, a measured herring eDNA-fraction of 0.40 from blood water would from modelling be predicted to correspond to a 0.54 weight fraction. In comparison, the same eDNA-fraction (0.40) measured from discharge water corresponded to a 0.45 of weight fraction (Figure 2.2B). The eDNA-to-biomass model based on blood water estimated weight fractions with a 0.064 error rate, whereas the eDNA-to-biomass model based on discharge water had an error rate of 0.080.

Examination of the three experimental replicates of the targeted mixture 90/10 indicated low variance in the measured herring-DNA fractions (0.081, 0.103, 0.117, respectively for 90/10a, 90/10b, and 90/10c). The small variation was in agreement with variation in the herring weight fractions: 0.113, 0.123, 0.154 in each replicate (respectively for 90/10a, 90/10b, and 90/10c).

2.4.3 Relationship between qPCR and allometrically scaled weights

The allometric scaling coefficients for blood and discharge water were estimated empirically for each treatment, as the progressing decomposition of the fish in the mixtures will affect the allometric scaling. For blood water, the allometric scaling coefficient *y* of 0.5 provided a very strong positive linear relationship between ASW and eDNA-based fractions (Figure 2.3A and Table 2.5).

Table 2.5. Overview of the fit of the models used to describe the different water type. For each generalized linear model (glm), we present the Akaike Information Criterion (AIC), (BIC) and likelihood (l) of the data. The "effect" refers to the effect that the weight fraction increase has on the DNA-fraction.

Water type	Model	Intercept	Effect (logit scale)	AIC	BIC	l
Blood water	weight	-0.55	0.92	-45.10	-43.91	25.55
	allometrically scaled weight	0.03	0.96	-47.04	-45.84	26.52
Discharge water	weight	-0.22	0.88	-318.20	-311.17	162.10
	allometrically scaled weight	0.00	0.90	-318.55	-311.52	162.28

Likewise, there was a similar positive linear relationship between estimated eDNA-fractions from discharge water and allometrically scaled weight, with a *y* of 0.8 (Figure 2.3B and Table 2.5). Using the allometric scaling model, a herring eDNA-fraction of 0.40 measured from blood water would result in an allometrically scaled weight fraction estimated at 0.39, which translates to a 0.47 weight fraction (Figure 2.3A). When applying allometric scaling coefficients to discharge water estimation, an eDNA fraction of 0.40 would likewise result in an allometrically scaled weight fraction of 0.39, which then translates to a 0.51 weight fraction. The difference in the translation of the allometrically scaled weight to weight fraction is due to different allometric scaling coefficient *y* used for each water type (Figure 2.3B). The models based on allometrically scaled weight increased the precision in weight estimates. Hence, when translating eDNA-to-biomass the error rate in estimates decreased from 0.064 (weight

based model) to 0.054 (allometrically scaled weight model) in blood water, and from 0.080 (weight based model) to 0.075 (allometrically scaled weight model) in discharge water.
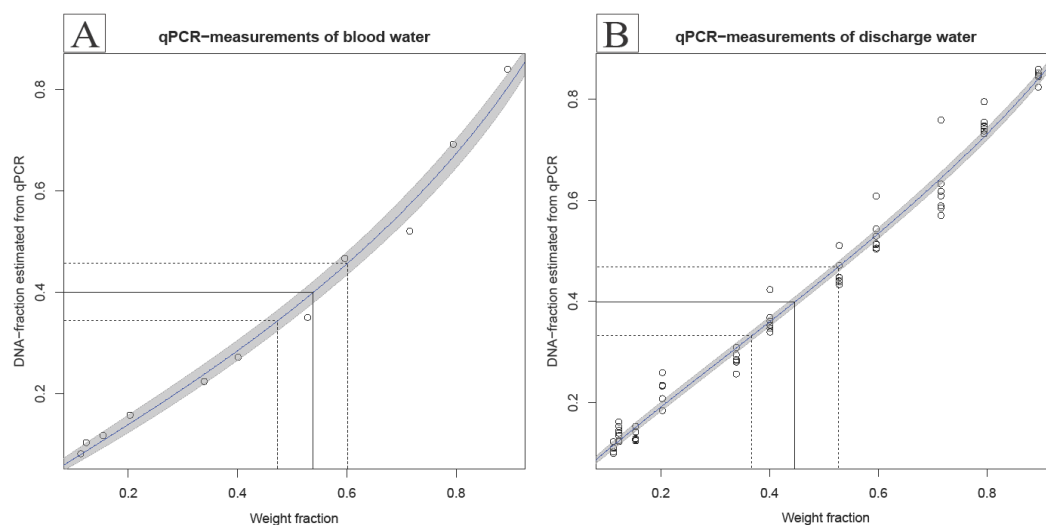
Figure 2.2. Relationship between DNA-based herring fractions and the weight-based fraction, for respectively, A) blood water; and B) discharge water. Blue line shows model prediction with its 95% confidence interval (CI – grey shaded area). The model can subsequently be used to estimate the weight fraction of herring from a measured DNA-fraction of the species. We estimated that a DNA-fraction of 0.4 (estimated with 95% CI) corresponds to 0.54 ± 0.07 weight fraction when using the blood water derived model (A), and to 0.44 ± 0.075 when using the discharge water derived model (B). Black solid lines are weight fraction estimates and the black dotted lines are the corresponding 95% confidence intervals for the estimates.

2.4.4   Effect of time on DNA fractions

Herring fractions in DNA shedding estimated from discharge samples were consistent over time (Figure showing that time of sampling did not affect results. In contrast, blood water decay and discharge water decay (Figure 2.4) samples exhibited some variation in the DNA fractions over time for all mixtures, especially within the first hours (> 10h). In the discharge water decay fractions estimated from the samples between 2-6h were significantly different from fractions estimated from samples collected after 12h (GLM, p < 0.001).

2.4.5   Natural contamination from seawater

We found only few DNA copies of herring (avg. 5.6 copies/µl in seawater blanks) and sprat (avg. 10 copies/µl in seawater blanks) in the natural seawater used for generating the blood water. Given the generally high DNA copy number detected in the experimental samples (between 967 to 15981 mean copies/µl), the average "natural contamination" was estimated to contribute with approx. 0.1 % to the results. In two out of ten DNA-extraction blanks, three copies/µl of sprat DNA were detected, respectively, and in one out of ten DNA-extraction blanks three copies/µl of herring DNA were detected.

The load of bacteria was similar in magnitude across all experimental units, with average BQ number (20 CFU/ml) of 2278668 and ranging between 1626553 and 2990912.

2.5   Discussion

This study showed that species-specific qPCR analysis of eDNA from production water surrounding sprat and herring mixtures is a precise and reproducible method for quantifying mixture fractions. In all experiments, we found strong correlations between weight input fraction and DNA fraction of herring with a relationship close to 1:1. Using a beta generalized linear model, we estimated herring

weight input fractions with very low error that can be further reduced when accounting for allometrically scaled weights in the mixtures. As regular landing duration is approx. 10h, it is shorter than the timespan tested. Hence, we assume that the shift in herring eDNA fraction observed in the discharge water decay experiment after 12h, will not affect biomass estimations in real fisheries samples. The experiments were conducted to mimic the processes related to realistic scenarios under catch, onboard storage, and discharge of pelagic fish to the factory. Our results thus illustrate the large potential for expanding the approach to eDNA based estimation of bycatch fractions in large-scale fisheries samples.

2.5.1 Assay performance

We used a combination of a previously published qPCR assay for herring, and a newly developed assay for sprat. The performance of the herring assay was thoroughly evaluated in Knudsen *et al.* (2019), but for completeness we decided to assess potential cross-amplification for both assays on tissue extracted DNA of the two target species. We found some cross-contamination but the amount of unspecific sprat amplification detected using the herring assay was six orders of magnitude lower than for herring, and will therefore have negligible effects on estimation of bycatch in real fisheries mixtures. Overall, the herring assay efficiency was bordering the lower threshold for being acceptable that is between 90-110 % (Applied Biosystems, 2015; Bustin *et al.*, 2009), but was consistent across all samples analysed. Accordingly, we are confident, that the apparent underrepresentation of herring DNA in the samples neither is the effect of the unspecific binding of the assay, nor of DNA concentration in samples, nor of the reduced efficiency compared to the sprat assay.

We detected low rates of technical contamination despite employing rigorous routines at all steps from the sampling of water and fish to the extraction of DNA and qPCR analysis. Likewise, we generally observed low levels of natural contamination from seawater and potentially also through leakage of stomach content, which may include DNA from target species (Jacobs*en et al*., 2019). However, in the context of estimating fractions of the two species in the catch, the levels of natural contamination are not of concern, due to the very high DNA content of target species in the samples, which swamped the minute signal from natural contamination. The high DNA yield found in the samples probably reflects increased DNA shedding of dead fish compared to live specimens (Tillotson *et al.*, 2018). Similar high DNA content can therefore be expected when working with fisheries samples. This is opposed to more classical eDNA samples in natural environments where target copy numbers can be very low (Goldberg *et al.*, 2016) like in our case 5-10 copies/mL compared > 950 copies/mL estimated in blood and discharge samples. Hence, except under special circumstances, like a very short emersion time of specimens in the water or the collection of seawater from spawning sites, the contribution from natural contamination can thus be considered negligible in similar fisheries samples.

2.5.2 Relationship between qPCR and weight input fractions

Overall, we found a very strong correlation between the eDNA-based estimation of mixture fractions and weight input fractions for sprat and herring. The proportional and predictable contribution of the two species to the mixed pool of DNA in samples is likely due to several factors. First, we thoroughly mixed water and fish for both blood water and discharge water treatments. This was done to mimic conditions onboard the fishing vessels and during landing at the factories, and assured that DNA from all fish in the experimental units was released and mixed at equal rates. Likewise, high biomass-to-water ratio (70% of biomass to 30% of water), and low and stable temperature conditions in the experimental units assured high concentrations of DNA. Finally, the observed near- equivalent DNA contributions of the two species, once allometric relationships were accounted for, suggests very similar DNA shedding (rates and DNA state) and decay rates likely due to their close phylogenetic relationship (Lavoué *et al.*, 2007). Other biological factors than species *per se*, such as the difference in

size between herring and sprat in the experiments are likely the cause for the under-representation of herring DNA in both blood and discharge water, as discussed below (*Relationships between qPCR and allometrically scaled weight*). Temperature is likely the main factor explaining the skewed estimates of species fractions in the two water types as reflected in the difference between the eDNA-to-biomass estimates. Thus, blood water was generated at a low temperature (4°C) and discharge water at a slightly higher temperature (7°C). Temperature can affect species-specific susceptibility to eDNA degradation (DNA-decay) thereby changing their fractions in the solution (Andruszkiewicz Allan *et al.*, 2021; Jo and Minamoto, 2021; Saito and Doi, 2021; Lamb *et al.*, 2022; Mauvisseau *et al.*, 2022). Higher temperatures can also lead to an increase in DNA release (DNA-shedding), thus affecting the availability of DNA in water (Lacoursière-Roussel *et al.*, 2016; Jo *et al.*, 2020). As the primary goal of the experiment was to mimic the fisheries process water, it was inevitable to maintain the two types of production water at different temperatures. However, the observed difference between the water types seems to be constant and thus predictable.

2.5.3   Relationship between qPCR and allometrically scaled weights

We found that using allometrically scaled weight improves the precision in estimating weight fractions. Changes within species physiology, morphology, etc., as a result of changes in the body size of the species are referred to as allometric changes (Gittleman, 2011). In a physical context, allometrically scaled weight refers to changes in the relationship between weight and surface area of the fish. Although no empirical data exist on a general relationship between surface area and DNA release, it is assumed that small fish shed more DNA per unit of weight than large fish due to larger relative surface area (Maruyama *et al.*, 2014; Hansen *et al.*, 2018; Yates *et al.*, 2021). This finds some indirect support as accounting for allometric differences has been shown to improve biomass estimations based on eDNA measurements (Stoeckle *et al.*, 2021; Yates *et al.*, 2021). Herring and sprat are closely related, and have similar biology (i.e. species distribution, feeding habitat, morphology) and could therefore *a priori* be assumed to have relatively similar DNA shedding rates. Still, the two species differ in size at maturity and commonly differ in size in mixed species catches, which in turn leads to differences in surface/weight relationships between the two species. Testing which allometric scaling coefficient provides the most accurate estimate of biomass fractions resulted in different coefficients for the two water types (blood water y = 0.5 and discharge water y = 0.8). This was somewhat surprising, as we estimated the allometric scaling coefficient for the same individuals. We speculate that the observed differences are the result of either different abiotic conditions (e.g. salinity/temperature) varying between the water types that affect the individual's DNA shedding rate, or a result of the intensified decomposition process of the fish leading to bloating (i.e. increased volume) and increased fluid sequestration from the body (Zhou *et al.*, 2021). Decomposition could e.g. lead to increasing the tissue surface that has contact with the water. Estimating allometrically scaled weight using the different coefficients lead to marginally different fractions (as an example, allometrically scaled weight-based fraction of the experimental unit 90/10a blood water, and discharge water was 0.070, and 0.093, respectively). At present, we do not have data that allow us to examine the specific factors causing the changes in the surface area but emphasize that this issue has to be taken into account for practical applications, as a single standard relationship for the two types of water does not appear. It seems most likely that application of an inaccurate coefficient would influence the accuracy of the herring weight fraction estimates. Following, further knowledge on size-based differences in DNA release and persistence of the coefficients for the different water types would be useful for improving quantitative eDNA-based estimates for closely related and morphologically similar species.
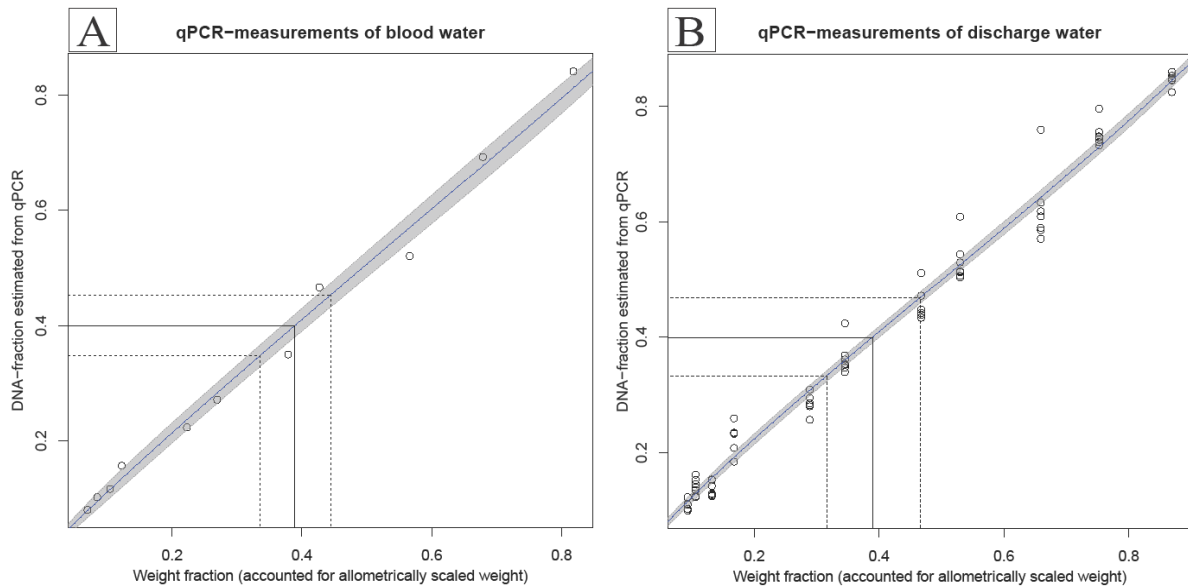
Figure 2.3. Relationship between DNA-based herring fractions derived from the experiment and allometrically scaled weights. "A" represents the relationship based on DNA-fractions derived from blood water samples; "B" is based on DNA-fractions derived from discharge water samples. The blue line shows the model prediction of fractions of allometrically scaled weight for a set of artificially created data of DNA-based fractions ranging from 0.01-0.99 (blue solid line) with a confidence interval (CI) of 95% (*grey shaded area*). The model can subsequently be used to estimate the allometrically scaled weights of herring and the corresponding weight fractions. For blood water (A) we estimate that a herring DNA-fraction of 0.4 (estimated with 95% CI) corresponds to 0.4 ± 0.054 allometrically scaled weight, which translates to approximately 0.47 ± 0.01 weight fraction of herring. For the discharge water derived model (B) the same fraction of herring DNA (0.4) corresponds to 0.39 ± 0.075 allometrically scaled weight, hence 0.51 ± 0.02 weight fraction of herring. Black solid lines estimates of the allometrically scaled weights and the black dotted lines are the corresponding 95% confidence intervals of the estimates.

2.5.4 Effect of time on DNA fraction estimation

Overall, estimated herring DNA fractions remained very stable over time in the blood water decay and discharge water experiments, with only small variations. The relatively small perturbations of the fractions could be associated with sampling variance, e.g. associated with incomplete mixing within samples, as this affects reliability in target species quantification (Rourke *et al.*, 2021). The pattern in discharge water decay, on the contrary, shows a significantly different development of DNA fractions over time. At the start of the discharge decay experiment the DNA abundance dropped for both species, and then increased again after 6h. Although this pattern is the same for both species, the magnitude of the process is different for herring and sprat, causing the observed fractions to vary. This pattern could be a result of unequal DNA degradation between species. To support this, sprat DNA would need to break down faster than herring DNA, which is unknown. Variance generated during sampling and analysis processes, although possible, is unlikely in this case. It is possible that unequal mixing prior to sampling could change the source of DNA (extracellular, intracellular, and tissue particles) sampled at a particular time (Hansen *et al.*, 2020; Mauvisseau *et al.*, 2022). However, it seems unlikely that it would lead to distinct temporal differences in estimated fractions. Such observation would entail that one of the species is more likely to release a different state of DNA, or that the DNA state of one of the species would suddenly change at one time point, which seems unlikely due to the high phylogenetic similarity of the two species. The observed species difference in DNA decay is likely not caused by the qPCR analysis either. Quantitative PCR analyses potentially suffer from plate specific/run specific differences, that need to be accounted for to obtain reliable quantitative analyses (Ruijter *et al.*, 2015). In this study, we analysed all samples collected between 2-12h on the same qPCR plate, which eliminated potential plate-effects causing the observed temporal change.
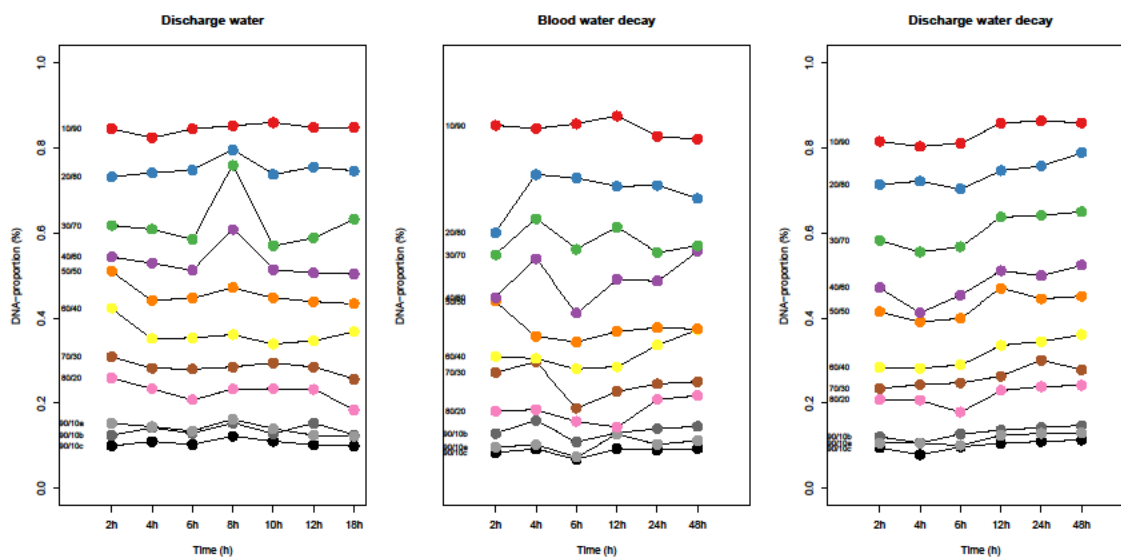
Figure 2.4. Herring DNA fraction over time for three treatments "Discharge water", "Blood water decay", "Discharge water decay". For the "DNA-shedding experiment", i.e. "Discharge water", fish were present in the water at the time point of sampling. During the DNA-decay i.e. "Blood water decay", "Discharge water decay" fish were absent in the water at the time point of sampling. Results from individual experimental units (Table 2.1) are shown in different colors connected with lines. Note that measurements took place at different time intervals depending on treatment.

In order to fully understand the cause of the significant increase of herring DNA fraction with time in the discharge water decay experiment would require a more targeted study. It is important to state that, under realistic settings, the duration of the discharge from ship to factory is, on average, 6h. Therefore the observed temporal differences will likely not have any effect on the precision of estimates of bycatch in true samples collected from fisheries.

2.5.5 Timeline for implementation to the sprat fishery

Our approach holds promise for scaling up to industrial size samples, such as derived directly from the Baltic Sea sprat fishery, and ultimately for being used to provide reliable information on bycatch quantities. The fact that biomass in the fishing tanks remains unchanged during transport and the constant mixing of the water around the fish assures the quality of the product (Refrigerated Sea Water systems onboard), provide a good basis for the method to yield reliable, robust quantitative data that could be much more cost-efficient than the currently used visual inspection of catches. In the light of difficulties this study faced with low efficiency in the qPCR, that could influence accurate estimates of DNA counts in samples, one could consider applying other molecular tools, such as digital droplet PCR (ddPCR) for the same purpose. Unlike qPCR, ddPCR does not rely on the correct amplification of a standard curve for the estimation of the species-specific DNA amounts in samples (Do*i et al*., 2015). The method relies on the concept that single DNA strands can be separated into oil-droplets were species-specific amplification can run. The amount of positively amplified droplets translates directly to the amount of species specific DNA present in samples (Doi *et al.*, 2015). The usefulness of this approach for the most accurate estimation of DNA counts is without doubt, however for the eDNA-based bycatch application to be truly applicable to fisheries other aspects, then superior accuracy, are valuable. The most important aspect it the feasibility to be applicable outside of modern laboratories to allow fast, on site species assessment. The second most important are the costs of the analysis per sample. Considering the speed and lower cost at which quantitative PCR can be per-

formed compared to ddPCR (Doi *et al.*, 2015) even on-site using portable equipment such as Franklin™ (portable qPCR machine from Biomeme) the method could prove to be better suited to the fisheries needs.

Before the eDNA-based method can be implemented in the fishing industry, remaining questions about if and how the allometric scaling coefficient can be estimated for each catch need to be answered. The most likely scenario is that, the eDNA-based method if implemented for the bycatch assessment routines, would be applied in parallel at least for a while to the traditional method based on subsampling and visual identification of the species (Urban et al 2022). This would allow to take additional measurements, like individual weight and length of each species collected in the subsamples, which would serve for the estimation of the allometrically scaled weight of the species caught. In that time, when both methods would run in parallel, one should repeated the experiments for different fishing seasons to verify the universality of these relationships. For our experiment, we used specimens from the same Baltic Sea catch, and thus relationships with other catches from other areas and seasons would need to be studied in order to assess the generality of the derived relationships for the sprat fishing industry. In conclusion, the experiments presented here hold great promise for the DNA-based catch assessment. Yet, further studies need to be performed to assess the robustness and allow upscaling of the results depending on e.g. geographical area, size distribution, and maturity of the catch, as well as abiotic conditions (season).

2.5.6   Considerations for future directions

In this study, we developed a method for assessing the species mixture fractions for a very simple catch composition scenario with just two species. Pelagic fisheries with comparable simplicity such as the North Atlantic boarfish fishery (with bycatch of horse mackerel), and blue whiting fishery (with bycatch of mackerel) (Fiskeristyrelsen, 2021), can benefit from these first insights, so that similar systems can readily be set up and tested. Further, research focusing on more complex species bycatch and mixed catch assemblages should be encouraged in order to fully understand the potential and limitations of the eDNA-based catch assessment approach. Many fisheries cover much more complex species assemblages (e.g. demersal round-fish, flatfish, Norway pout, and prawn fisheries), commonly characterized by multiple bycatch species with distant phylogenetic relations (e.g. crustaceans and fish), with strongly varying morphology and life histories (Storr-Paulsen *et al*., 2012). These variables can affect DNA release and degradation and thus the individual species "eDNA signatures", i.e. the DNA to biomass weight ratio (Hansen *et al*., 2020; Shelton *et al*., 2022). With increasing complexity in fisheries (higher number of species) it is most likely that another molecular based method, DNA-metabarcoding, would need to be used for the estimation of eDNA fractions. This method can introduce more bias in the estimation of eDNA fractions (Hansen et al., 2020), thus its suitability for the eDNA-to-biomass conversion would need to be experimentally tested.

Insights gained from such studies under controlled conditions can also be of value for more classical eDNA studies that focus on providing quantitative estimates of species abundance in natural systems. Unlike our study, the majority of publications on eDNA, if not all, investigate the DNA-biomass relationship using raw DNA abundance (i.e. DNA-copies, sequence reads, etc.) (Thomsen *et al*., 2016; Knudsen *et al*., 2019; Salter *et al*., 2019; Stoeckle *et al*., 2021; Yates *et al*., 2021). However, comparability of these data derived directly from DNA- metabarcoding or qPCR analysis might be misleading, in particular if interspecific comparisons are in focus (Shelton *et al*., 2022; Urban *et al*., 2022). Our study highlights the usefulness of working with fractions/proportional outputs for obtaining reliable eDNA and thus biomass estimates.

2.6 References

Andruszkiewicz Allan, E., Gordon Zhang, W., Lavery, A. C., and Govindarajan, A. F. 2021. Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. Environmental DNA: 492–514.

Beng, K. C., and Corlett, R. T. 2020. Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. Biodiversity and Conservation, 29: 2089–2121. Springer. https://link.springer.com/article/10.1007/s10531-020-01980-0 (Accessed 5 August 2022).

Biosystems, A. 2015. Guidelines for comparing SYBR Green master mixes. https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fbrochures%2Fguidelines-comparing-sybr-green-master-mixes.pdf&title=R3VpZGVsaW5lcyBmb3IgY29tcGFyaW5nIFNZQlIgR3JlZW4gbWFzdGVyI.

Boussarie, G., Bakker, J., Wangensteen, O. S., Mariani, S., Bonnin, L., Juhel, J.-B., Kiszka, J. J., *et al.* 2018. Environmental DNA illuminates the dark diversity of sharks. Science advances, 4: eaap9661.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., *et al.* 2009. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry, 55: 611–622. Oxford Academic. https://academic.oup.com/clinchem/article/55/4/611/5631762 (Accessed 18 August 2022).

Clark, K., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., and Sayers, E. W. 2016. GenBank. Nucleic Acids Research, 44: 67–72. https://academic.oup.com/nar/article/44/D1/D67/2503088.

Collins, R. A., Wangensteen, O. S., O'Gorman, E. J., Mariani, S., Sims, D. W., and Genner, M. J. 2018. Persistence of environmental DNA in marine systems. Communications Biology, 1: 185.

Deutschmann, B., Müller, A. K., Hollert, H., and Brinkmann, M. 2019. Assessing the fate of brown trout (Salmo trutta) environmental DNA in a natural stream using a sensitive and specific dual-labelled probe. Science of The Total Environment, 655: 321–327. Elsevier.

Doi, H., Uchii, K., Takahara, T., Matsuhashi, S., Yamanaka, H., and Minamoto, T. 2015. Use of droplet digital PCR for estimation of fish abundance and biomass in environmental DNA surveys. PLoS One, 10: e0122763.

European Commission. 2020. Council Regulation (EU) 2020/1579. Official Journal of the European Union, 362: 12 pp.

European Union. 2009. Council Regulation (EC) No 1224/2009 of 20 November 2009 establishing a Community control system for ensuring compliance with the rules of the common fisheries policy, amending Regulations (EC) No 847/96, (EC) No 2371/2002, (EC) No 811/2004, (EC) No 768/2. 50 pp.

Fiskeristyrelsen. 2021. Fiskeristyrelsens prøvetagningsplan for vejning af fiskevarer, der landes usorteret til industriformål (Case nr.:19-4000-000459). 1–5 pp.

Gittleman, J. 2011. Allometry.

Goldberg, C. S., Turner, C. R., Deiner, K., Klymus, K. E., Thomsen, P. F., Murphy, M. A., Spear, S. F., *et al.* 2016. Critical considerations for the application of environmental DNA methods to detect aquatic species. Methods in Ecology and Evolution, 7: 1299–1307.

Hansen, B. K., Bekkevold, D., Clausen, L. W., and Nielsen, E. E. 2018. The sceptical optimist: challenges and perspectives for the application of environmental DNA in marine fisheries. Fish and fisheries, 19: 751–768.

Hansen, B. K., Farrant, G. K., Ogden, R., Humble, E., Olafsdottir, G. bjorg, Bekkevold, D., Knudsen, S. W., *et al.* 2020. From DNA to biomass: opportunities and challenges in species quantification of bulk fisheries products. ICES Journal of Marine Science, 77: 2557–2566. Oxford Academic. https://academic.oup.com/icesjms/article/77/7-8/2557/5894067 (Accessed 17 November 2021).

Hongo, Y., Nishijima, S., Kanamori, Y., Sawayama, S., Yokouchi, K., Kanda, N., Oori, S., *et al.* 2021. Fish environmental DNA in Tokyo Bay: A feasibility study on the availability of environmental DNA for fisheries. Regional Studies in Marine Science, 47: 101950. Elsevier.

ICES. 2020. Baltic Sea Ecoregion–Fisheries overview. Report of the ICES Advisory Committee: Section 4.1. https://doi.org/10.17895/ices.advice.7635.

Jacobsen, M. W., Hansen, B. K., and Nielsen, E. E. 2018. Possible uses of genetic methods in fisheries under the EU landing obligation. The European Landing Obligation: Reducing Discards in Complex, Multi-Species and Multi-Jurisdictional Fisheries: 407–427. Springer International Publishing. https://link.springer.com/chapter/10.1007/978-3-030-03308-8_20 (Accessed 24 August 2022).

Jacobsen, M. W., Hansen, B. K., and Nielsen, E. E. 2019. Possible uses of genetic methods in fisheries under the EU Landing Obligation. *In* The European Landing Obligation, pp. 407–427.

Jo, T., Arimoto, M., Murakami, H., Masuda, R., Minamoto, T., and Toshiaki Jo, C. 2020. Estimating shedding and decay rates of environmental nuclear DNA with relation to water temperature and biomass. Environmental DNA, 2: 140–151.

Jo, T., and Minamoto, T. 2021. Complex interactions between environmental DNA (eDNA) state and water chemistries on eDNA persistence suggested by meta- analyses. Molecular Ecology Resources: 1490–1503.

Kelly, R. P., Shelton, A. O., and Gallego, R. 2019. Understanding PCR Processes to Draw Meaningful Conclusions from Environmental DNA Studies. Scientific Reports, 9: 1–14.

Knudsen, S. W., Ebert, R. B., Hesselsøe, M., Kuntke, F., Hassingboe, J., Mortensen, P. B., Thomsen, P. F., *et al.* 2019. Species-specific detection and quantification of environmental DNA from marine fishes in the Baltic Sea. Journal of Experimental Marine Biology and Ecology, 510: 31–45.

Knudsen, S. W., Hesselsøe, M., Thaulow, J., Agersnap, S., Hansen, B. K., Jacobsen, M. W., Bekkevold, D., *et al.* 2022. Monitoring of environmental DNA from nonindigenous species of algae, dinoflagellates and animals in the North East Atlantic. Science of the Total Environment, 821. Elsevier B.V.

Lacoursière-Roussel, A., Rosabal, M., and Bernatchez, L. 2016. Estimating fish abundance and biomass from eDNA concentrations: variability among capture methods and environmental conditions. Molecular Ecology Resources, 16: 1401–1414. John Wiley & Sons, Ltd. https://onlinelibrary.wiley.com/doi/full/10.1111/1755-0998.12522 (Accessed 9 December 2021).

Lamb, P. D., Fonseca, V. G., Maxwell, D. L., and Nnanatu, C. C. 2022. Systematic review and meta-analysis: Water type and temperature affect environmental DNA decay. Molecular Ecology Resources. John Wiley & Sons, Ltd. https://onlinelibrary.wiley.com/doi/full/10.1111/1755-0998.13627 (Accessed 24 August 2022).

Lavoué, S., Masaki, M., Kenji, S., Naoya, B. I., and Mutsumi, N. 2007. Phylogenetic relationships among anchovies, sardines, herrings and their relatives (Clupeiformes), inferred from whole mitogenome sequences. Molecular phylogenetics and evolution, 43: 1096–1105.

Maruyama, A., Nakamura, K., Yamanaka, H., Kondoh, M., and Minamoto, T. 2014. The Release Rate of Environmental DNA from Juvenile and Adult Fish. PLoS One, 9. http://www.jst.go.jp/ (Accessed 28 February 2022).

Mauvisseau, Q., Parrondo, M., Fernández, M. P., García, L., Martínez, J. L., García-Vázquez, E., and Borrell, Y. J. 2017. On the way for detecting and quantifying elusive species in the sea: The Octopus vulgaris case study. Fisheries Research, 191: 41–48. Elsevier B.V.

Mauvisseau, Q., Harper, L. R., Sander, M., Hanner, R. H., Kleyer, H., and Deiner, K. 2022. The Multiple States of Environmental DNA and What Is Known about Their Persistence in Aquatic Environments. Environmental Science and Technology, 56: 5322–5333. American Chemical Society. https://pubs.acs.org/doi/full/10.1021/acs.est.1c07638 (Accessed 2 August 2022).

Merkes, C., Klymus, K., Allison, M., Goldberg, C., Helbin, C., Hunter, M., Jackson, C., *et al.* 2019. Generic qPCR Limit of Detection (LOD) / Limit of Quantification (LOQ) calculator. Data: U.S. Geological Survey data release.

Nielsen, J. M., Clare, E. L., Hayden, B., Brett, M. T., and Kratina, P. 2018. Diet tracing in ecology: Method comparison and selection. Methods in Ecology and Evolution, 9: 278–291. John Wiley & Sons, Ltd. https://onlinelibrary.wiley.com/doi/full/10.1111/2041-210X.12869 (Accessed 17 January 2023).

Nøttestad, L., Utne, K. R., Óskarsson, G. J., Jónsson, S. P., Jacobsen, J. A., Tangen, Ø., Anthonypillai, V., *et al.* 2016. Quantifying changes in abundance, biomass, and spatial distribution of Northeast Atlantic mackerel (Scomber scombrus) in the Nordic seas from 2007 to 2014. ICES Journal of Marine Science, 73: 359–373. Oxford Academic. https://academic.oup.com/icesjms/article/73/2/359/2614456 (Accessed 14 June 2022).

O'Shea, B., Mordue-Luntz, A. J., Fryer, R. J., Pert, C. C., and Bricknell, I. R. 2006. Determination of the surface area of a fish. Journal of Fish Diseases, 29: 437–440.

Perez, C. R., Bonar, S. A., Amberg, J. J., Ladell, B., Rees, C., Stewart, W. T., Gill, C. J., *et al.* 2017. Comparison of American Fisheries Society (AFS) Standard Fish Sampling Techniques and Environmental DNA for Characterizing Fish Communities in a Large Reservoir. North American Journal of Fisheries Management, 37: 1010–1027. https://doi.org/10.1080/02755947.2017.1342721 (Accessed 21 November 2022).

Rees, H. C., Maddison, B. C., Middleditch, D. J., Patmore, J. R. M., Gough, K. C., and Crispo, E. 2014. REVIEW: The detection of aquatic animal species using environmental DNA - a review of eDNA as a survey tool in ecology. Journal of Applied Ecology, 51: 1450–1459.

Reeslev, M., Nielsen, J., and Rogers, L. 2011. Assessment of the bacterial contamination and remediation efficacy after flooding using fluorometric detection. Surface and Dermal Sampling, ASTM Inter.

Rodríguez-Ezpeleta, N., Morissette, O., Bean, C. W., Manu, S., Banerjee, P., Lacoursiere-Roussel, A., Beng, K. C., *et al.* 2021. Trade-offs between reducing complex terminology and producing accurate interpretations from environmental DNA: Comment on 'Environmental DNA: What's behind the term?' by Pawlowski et al THE TOTAL P OOL OF DNA ISOL ATED FROM THE ENVIRONMENT.

Rourke, M. L., Fowler, A. M., Hughes, J. M., Broadhurst, M. K., DiBattista, J. D., Fielder, S., Wilkes Walburn, J., *et al.* 2021. Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. Environmental DNA. Wiley.

Ruijter, J. M., Ruiz Villalba, A., Hellemans, J., Untergasser, A., and van den Hoff, M. J. B. 2015. Removal of between-run variation in a multi-plate qPCR experiment. Biomolecular Detection and Quantification, 5: 10–14. Elsevier.

Ruppert, K. M., Kline, R. J., and Rahman, M. S. 2019. Past, present, and future perspectives of environmental DNA (eDNA) metabarcoding: A systematic review in methods, monitoring, and applications of global eDNA. Global Ecology and Conservation: e00547.

Russo, T., Maiello, G., Talarico, L., Baillie, C., Colosimo, G., D'Andrea, L., Di Maio, F., *et al.* 2021. All is fish that comes to the net: metabarcoding for rapid fisheries catch assessment. Ecological Applications, 31: e02273. John Wiley & Sons, Ltd. https://onlinelibrary.wiley.com/doi/full/10.1002/eap.2273 (Accessed 17 November 2021).

Saito, T., and Doi, H. 2021. A Model and Simulation of the Influence of Temperature and Amplicon Length on Environmental DNA Degradation Rates: A Meta-Analysis Approach. Frontiers in Ecology and Evolution, 9: 139. Frontiers Media S.A.

Salter, I. 2018. Seasonal variability in the persistence of dissolved environmental DNA (eDNA) in a marine system: The role of microbial nutrient limitation. PLOS ONE, 13: e0192409. Public Library of Science. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0192409 (Accessed 15 August 2022).

Salter, I., Joensen, M., Kristiansen, R., Steingrund, P., and Vestergaard, P. 2019. Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. Communications Biology 2019 2:1, 2: 1–9. Nature Publishing Group. https://www.nature.com/articles/s42003-019-0696-8 (Accessed 17 November 2021).

Sassoubre, L. M., Yamahara, K. M., Gardner, L. D., Block, B. A., and Boehm, A. B. 2016. Quantification of Environmental DNA (eDNA) Shedding and Decay Rates for Three Marine Fish. https://pubs.acs.org/sharingguidelines (Accessed 28 February 2022).

Segelbacher, G., Cushman, S. A., Epperson, B. K., Fortin, M. J., Francois, O., Hardy, O. J., Holderegger, R., *et al.* 2010. Applications of landscape genetics in conservation biology: Concepts and challenges. Conservation Genetics, 11: 375–385. Springer. https://link.springer.com/article/10.1007/s10592-009-0044-5 (Accessed 17 January 2023).

Shelton, A. O., Ramón-Laca, A., Wells, A., Clemons, J., Chu, D., Feist, B. E., Kelly, R. P., *et al.* 2022a. Environmental DNA provides quantitative estimates of Pacific hake abundance and distribution in the open ocean.

Shelton, A. O., Gold, Z. J., Jensen, A. J., D'Agnese, E., Andruszkiewicz Allan, E., Van Cise, A., Gallego, R., *et al.* 2022b. Toward quantitative metabarcoding. Ecology. https://onlinelibrary.wiley.com/doi/10.1002/ecy.3906 (Accessed 21 November 2022).

STECF. 2013. Landing obligation in EU fisheries (STECF-13-23). 115 pp. pp.

Stoeckle, M. Y., Adolf, J., Charlop-Powers, Z., Dunton, K. J., Hinks, G., and Vanmorter, S. M. 2021. Trawl and eDNA assessment of marine fish diversity, seasonality, and relative abundance in coastal New Jersey, USA. ICES Journal of Marine Science, 78: 293–304. Oxford Academic. https://academic.oup.com/icesjms/article/78/1/293/6017541 (Accessed 17 November 2021).

Storr-Paulsen, M., Håkansson, K. ., Egekvist, J., Degel, H., and Dalskov, J. 2012. Danish Sampling of Commercial Fishery. Overview with special attention to discards. 2010 data. 87 pp.

Taberlet, P., Coissac, E., Hajibabaei, M., and Rieseberg, L. H. 2012. Environmantal DNA. Molecular Ecology, 21: 1789–1793.

Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P. R., and Rasmussen, M. 2012. Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. PLoS ONE, 7: 41732. www.plosone.org (Accessed 19 July 2021).

Thomsen, P. F., Moller, P. R., Sigsgaard, E. E., Knudsen, S. W., Jorgensen, O. A., and Willerslev, E. 2016. Environmental DNA from Seawater Samples Correlate with Trawl Catches of Subarctic, Deepwater Fishes. PLoS One, 11: e0165252. https://www.ncbi.nlm.nih.gov/pubmed/27851757.

Tillotson, M. D., Kelly, R. P., Duda, J. J., Hoy, M., Kralj, J., and Quinn, T. P. 2018. Concentrations of environmental DNA (eDNA) reflect spawning salmon abundance at fine spatial and temporal scales. Biological Conservation, 220: 1–11.

Uhlmann, S. S., Ulrich, C., and Kennelly, S. J. 2019. The European Landing Obligation. The European Landing Obligation: Reducing Discards in Complex, Multi-Species and Multi-Jurisdictional Fisheries: 431. Springer Nature.

Urban, P., Bekkevold, D., Hansen, B. K., Jacobsen, M. W., Nielsen, A., and Nielsen, E. E. 2022. Using eDNA to estimate biomass of bycatch in pelagic fisheries. Environmental DNA.

Wickham, H., Averick, M., Bryan, J., Chang, W., D', L., Mcgowan, A., François, R., *et al.* 2019. Welcome to the Tidyverse. Journal of Open Source Software, 4: 1686. The Open Journal. https://joss.theoj.org/papers/10.21105/joss.01686 (Accessed 29 November 2021).

Yates, M. C., Glaser, D. M., Post, J. R., Cristescu, M. E., Fraser, D. J., and Derry, A. M. 2021. The relationship between eDNA particle concentration and organism abundance in nature is strengthened by allometric scaling. Molecular Ecology, 30: 3068–3082. John Wiley & Sons, Ltd. https://onlinelibrary.wiley.com/doi/full/10.1111/mec.15543 (Accessed 13 December 2021).

Zhao, B., Bodebom, P. M. van, and Trimbos, K. 2021. The particle size distribution of environmental DNA varies with species and degradation. Science of The Total Environment, 797: 149–175.

Zhou, R., Wang, Y., Hilal, M. G., Yu, Q., Feng, T., and Li, H. 2021. Temporal succession of water microbiomes and resistomes during carcass decomposition in a fish model. Journal of Hazardous Materials, 403: 123795. Elsevier.

3. A study of the potential use of DNA from catch water to assess biodiversity of fishing catches in the consumption fisheries

Authors

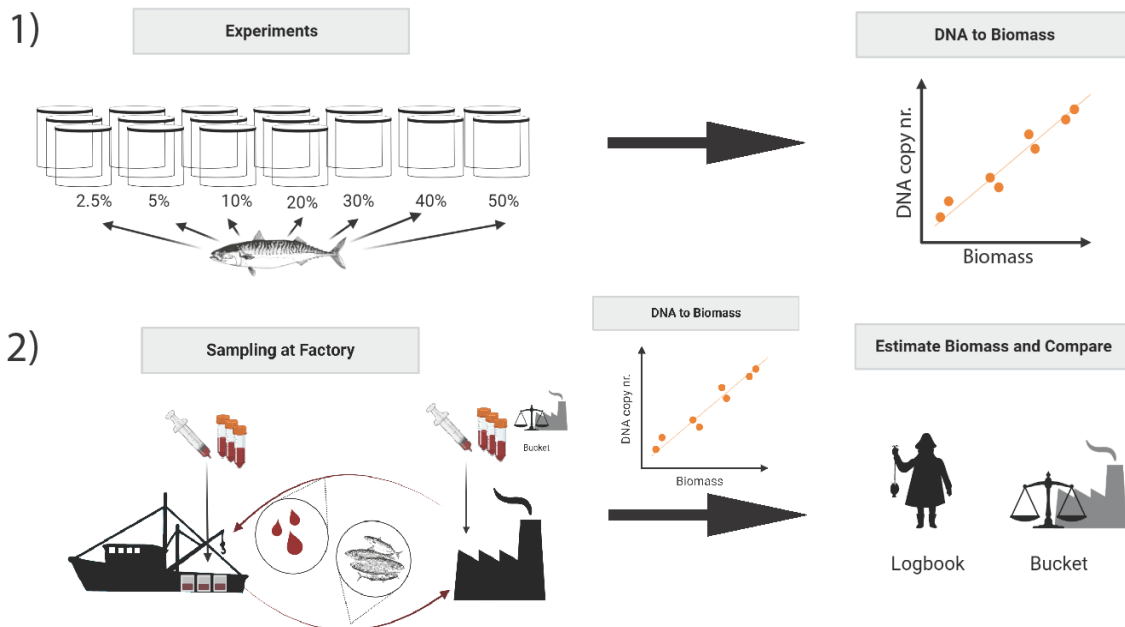Paulina Urban[1], Dorte Bekkevold[1], Brian Klitgaard Hansen[1], Magnus Wulff Jacobsen[1], Anders Nielsen[1] & Einar Eg Nielsen

Affiliations

[1]Section for Marine Living Resources, DTU Aqua, Technical University of Denmark, Vejlsøvej 39, 8600 Silkeborg, Denmark

3.1 Abstract

In recent years, the analysis of environmental DNA (eDNA) has significantly improved, allowing for high-resolution species identification and possible biomass quantification from water samples. Fisheries management typically requires monitoring of catches, including precise information about bycatch quantities to make sound assessments of exploitation rates. Bycatch assessment is particularly challenging in large catches (>500 T), and the current practice of visual assessment of sub-sampled catches is time-consuming, requires extensive labour, and often has low precision. We explored the feasibility for applying eDNA-based methods for studying catch composition using the pelagic North Sea herring fishery with bycatch of mackerel as a case. First, we experimentally simulate a series of catches using a range of herring and mackerel weight proportions to establish relationships under real fisheries scenarios. The relationship is subsequently used to estimate the biomass of mackerel bycatch from eDNA from three herring catches, by sampling and comparing processing water both onboard ships and at the processing factory. All samples are analyzed using species-specific quantitative PCR (qPCR). The experiments reveled a strong correlation between DNA and weight fractions characterized by a constant overrepresentation of mackerel DNA compared to expected mackerel weight. We found that eDNA-based and visual methods applied to the same landing reflect the within catch variability in species composition alike, however the methods can show disparity in total estimates of mackerel biomass. Accounting for haul mixing within total landed catches increases the precision of the factory and ship eDNA-based estimates for the same catch. We show that eDNA-based bycatch estimates provide coherent quantitative data, and likely improve quality and reduce costs of collecting fisheries-dependent data and thereby contribute to securing sustainable fisheries.

3.2 Introduction

Over the past decade, environmental DNA (eDNA) has evolved into an effective, non-invasive tool for species monitoring in natural environments (Hongo et al., 2021; Salter, Joensen, Kristiansen, Steingrund, & Vestergaard, 2019; Taberlet, Coissac, Hajibabaei, & Rieseberg, 2012; Thomsen et al., 2016; Yates et al., 2021). The basis for the eDNA concept is that all organisms constantly release DNA into their surroundings via the skin, mucus, defecation, and other processes (Rodríguez-Ezpeleta et al., 2021; Taberlet et al., 2012). The DNA left in the surrounding environment can be retrieved and analyzed to deduce the species' identity. Environmental DNA has been studied in different environments, including water (Ficetola, Miaud, Pompanon, & Taberlet, 2008; Knudsen et al., 2022; Stoeckle et al., 2021; Thomsen, Kielgast, Iversen, Møller, & Rasmussen, 2012; Thomsen et al., 2016), air (Clare et al., 2022; Roger et al., 2022), and soil (Buxton, Groombridge, & Griffiths, 2018; Ryan, Bateman, Fernandes, van der Heyde, & Nevill, 2022). Typically, it is used to assess the presence of either a single target species (Ficetola et al., 2008; Knudsen et al., 2022; Yates et al., 2021) or the composition of species from larger taxonomic groups i.e. biodiversity (Bakker et al., 2019; Boussarie et al., 2018; Hongo et al., 2021; Roger et al., 2022; Russo et al., 2021). Because eDNA allows for easier, cheaper, and faster species monitoring compared to the labour-intensive traditional visual methods (Fediajevaite, Priestley, Arnold, & Savolainen, 2021; Goldberg, Sepulveda, Ray, Baumgardt, & Waits, 2013; Lugg, Griffiths, van Rooyen, Weeks, & Tingley, 2018; Thomsen et al., 2012), there is an ongoing effort to explore the potential of eDNA studies beyond species detection towards estimation of biomass and abundance (Russo et al., 2021; Stoeckle et al., 2021; Thomsen et al., 2012; Yates et al., 2021).

The fisheries management sector has a long tradition of high need for extensive monitoring, with high associated costs, in relation to assessment of fisheries resources and food safety (Arnason, Hannesson, & Schrank, 2000; Link et al., 2002; Richards et al., 2022; Wallis & Flaaten, 2003). With the development of DNA-based monitoring tools, the sector is looking towards the potential for practi-

cal application of eDNA for some monitoring purposes such as in stock assessment, product traceability, and quality assurance (Cusa et al., 2022; Hansen et al., 2020a; Helyar et al., 2014; Jacobsen et al., 2019; Roungchun et al., 2022; Salter et al., 2019; Stoeckle et al., 2021). However, for these applications there is so far an insufficient understanding of the quantitative aspects of the DNA signal measured. In natural open environments such as the sea, the reliability of quantitative applications of eDNA is tightly coupled to an understanding of the origin, production rates, degradations rates, and transport of DNA which are to some degree specific to the environment the DNA particle has been sampled from (Hansen et al. 2018). Therefore, establishing very tight relationships between fish abundance/biomass and DNA copy number in ambient water samples is generally considered difficult (Beng & Corlett, 2020; Hansen et al., 2018; Knudsen et al., 2019). Instead, a potentially more tangible application of eDNA in fisheries is for reconstructing catch composition (Russo et al., 2021) including bycatch estimation. Unlike in natural environments, where species biomass and water movements (i.e. flow intensity and direction) can change fast, thereby affecting the retrieved eDNA-signal and making interpretations difficult, the hold of a fisheries vessel represents a confined environment. The composition and biomass of fish in the holdings tanks are unchanged from when the fishing operation ends until the catch is landed to a factory. Onboard modern pelagic trawlers, seawater is used for cooling the catch. For this, the seawater is stored in tanks with the fish and mixed constantly leading to a well-mixed solution (del Valle & Aguilera, 1991), that potentially can provide an integrated signal of the catch composition (Fig. 3.1). Factors associated with fish physiology (i.e. metabolism) and fish movement can also be neglected, as all fish are dead once stored onboard the ship. This controlled setting allows for studying the relationship between eDNA and species abundance/biomass that is far less complex than under ambient conditions at sea.

Bycatch is defined as the accidental intake of non-target species during fisheries operations. Bycatch is often of low economic value and limited interest for the fisherman, leading not seldom to discarding or slipping and thus poor overall catch records (Tenningen, Zimmermann, & Enberg, 2021). In pelagic fisheries even small bycatch rates (~1%) can result in high biomass of non-target species caught due to the generally large size of the catches (>500 t) (Nøttestad et al., 2016). Thus, the information about the quantity of the unwanted part can still be of high value for fisheries management as it serves for predicting catchable amounts. Poorly done bycatch assessment can lead to issues related to spawning stock biomass and associated recruitment and thus lead to instability of the harvested populations and in turn affect the future economic profit (Dickey-Collas et al., 2007; Rudd & Branch, 2016). A poor assessment also potentially prevents appropriate conservation actions in the understanding of the real impact of fisheries on the ecosystem (Gray & Kennelly, 2018) and verifications of eco-labeling (Clegg, Steven, Geir, & Kjell, 2021). Thus, to ensure the long-term economical profitability of fisheries operations without jeopardizing marine biodiversity all catch components need to be reliably monitored (Booth, Arlidge, Squires, & Milner-Gulland, 2021).

The Atlantic herring (*Clupea harengus*) fishery has historically been one of the most economically important fisheries in the North Sea region. Accordingly, the herring fishery is one of the most data-rich and after its collapse in 1970s, a species with a particularly high management focus in the North Sea (Tenningen et al., 2021). Bycatch species in the herring fishery can be diverse, but with the most likely bycatch of Atlantic mackerel (*Scomber scombrus*), whiting (*Merlangius merlangus*), haddock (*Melanogrammus aeglefinus*), and horse mackerel (*Trachurus trachurus*) (ICES, 2017). Out of these, mackerel is a quota-regulated and economically very profitable species (Trenkel et al., 2014). As such, mackerel biomass caught and reported as bycatch is directly subtracted from the species' main quota. Accordingly, there is a need for methods, which can effectively monitor mackerel bycatch, even when at low abundance (> 50 kg) in large pelagic catches of herring.

We explored the opportunity to apply eDNA-based methods for studying catch composition and thereby derive quantitative bycatch information for mackerel with high precision and at a low cost. In Denmark, bycatch quantities in the herring fishery are estimated for all landings using a so-called "bucket method". In brief, the method consists of taking a 20-30 kg subsample of the catch for every 25 tons as the catch is flushed from the ship to the processing factory via chutes. For each bucket sample, all species are identified and weighed. Subsequently, the species-specific weight composition in the buckets is extrapolated to the total catch and the overall bycatch rate is determined (Fiskeristyrelsen, 2021). The method and its results can be a source of conflict among fishers, fisheries agencies, and industry officials, because of the methods' large and inevitable variation and thus uncertainty (approx. 10 %).

The catch onboard a fishing vessel can be seen as a stand-alone entity from which species composition could be determined through eDNA sampling and analysis. We use the pelagic North Sea herring fishery with bycatch of mackerel as a case study to test the application of DNA analysis from blood water onboard fisheries vessels and at the processing factory for its suitability for reliable eDNA-based bycatch quantification. This is the first study to determine relationships between eDNA copy number fraction and weight fraction for different artificial herring/mackerel mock samples where fractions of mackerel varied. Results were used to establish a model to estimate mackerel biomass from eDNA estimates. Secondly, we applied inference from the model to estimate the weight of mackerel in three landings from the herring fishery. For these landings, eDNA was collected and analyzed from blood water both on board fishing vessels (hereafter eDNA at ship) and in factories (hereafter eDNA at factory). These eDNA based weight estimates were compared with the estimates derived from the logb ook (hereafter visual (log book)) and the bucket method (hereafter visual (bucket)). We also investigated if and how eDNA-based estimates of catch composition are affected by the distribution of fish in the individual hauls in different holding tanks onboard the ship, and the mixing of the process water, during the unloading process. Finally, we evaluated the potential of the method for routine implementation to document bycatch and for control and enforcement.

3.3 Material and methods

3.3.1 Experiment: weight to eDNA relationship

The relationship between species biomass and DNA fractions was studied in experimental mixtures of herring and mackerel. Two types of experiments were conducted, DNA-shedding (i.e. DNA production) and DNA-decay (i.e. DNA degradation). Two "DNA-shedding" experiments were performed for this purpose, 1) a laboratory-based DNA-shedding experiment to test the DNA release of species under controlled temperature conditions and 2) a ship-based DNA-shedding experiment that simulated real fisheries conditions. In the laboratory-based DNA-shedding experiment each of 2x7 experimental units was constructed with 5kg mixed, freshly caught herring-mackerel, and 2.5L of seawater. Mixtures were prepared based on weight exploring from low (2.5%) to substantial (50%) bycatch contribution (Table 3.1). The ship based experiment consisted of four experimental units, each consisting of 5kg mixed fresh herring and thawed mackerel, and 2.5L of seawater, with mixtures ranging from 2.5% to 20% of mackerel bycatch (Table 3.1). For both experiments, only whole specimens were used and thus the actual gram-to-gram proportion in each mixture deviated slightly from the target (Supporting Information 4.9). All experimental units were prepared in separate containers double wrapped with plastic bags (Cater Line, Freezer bags, 40L). Before starting the experiments, fish were briefly washed with seawater. The DNA shedding experiments started with placing the predefined herring-mackerel mix (Table 3.1) and seawater into the container and closing the plastic bags to prevent contamination. The experimental units were then subject to either 1) temperature-controlled laboratory settings simulating real fishery conditions (average temp. -1.25 °C ± 1.43, more details see Supporting Information 4.7 and 4.8), or 2) real fisheries conditions onboard a ship. During both DNA shedding

experiments, up to 6 ml of blood water was collected at regular intervals (Table 3.1) using a sterile 20 ml syringe (Inject® Solo, B. Braun), into a sterile collection tube (Sarstedt, Screw cap tube, 10 ml). All samples were frozen at -20°C immediately after sampling. In the lab, after the DNA-shedding experiment ended, the "DNA-decay experiment" was initiated to study the potential effect of decay of DNA, after removal of the fish from the water, on the estimated fraction of herring and mackerel. The experiment started by separating the fish from the blood water and subsequently maintaining the blood water at same experimental conditions for additional 48h. Also during this experiment, up to 6ml of the decaying blood water was sampled at regular intervals (Table 3.1) using a sterile 20 ml syringe (Inject® Solo, B. Braun), into a sterile tube (Sarstedt, Screw cap tube, 10 ml). All samples were frozen at -20°C immediately after sampling. At all times, all experimental units were thoroughly mixed prior to sampling.

Table 3.2. Overview of the experiments performed to study the eDNA to weight relationship of herring and mackerel mock samples. In total, three experiments were performed from which two under controlled conditions in the lab and one under "real" conditions onboard a ship, as indicated in "Experiment" column. In the lab-based experiments, for each anticipated proportion we prepared two mock units (replicates), indicated in the "Replicates" column. The end-proportions in the experimental units can deviate from the anticipated because only whole specimens were used. Each experiment was sampled at different time intervals, in total ranging from 2-120 hours. The specimen used in the experiments were commercially caught at different places, as indicated in "catch region". The region of the catch was identified following the ecoregion description from ICES ("2A" is in the Norwegian Sea/North Atlantic, "3A" is the Skagerrak-Kattegat area and "4B" is the southern North Sea).

Experiment	Replicates	Proportions anticipated in mock units (herring/mackerel)	Sampling time points	Catch region			Comments
				herring	mackerel	water	
Shedding experiment in the lab	2	97.5/2.5 95/5 90/10 80/20 70/30 60/40 50/50	24h 48h 72h 96h 120h	2A	3A	artificial seawater (32 PSU)	herring 5 days older than mackerel (kept chilled until setup)
Decay experiment in the lab	2	97.5/2.5 95/5 90/10 80/20 70/30 60/40 50/50	2h 4h 6h 12h 24h 24h 48h	2A	3A	artificial seawater (32 PSU)	
Shedding experiment onboard	1	97.5/2.5 95/5 90/10 80/20	24h 48h	4B	3A	4B	thawed mackerel used for experiment

3.3.2 Application to fisheries samples: estimating bycatch weight

General description of the fishery process

We define three phases during herring fisheries: the before fishing, the fishing, and the landing phase (Fig. 3.1). In the first phase, the holding tanks (separate compartments of a ship designed to keep water and fish) onboard the ship are filled with seawater up to 1/3 of their volume. The uptake of seawater happens on the voyage to the fishing grounds. During fishing, each casting and pulling in of the fishing net is called a "haul". Depending on the size of the haul, its content is transferred to one or more tanks. One to several hauls are performed to fill the capacity of the ship. Once all tanks are

filled, the total amount of fish stored is referred to as "total catch" or "catch". In the logbook, the fishermen records the species composition as species (kg)/ haul. The composition is usually determined by the fishermen using the bucket method. Here, each haul is periodically subsampled and species composition is assessed through visual identification and weighing of each species separately. The estimated proportions are used to extrapolate the species composition to the total haul and catch, and reported to the fisheries authorities in a log book. The frequency of this subsampling is not recorded.

While at sea, the seawater inside the tanks keeps the catch fresh until landed. With time, fish remains (i.e. blood, urine, skin cells, gametes, and scales) accumulate and change the appearance of the water, which is subsequently called "blood water". When the ship reaches the processing factory, commonly 72 hours, the landing phase begins. In this phase, catch and blood water are transported via chutes from the ship to the factory (discharged). During transport, the blood water is constantly reused by pumping to lift the next portion of the catch into the factory. During the discharge, the bucket method is applied at the factory to determine the species composition. Here, the method consists of subsampling 20-30 kg of the catch at regular intervals (every 25 tons). The species composition in the subsamples is assessed through visual identification and weighing of each species separately, and these proportions are then used to extrapolate the bycatch fraction in the subsamples to the total catch and reported to both, fishermen and fisheries control.
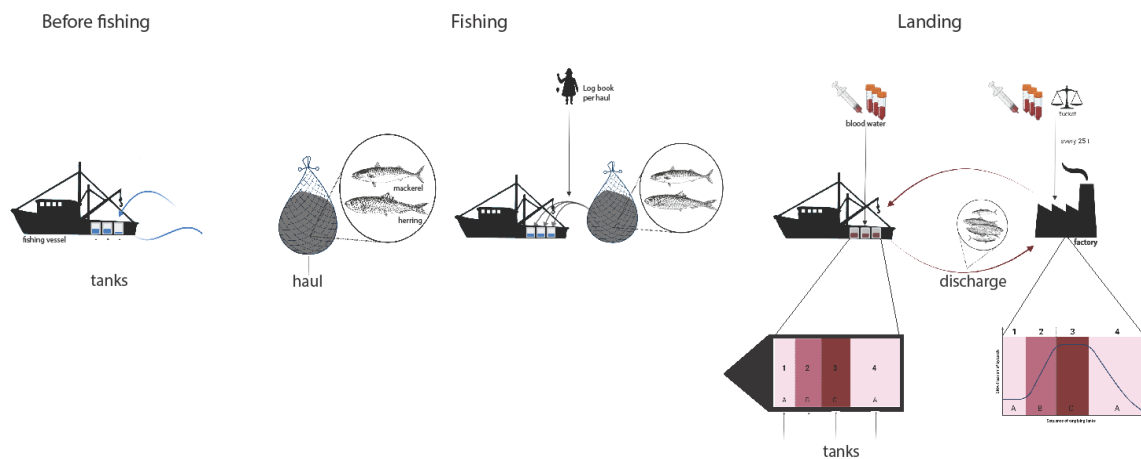
Figure 3.5 A. visualization of the three phases in a pelagic industrial fisheries. The first phase, "before fishing" shows the uptake of seawater into tanks on board the ship. The seawater is used during the fishing stage to keep the catch fresh and it is used to transfer the catch from the ship to the factory. During fishing, separate casts of the fishing net are performed ("hauls") to collect the total catch. The letter "A", "B", and "C" represent three different hauls distributed among four ship tanks. The species composition from each haul is determined and noted in the logbook. During landing, fish and the seawater (now called blood water) are transported from the ship to the factory. Each tank is emptied once at the time in a predefined sequence (1-4). During the discharge, the species composition is further determined using the bucket method. The genetic sampling took place on the ship (before the discharge started) and at the factory at the same rate as the bucket method. Importantly, during landing blood water is constantly recirculated between the factory and the ship.

3.3.3 Sampling blood water from landings

We sampled data from a total of three landings ("landing 1-3"). Each sampling started with collecting blood water from the ship. We collected three replicates (one sample = three replicates) of blood water from each holding tank of the ship (individual ships had 8-11 tanks). All replicates were collected at the opening ("top-part") of each tank. Once the landing process started, we collected three replicates

of blood water for every 25t at the factory, synchronized to the visual (bucket) method. Both sampling onboard and at the factory consisted of collecting up to 50 ml of the blood water using a sterile 60 ml syringe (Codan™), into a sterile 50 ml falcon tube (Sarstedt, Screw cap tube, 50 ml). All samples were kept on ice during the sampling and frozen at -20C immediately after the end of the landing. In each landing bycatch was also estimated using visual methods (logbook and bucket). For one landing (no. 3), additional information was recorded about the volume of each of the 11 tanks of the ship, which haul was placed in which tank and the sequence of discharging individual tanks to the factory.

Table 3.3. Overview of the three landings from which blood water was collected for eDNA-based estimation of mackerel biomass. Only a part of samples from landing 1 was analyzed, i.e. only 9 out of 11 samples collected from the ship were analyzed using the eDNA-based approach to explore the possibility of mackerel being present in the catch. Estimates of mackerel biomass for each landing were obtained from the logbook and buckets method.

Landing ID	Time between catch and the landing (h)	Total catch size (t)	Size of the ship (m)	Number of hauls	Number of tanks holding	Number of eDNA at ship samples	Number of eDNA at factory samples
1	48-72	1185.4	75	5	11	9 (out of 11 collected)	6 (out of 36 collected)
2	120	940.2	63	3	8	8	42
3	72-144	902.1	75	3	11	11	37

3.3.4 DNA-extraction

DNA was extracted from 1 ml of each water sample using the Omega Bio-tek E.Z.N.A. Tissue DNA kit (Omega Bio-tek, USA). The standard extraction protocol was adjusted for higher sample volume (with 2.5X the recommended volume of buffers during DNA lysis and adjustment before silica-membrane binding). DNA extraction took place in a molecular lab. Blank samples were collected throughout the sampling (using DNA-free water that did not get into contact with either catch or blood water), DNA-extraction and analysis process (non template controls) to monitor possible contamination.

3.3.5 Species-specific qPCR

Species-specific assays targeting herring and mackerel cytochrome b sequence of the mitochondrial genome were used for DNA quantification in the samples (Supporting Information 4.1). Assays used were optimized and tested on a StepOnePlus Real-Time PCR System (Life Technologies, USA). Assay specificity was assessed using genomic DNA from the two target species (herring and mackerel) cross-tested on both assays. Each qPCR reaction was conducted in 10 µl volume with 3 µl of sample and 4 µl TaqMan Universal PCR Master Mix (Thermo Fisher Scientific) and assay specific volumes of primers and probes to obtain optimal reaction conditions (Supporting Information 4.1). Thermal cycling conditions for both assays were the same (5°C for 5 min and 95°C for 10 min followed by 50 cycles at 95°C for 30 s and 60°C for 1 min). Samples were analysed in duplicates with triplicate negative controls and a triplicate standard curve ranging from 3×10^{6} to 3×10^{0} copies per reaction in each run. The experimental samples were analysed in a multiplex (duplex) setting, while factory samples were analysed as singleplex reactions. Primer concentration was the same for both setups with overall higher concentration of mackerel primer, to prevent dominance of the herring assay (see Supporting Information 4.2). The herring assay was modified for the multiplexing approach by using a NED-dye for the herring probe to separate its fluorescence signal from the FAM-dyed mackerel probe (see Supporting Information 4.2). To increase delta-fluorescence assays used double-quencher probes (singleplex: 5'FAM/ZEN/3'IB®FQ (Integrated DNA Technologies, USA; multiplex: 5'TAMRA/ZEN/3'BHQ-2® (Integrated DNA Technologies, USA), in all reactions. The singleplex and multiplex approaches were compared for 36 out of 162 experimental samples to ascertain estimate consistency. An internal positive control (hereafter IPC) dyed with VIC was used in each reaction to monitor inhibition. For the

multiplexing we used the VetMAX Xeno Internal Positive Control containing the BHQ-3 quencher (Applied Biosystems) and for the singlplex approach we used the TaqMan™ Exogenous Internal Positive Control containing the TAMRA quencher (Applied Biosystems).

3.3.6   Modeling of the catch composition

Model 1

The experimental data served for establishing an eDNA-to-biomass model, which subsequently was used to predict the biomass of mackerel from eDNA measurements from fisheries samples. We used generalized linear mixed models (GLM) built using the package *glmmTMB* 1.0.2.9 (Brooks et al., 2017). The DNA quantities estimated from herring and mackerel were converted into mackerel-to-herring fractions; hence the genetic observations are continuous numbers between 0 and 1, naturally described by a beta distribution:

$O_i \sim$ Beta(μ_i, ϕ) independent where:
logit(μ_i) = $\alpha + \beta \cdot$ logit(true weight fraction$_i$) + δ(*treatment$_i$*) + $\gamma \cdot$ time$_i$ + E(Replicate$_i$) + ε_i, and
    E(Replicate$_i$) $\sim N(0, \sigma^2_E)$ and $\varepsilon_i \sim N(0, \sigma^2)$ independent.

The model parameter ϕ is a precision parameter scaling the variance of Oi as Var(Oi) = µi(1 − µi)/(1 + ϕ). The logit-scale genetic fractions (Oi) are described as a linear function of time and logit of the true weight-based fractions with separate levels corresponding to each level of treatment (DNA-shedding lab, DNA-shedding ship, DNA decay). The uncertainty structure of the model accounts for variations among replicates of mixtures and individual measurement noise.

To describe the mackerel DNA fraction to biomass relationship with resemblance to the conditions of the catch during landing (Table 3.2) we chose a specific treatment ('DNA-shedding experiment' from laboratory), and the results from the time period of 72h, as well as a range of true weight fraction from 0.001 to 0.05.

$O_i \sim$ Beta(μ_i, ϕ) independent where:
logit (μ_i) = $\alpha + \beta$ * logit(true weight fraction$_i$)

Model 2

For landing 3, additional information was collected about total catch separation into individual hauls and holding tanks. We explored this in an analysis where the mackerel weight fractions estimated from all four methods (logbook, eDNA- from ship, eDNA from factory, and factory bucket method) were modelled to understand how the variation in mackerel fractions in individual hauls and the mixing of the water during the landing process influence the estimations of the total mackerel bycatch. Again, eDNA-based weight fractions and logbook fractions are continuous numbers between 0 and 1, naturally described by a beta distribution. Fractions from the bucket method have a higher occurrence of zero observations than can be explained by a beta distribution, due to sampling of whole fish, hence the bucket observations are described by a zero inflated beta distribution.

$$P(O_i = o_i) = \begin{cases} p_i & \text{if } o_i = 0 \\ (1 - p_i)f(o_i, \mu_i, \phi_{type_i}) & \text{otherwise} \end{cases} \qquad (1)$$

Here, the function *f* is the density of a beta distribution with mean µi and measurement-type specific precision parameter ϕ_{type_i}, such that variance of the observations Oi scales as *Var*(Oi) = µi(1 − µi)/(1 + ϕ_{type_i}).

As zero inflation is only relevant for measurements originating from the bucket method it is assumed that p_i = 0 unless measurements are from the bucket method. The probability of zero bycatch could further be haul specific, so the model assumed for the zero probability is:

$$\text{logit}(p_i) = \alpha(haul_i) \qquad (2)$$

if i'th observation is from the bucket method.

The conditional expected non-zero fractions μ_i are assumed to be:

$$\text{logit}(\mu_i) = \sum_{tank=1}^{n_{tank}} \beta(type_i, tank_i)W_{i,tank} \qquad (3)$$

W is matrix of data weights, which is intended to describe how the sample is composed of fish from different tanks. If e.g. the i'th sample is exclusively from the first tank then the first $W_{i,1}$ = 1 and the following $W_{i,2}$ = 0, $W_{i,3}$ = 0,.... If the j'th sample is taken from an even mixture of tank 1 and tank 2, then $W_{j,1}$ = 0.5, $W_{j,2}$ = 0.5 and the rest zero.

Using W we tested three different mixing approaches between tanks that contain different fish hauls. We tested the "no mixing", "100T mixing" and "full mixing" scenario. In "100T mixing" W describes the changing composition of each sample collected within the first 100T of the discharge of a tank, after 100T all subsequent samples collected from the same tank were not considered under W, hence did not mix with the previous tank. In "Full mixing" W describes the changing composition of each sample taken during the discharge at the factory, hence every sample collected is a mixture of previously discharged fractions, and thus subject to W.

Finally, the eDNA-based weight estimates (ship and factory) were analysed in triplicates and hence those measurements can be expected to be correlated, which was accounted for by introducing sample-level as a random effect.

The generalized linear mixed model 2 was created in C++ and imported into R (4.1.0) using the package *TMB* (Kristensen, Nielsen, Berg, Skaug, & Bell, 2016).

3.4 Results

3.4.1 qPCR assay performance

The study used previously published and verified qPCR assays to target both species, herring and mackerel (Hansen et al., 2020b; Knudsen et al., 2019) (Supporting Information 4.1). The standard curves used for the quantitative estimation of DNA copies from the fisheries samples showed comparable efficiencies (herring average efficiency: 91.08%, mackerel average efficiency: 92.56%, multiplex: 94.91%) and correlation coefficients (R^2 >0.997 for all) between species (See Supporting Information 4.2 and 4.3). Similarly, the standard curves used for the estimation of the DNA quantity in experimental samples also showed comparable efficiencies between species (herring average multiplex: 98.68%, mackerel average efficiency multiplex: 94.91%). None of the assays showed unspecific amplification (See Supporting Information 4.4). Estimates of contamination were extremely small throughout the analytical process, and did not reach quantifiable amounts (above LOQ, (Merkes et al., 2019)). A subset of the experimental data was analysed using respectively singleplex and multiplex approach results, and showed highly comparable efficiencies E% (for mackerel, multiplex = 92.89% and singleplex = 95.49%, for herring, multiplex = 99.06 %and singleplex = 92.29%) and precision (for all $R^2 \geq 0.996$) derived from the standard curves. Nevertheless, these samples showed significant differences in Ct-value (Wilcoxon signed-rank for herring N = 36, V = 493, $p < 0.05$ and for mackerel N =

36, V = 42, *p* < 0.001) and estimated species fraction (Wilcoxon signed-rank for herring N = 36, V = 164, *p* = < 0.001 and for mackerel N = 36, V = 502, *p* = < 0.001).

3.4.2 Mackerel-herring weight to eDNA relationship

The shedding experiment in the lab, the shedding experiment onboard and the decay experiment all showed a strong relationship between estimated DNA fractions in the blood water and input weight fractions for all mock samples (Fig. 3.2). DNA fractions of mackerel were at all times overrepresented compared to the weight-based input fractions. The relationship between DNA fractions and input weight fractions was influenced by the time span of DNA release, and by the different treatments tested (DNA-shedding in the lab, DNA-shedding on the ship, and DNA-decay in the lab). In general, the mackerel DNA fraction showed a tendency to increase with time within the mock units (Fig. 3.2A and B). The difference in fractions over time was not statistically significant in any treatment, but was marginally non-significant for the lab-based DNA-shedding experiment (*p* = 0.0519; ship-based DNA-shedding experiment *p* = 0.8128, DNA-decay experiment *p* = 0.1557). Overall, the three different treatments tested show statistically significant differences in estimated mackerel fractions (for all, *p* < 0.001). Because the weight fractions and the DNA fractions are logit transformed, it is difficult to translate the change into a sinlge value per treatment. Hence, the Figure 3.2C shows how the change in weight fraction translates into changes in DNA fractions. We would like to highlight two examples that visualize the extent of the differences recorded by the different treatments. I.e. a mackerel weight fraction of 0.1 (10 %) corresponds to 0.456 (45.6 %), 0.329 (32.9 %), and 0.291 (29.1 %) DNA fraction, respectively when estimated from a DNA-decay, lab-based DNA-shedding experiment, and ship-based DNA-shedding experiment. A mackerel weight fraction of 0.5 (50 %) corresponds to 0.858 (85.8 %), 0.774 (77.4 %), and 0.747 (74.7 %) DNA fraction respectively when estimated from a DNA-decay, lab-based DNA-shedding experiment, and ship-based DNA-shedding experiment. The DNA-shedding experiments showed a higher mackerel fraction to weight fraction relationship when using fresh (lab-based DNA-shedding experiment) compared to thawed fish (ship-based DNA-shedding experiment) (Fig. 3.2C). Overall, the model outcomes are more precise when using the DNA-shedding data compared to using DNA-decay data (Fig. 3.2D).
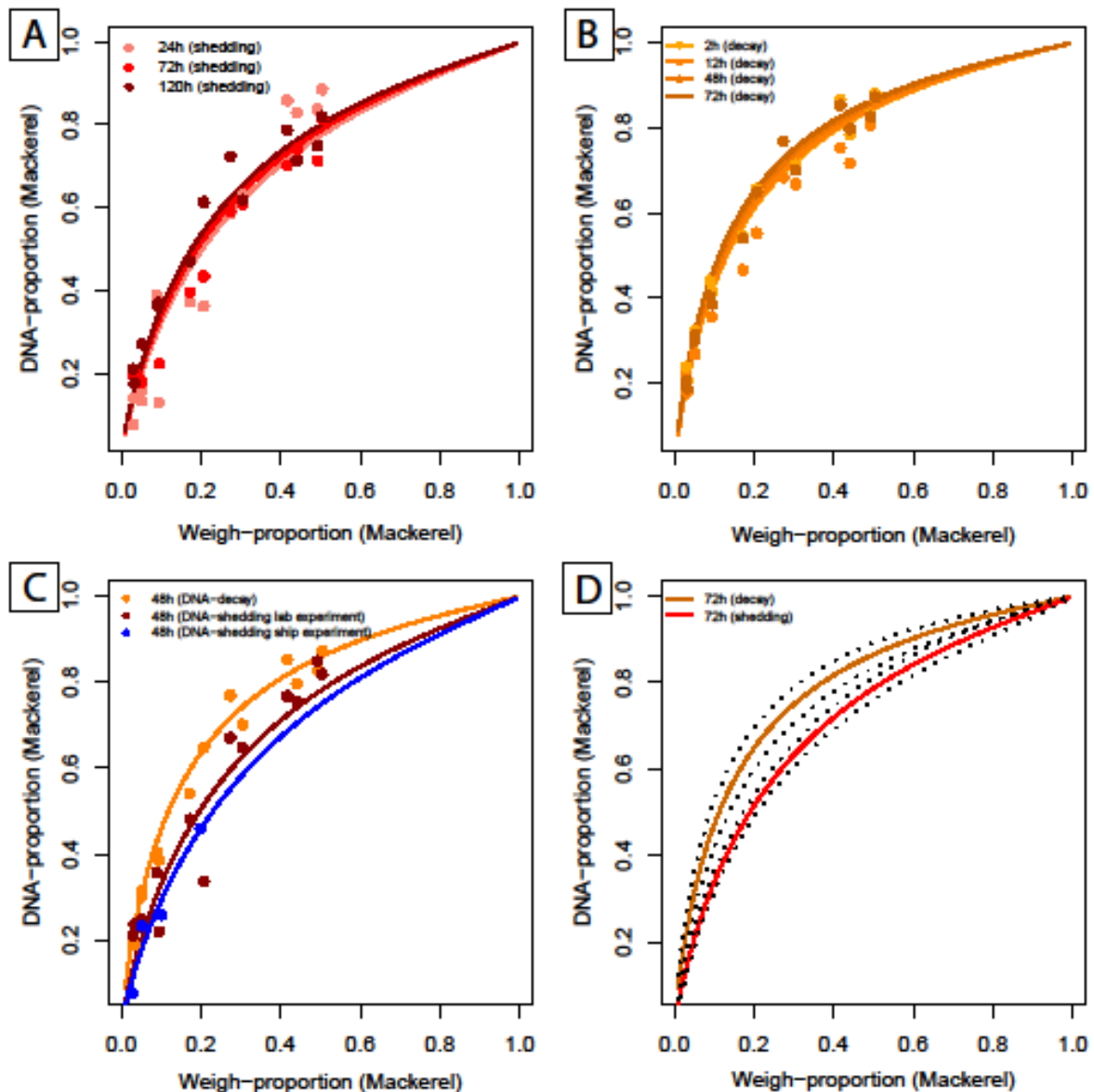
Figure 3.6. Predictions of the DNA to biomass relationship using model 1 for A: the effect of time on "DNA-shedding estimated from the lab-based DNA shedding experiment", B: the effect of time on "DNA-decay". C: the effect of three treatments ("Shedding experiment in the lab", "Shedding experiment on board a ship","Decay experiment in the lab"). The two shedding experiments besides of being conducted at different places, used mackerel of different quality, i.e. in the lab fresh mackerel was used whereas on the ship defrosted mackerel. D shows the assessment of the precision of the model-prediction for "DNA-shedding (lab-based experiment)" and "DNA-decay". Solid lines in each graph show the model predictions of weight-based fractions for DNA-based fractions ranging from 0.01-0.99, dotted lines in D show the 95% confidence intervals for the predictions. The dots in A-C reflect the measured mackerel eDNA-fractions in the different experiments.

3.4.3 Estimating weight of mackerel bycatch in three fisheries landings

For landing 1, a part of the samples collected was analysed using the qPCR approach (9 out of 11 samples from the ship and 6 out of 36 samples from the factory). Very low raw DNA copy numbers of mackerel were recorded in some samples. No samples analysed from landing 1 reached LOQ for mackerel (see Supporting Information 4.2), thus, all measurements were regarded as un-quantifiable detections. In this landing, the average raw DNA copy number for mackerel in both eDNA at ship and eDNA at factory samples was 5.19 copies/reaction (in the range of 0-41 DNA copies/reaction). The

highest raw DNA copy number was recorded in one out of three replicates of a eDNA at factory sample analysed (41 copies). In contrast to this, the average herring raw DNA copy number was 50084 (ranging from 1433-238734 DNA copies in all samples analysed). Thus mackerel fraction based on raw DNA in both, eDNA at factory and eDNA at ship samples, was on average 0.0001 (ranging from 0-0.0025). The highest mackerel fraction (based on raw DNA) of 0.0025 (i.e. 0.25%) was recorded in one replicate of an eDNA at ship sample. This replicate had generally one of the lowest outputs of DNA (sum of raw DNA copies of both species = 1692) among all samples, with a mackerel DNA copy number estimated at four (resulting in a fraction of 0.0025 (i.e. 0.25 %)). When translating the raw DNA fractions to biomass using model 1, all factory samples ended with negative biomass estimations (hence, no biomass of mackerel detected). For the eDNA at ship samples, five of the analysed replicates (out of 27 replicates analysed) yielded positive biomass fraction estimates, with the highest biomass fraction estimated to 0.00029 (i.e. 0.029 %, corresponding to 346.0 kg of mackerel). However, the arithmetic mean of all ship observations resulted in -0.000012 (i.e. - 0.0012%); hence no biomass. The absence of mackerel biomass estimates in samples from landing 1 assessed using the eDNA-based approach was in agreement with both visual methods (log book mackerel fraction = 0.0, bucket mackerel fraction = 0.0) (Fig.3.3). Small fractions of mackerel DNA were detected in samples from landing 2 and 3 in both eDNA at ship and eDNA at factory samples, allowing total weight fractions to be estimated using model 1. In both landings, the total mackerel fractions estimated using eDNA-based analysis were lower from the estimates derived from the visual (bucket) method, however to a large extent aligned with the visual (log book) estimates derived for the same catch (Table 3.3). The arithmetic mean of fractions estimated from all eDNA at ship and eDNA at factory samples was 0.00032 (i.e. 0.032 %) and 0.00056 (i.e. 0.056 %) for landing 2 and 0.00096 (i.e. 0.096 %) and 0.00111 (i.e. 0.111 %) for landing 3 (Fig. 3.3 and Table 3.3). This corresponded to total mackerel biomas of 300.9 kg and 526.5 kg in landing 2, based on, respectively, eDNA at ship and eDNA at factory, and to 866.0 kg and 1001.3 kg of mackerel in landing 3 based on, respectively, eDNA at ship and eDNA at factory (Fig. 3.3 and Table 3.3). The mean mackerel fraction estimated using the visual (bucket) method was larger for both landings with 0.002 (i.e. 0. 2 %) for landing 2 and 0.0034 (i.e. 0.034 %) in landing 3, which corresponds to 1899.7 kg and 3067.1 kg mackerel, respectively (Fig. 3.3 and Table 3.3) (see also Supporting Information 4.9). In comparison, mackerel bycatch weights reported by the visual (log book) method for landing 2 and landing 3 were 950 kg and 800 kg (that would correspond to a fraction of 0.001 (i.e. 0.1 %) and 0.0009 (i.e. 0.09 %) for landing 2 and 3). The results from the visual (log book) method thus either over-estimated the mackerel fraction or aligned with the data from the eDNA-based methods (Fig. 3.3 and Table 3.3).
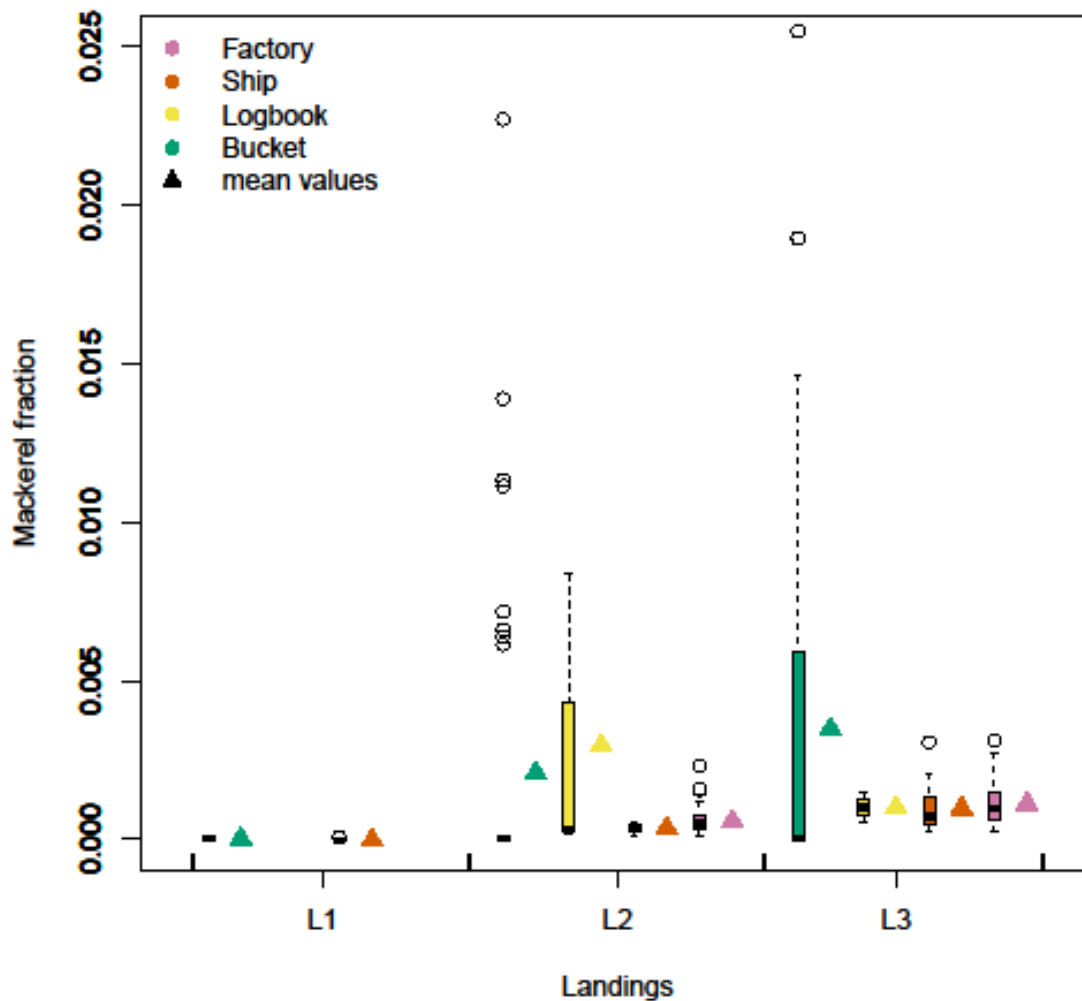
Figure 3.7. Box and whisker plots showing the estimated mackerel biomass per method for landing 1-3. Box and whisker plots show the median, 1st quantile and 3rd quantile as a box and whiskers (1.5 times the interquartile range i.e. difference between 3rd quantile and 1st quantile, above and below the 3rd quantile and 1st quantile respectively) display as dashed lines; the open circles show oultliars. The results from the eDNA-based method are shown as "eDNA at ship", and "eDNA at factory". The visual methods (visual (log book) and visual (bucket)) are both estimated using the bucket method applied at different time points during the industrial fisheries. The visual (log book) data consists of fractions assessed per haul (each catch within the landing consisted of 3-5 hauls). The triangles in the graph show the mean fractions recorded per method per landing. The mean fraction is converted to the total mackerel biomass (Table 3.3), which subsequentially would be recorded to authorities.

Table 3.3. Overview of the estimates of the total mackerel biomass in each of the landings analyzed following the methods. This estimate is based on the arithmetic mean of all subsamples collected per method per landing. The true mackerel biomass in each landing is not known.

Method	Landing 1	Landing 2	Landing 3
eDNA at factory	0 kg	526.5 kg	1001.3 kg
eDNA at ship	0 kg	300.9 kg	866.0 kg
Logbook	0 kg	950 kg	800 kg
Bucket	0 kg	1899.7 kg	3067.1 kg

3.4.4 Assessing variability within catch with the different methods

Additional insights emerged when analyzing results for the different tank, haul and factory discharge periods of landing 3 (Fig. 3.4). Thus, when discharging haul A, both at the beginning and the end of the discharge, (0.00045 (i.e. 0.045 %) mackerel fraction indicated from visual (logbook) data), all methods resulted in relatively low mackerel estimates (Fig. 3.4). In contrast, bycatch estimates increased in haul B and C (0.00149 (i.e. 0.149 %) and 0.00099 (i.e. 0.099 %) from the visual (logbook) data respectively) discharged in the middle of the discharging process (Fig. 3.4). Throughout the discharge, process we observe continuous and relatively low mackerel fractions in all eDNA at factory samples, hence we observed smooth transitions between discharging of different tanks consisting of different hauls (Fig. 3.4). On the contrary, the visual (bucket) method yielded highly fluctuating (from 0 to 0.025) pattern in fractions throughout the discharge. Both visual and eDNA methods indicated an increase in the mackerel fraction when 300-500 tons of the total catch (in total 902.1 tons) was discharged to the factory (Fig. 3.4). The two eDNA estimates (ship and factory) indicated the same trend in the mackerel fraction within the catch. However, the integrated estimate (arithmetic mean) for the total catch differed between eDNA at ship vs. eDNA at factory, coming out at 0.00096 (i.e. 0.096%) vs. 0.00111 (i.e. 0.111%), translating into 866.0 kg or 1001.3 kg of mackerel (Fig. 3.3).
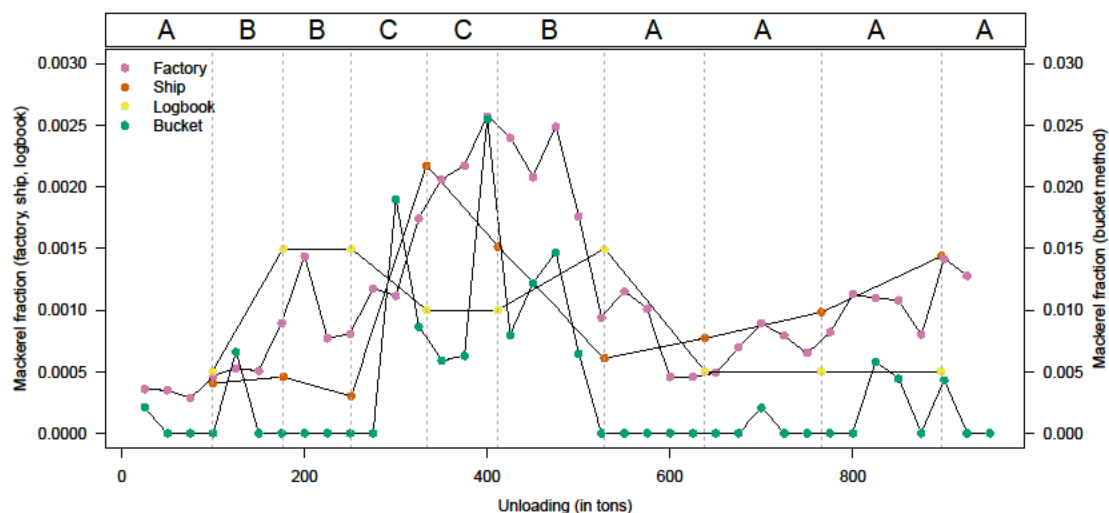

Figure 3.4. Variation in estimated mackerel fractions across landing 3 based on the different methods used: "eDNA at factory", "eDNA at ship", "visual (log book), "visual (bucket) . The y-axis scale of the eDNA-based results from ship factory is different from the scale used for the visual (log book and bucket) methods. The difference is because the eDNA-based methods provide a continuous, relative low mackerel fraction in all samples. On the contrary, the visual methods yield highly fluctuating (from 0 to 0.025) fractions. The eDNA-based results (eDNA at factory and eDNA at ship) are shown as means of the three replicates collected at each sampling point. For each method, the sampling points are connected using solid black line to ease the visualization. The vertical, dashed lines indicate a change in discharge of fish from different tanks onboard the vessel (1-10), filled with fish from one of 3 hauls. "A", "B", and "C". Each haul has potentially different bycatch fraction. According to the visual (logbook) data haul A has a mackerel fraction of 0.00045, haul B 0.00149, and haul C 0.00099.

3.4.5 The effect of mixing of water on the eDNA-based estimates

The distribution of the individual hauls into different holding tanks on board influenced the eDNA-based estimates. Only eDNA at factory samples were influenced by the sequential mixing of the water from different holding tanks, as a result of continued re-usage of the blood water for the transport of fish from ship to factory. Accounting for mixing of water further increased the similarity of the two eDNA–based estimates. Following the model, the estimated fraction of mackerel from the eDNA at

ship samples in the total catch was 0.00085 (i.e. 0.085 %) while for the eDNA at factory samples the estimates were, respectively, 0.0099 (i.e. 0.099 %), 0.0010 (i.e. 0.10 %), and 0.0009 (i.e. 0.09 %) in the three different mixing scenarios tested ("no mixing", "100T-mixing" and "full-mixing") (Fig. 3.5,). This corresponded to a total of 804.0 kg of mackerel in the total catch estimated using eDNA at ship, and to 934.294 kg, 950.686 kg and 849.343 kg of mackerel estimated using eDNA at factory (respectively, "no mixing", "100T-mixing" and "full-mixing"). Under the hypothesis that the entire blood water was mixed thoroughly during the discharge ('full-mixing'), the last sample taken from the blood water at the factory should reflect the integrated signal of the total mackerel weight in the whole catch. However, according to the last eDNA sample collected at the factory 1136.646 ± 27 kg mackerel were in the catch (mean fraction estimate of 0.00126 ± 0.00003 i.e. 0.126 % ± 0.003%) (Fig. 3.5). This weight estimate was different from the factory-based estimate of 849.343 kg that takes into account "full mixing".
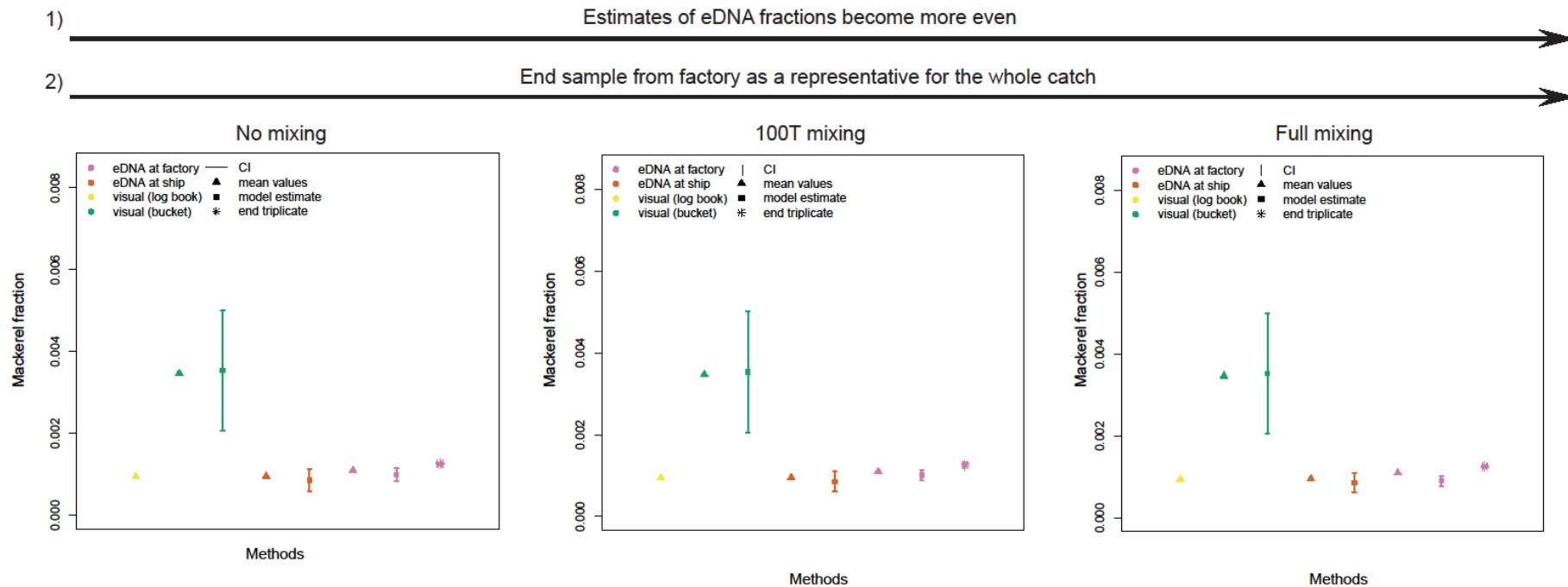
Figure 3.5. Sequential effect of blood water re-usage during discharge of the catch. The effect of re-usage of the blood water largely affects the eDNA at factory derived samples in the total mackerel estimation (the model estimate). In the "no mixing" scenario, no re-usage of the water is taken into account, thus the total mackerel estimate (the model estimate) is an arithmetic mean of eDNA at factory measurements regardless of succession point in discharge. "100 T mixing" assumes that when changing from one haul to the other during discharge, blood water from the first 100T represents a mixture of mackerel eDNA fractions of both hauls. After 100T the assumption is that the eDNA results corresponds solely to the currently unloaded haul. In "Full mixing" we assume that samples taken at any point in the discharge process (so any eDNA sample collected at the factory) give an integral value of for the previous and current haul being dicharged (i.e. all water was re-reused at all times). Following the idea of the "Full mixing", the last eDNA at factory sample collected should have the same mackerel fraction as the model estimate derived from all eDNA at factory samples.

3.5 Discussion

A tangible, large scale application of eDNA for fisheries purposes, like the monitoring of catch composition, was for a long time a scientific vision (Gilbey et al., 2021; Hansen et al., 2020a; Rourke et al., 2021; Russo et al., 2021). Turning possibility into reality, we highlight how quantification of bycatch using eDNA can be achieved. In this study, we undertook experimental work to establish an eDNA-to-biomass model, which subsequently was used to estimate the weight of bycatch using actual fisheries process water from three landings. The fractions/weights of mackerel estimated with DNA analysis were comparable to routinely used visual based estimation metrics, moreover the eDNA-based method stood out in precision of the estimates. Still, there appear to be some systematic differences related to the accuracy of the eDNA-based and the precision of the visual methods, which we discuss below. eDNA-based bycatch estimates conducted for the same catch at the ships and at the factory showed sufficient similarity and robustness to reject potentially confounding factors relating to the distribution of different fishing hauls within the total landing. In contrast, the same confounding factors did apparently affect the visual based estimates, where subsampling the catch onboard the vessel and at the factories returned highly divergent estimates. All in all, eDNA-based assessments, like the here presented bycatch estimation, have realistic prospects to be applied for monitoring activities within fisheries science.

3.5.1 Mackerel-herring weight to eDNA relationship

We found weight fractions and DNA fractions from mock samples to correlate strongly. However, mackerel DNA fractions were consistently higher than the expected fractions based on weight. Because of the stable abiotic conditions in the experiment, we expect biotic factors to be the main driver of the difference observed. Shedding is known to vary between species based on external features such as body shape (Andruszkiewicz Allan, Gordon Zhang, Lavery, & Govindarajan, 2021; Wood et al., 2020) and size (Yates et al., 2021). However, since the two species were in general similar in size and shape, other biological factors, such as the mtDNA content in cells and the type and rate of material shed into the environment were considered to be the primary drivers of the differences (Hansen et al., 2020a; Sassoubre et al., 2016). Herring and mackerel belong to distinct phylogenetic families (mackerel: Scombridae, herring: Clupeidae). Mackerel are swift, active predators related to tuna-like species and their high needs for energy could be responsible for a higher amount of mitochondria, and thus higher mtDNA content in cells shed from mackerel, identified using the qPCR approach. From studies on Pacific Chub Mackerel and Pacific Clupeid species, it was observed that mackerel tend to release slime in the water, in contrast to Clupeids, which shed scales (Sassoubre et al., 2016). Thus, the difference in the material and cell content shed into the environment could also be responsible for the observed higher mackerel DNA fraction. However, little specific information is available on these biotic differences between species and their effect on the qPCR results. Thus, for now, we only speculate that a combination of factors could be responsible for discrepancies between weight and eDNA fractions observed.

The DNA-shedding experiment conducted onboard the ship, using thawed mackerel yielded slightly lower mackerel DNA fractions than the laboratory experiment on fresh fish. Because the results were significantly different, one would need to account for this in using the appropriate model, in case freezing of the fish would occur during fisheries. During fisheries operations, blood water is usually cooled down close to the freezing point of the water to maintain a good quality of the fish (-1 to -1.7°C), but the freezing of fish is prevented (Sampels, 2014). The magnitude of difference measured between fresh and thawed fish even though significant, was very small. Thus, if unequal cooling would be observed at all, the overall effect of it on the measured DNA fractions of bycatch would be of little impact to the end estimates.

3.5.2   Estimating weight of mackerel bycatch in three fisheries landings

The eDNA tool for bycatch estimation worked equally well for the quantitative detection of species as the visual methods applied. Some of the eDNA at ship and eDNA at factory samples from landing 1 had very low levels of mackerel DNA, with quantities too low to be quantifiable using our species-specific PCR setup. In these samples, the measured copy numbers were too low to result in estimates of mackerel biomass in the modeling approach, hence returning the result that the catch did not contain mackerel bycatch. The low levels of mackerel DNA detected were possibly contamination from previous catches. Fishing vessels pump new seawater into their holding tanks during each sail-out, before fishing. However, for as long as vessels fish for the same species, the holding tanks are only rinsed with fresh seawater after each landing, and are not thoroughly cleaned with detergents. Thus, DNA remains from any previous bycatch can be carried over to the next catch likely in such small quantities that it has little to minimal effect on eDNA-based bycatch biomass estimates for subsequent catches. Overall, this illustrates the robustness of the DNA-based method towards contamination from fishing operations and likely also from natural contamination from the seawater used for holding the fish, as well as from potential contamination from stomach content (Russo et al., 2021). The underlying cause is that the fresh DNA from the catch is so much in excess compared to potential sources of contamination.

For landing 2 and 3, where non-zero mackerel fractions were estimated from the eDNA-based approach, the estimates showed comparable fractions to visual (log book) estimates, and similar but consistently lower fractions compared to estimates from the visual (bucket) method. The estimation of catch fractions from the eDNA-based method is dependent on the model prediction following the DNA shedding experiments. One limitation of our experimental setup was the range of tested catch-by-catch fractions, with the lowest tested mackerel fraction of 0.025 based on weight. In the analysed landings, mackerel bycatch hardly reached 0.01 of the total weight. Therefore, to predict mackerel weight from the eDNA-based fractions below 0.025 we used the extrapolation of the modeled experimental outcome. As with any predictions made outside the range of empirically gathered data, interpretations need to be made cautiously. Eventough it was unrealistic to prepare mock samples in the composition needed (i.e. for a 0.001 fraction we would need to mix 50g mackerel with 49.95kg of herring), we expect the model 1 to robustly convert DNA fractions to weight fractions outside the range investigated, as the low range mackerel DNA fractions (0.025 – 0.05) showed a good fit to the model.

The difference in total estimates of mackerel fraction between the eDNA-based and the visual (bucket) method can also be due to the limitations of the latter. The bucket provides an accurate, however less precise estimation of catch composition. The uncertainty of the estimates provided using the bucket method is directly related to the number of subsamples collected (with a higher frequency of sampling, chances of finding bycatch are higher) (Fiskeristyrelsen, 2021). Following the methods description, the bycatch estimates derived from the visual methods should be within ± 10%, i.e. for a catch with 100 kg of mackerel, the estimates is somewhere between 90-110 kg (Fiskeristyrelsen, 2021). For landing 3 this variation corresponds to a difference in estimated bycatch of 3067.1 kg ± 306.7 kg. On top, the difference between the two visual assessments is considerably higher (bucket method 3067 kg, log book 800 kg) then the allowed 10% margin in difference between the two assessments following the Council Regulation (EC) No 1224/2009 (European Union, 2009). The high uncertainty of the visual assessments is often the source of the conflict between the fisherman and control authorities.

3.5.3   Assessing variability within catch with the different methods

Fisheries catches typically consist of discrete hauls, which can vary in species composition. For fisheries management, information about variation among individual hauls is unimportant, and thus only

estimates of species quantities from total catches are reported in the logbook system. However, to assess the precision of the different methods used for bycatch estimation it is necessary to investigate which method can reliably reflect species distribution within and between hauls, regardless of whether the species distribution within the catch is uniform, random, or clumped. The eDNA-based method provides an integrated signal of the total catch and shows high sensitivity and precision in estimates regardless of the distribution of bycatch. At the same time, it allows studying very subtle differences in the catch composition from different hauls and tanks, as opposed to the bucket method, that only coarsely reflects the variation in bycatch distribution. The reason for the difference is that the eDNA-based method is a continuous measurement of the mackerel fraction, in contrast to the bucket method that measures whole fish, which as such appear at random in the buckets (Fiskeristyrelsen, 2021). Even though the bucket method could be accurate, the high uncertainty of the measurements (10 %) lowers the precision and thus limits the reliability and replicability of the method to the same catch. For instance, changing the sequence of emptying the holding tanks could very likely result in a very different bycatch estimate when using the bucket method, in contrast to the eDNA-based method. The aspect of reliability of estimates is important, as ideally, the estimates of the bycatch fraction should be within a 10 % margin, following the legislation on the allowed derivations of visual estimates (log book and bucket) (Article 14(3), European Union, 2009). Following this, we showed that the visual methods, logbook and bucket are not in accordance to the legislation, because in both landings (landing 2 and landing 3) mackerel biomass estimates differ more than 10%. Alongside this, we show that the eDNA-based approach performed on the ship and at the factory for the same catch result in estimates following the margin of 10% to one another. In the case of landing 3, both eDNA-based estimates are accordance to visual log book, hence align with the 10% margin defined in the legislation (Article 14(3), European Union, 2009). Since the true weight of mackerel is unknown, it is impossible to tell if the violation of the legislation between the two visual methods is because of human error or the error of the methodological approach. However, the precision of the eDNA based method gives enough support to believe in the methods robustness. It is of central importance that if a new method is implemented, it would provide robust estimates within the scope of the legal regulation of fisheries activities that ensures a sustainable usage of maritime resources.

3.5.4   The effect of mixing of water on the eDNA-based estimates

The goal of this study was to evaluate if an eDNA-based tool can be more reliable in bycatch estimation than the visual assessments used currently. We observed deviations between eDNA at ship and eDNA at factory assessed mackerel bycatch estimates. These discrepancies, however, are of a much smaller magnitude than the ones observed between visual assessments, i.e. logbook and bucket method. The differences between the eDNA-based approaches can be the result of the restricted potential for true replication of the ship-derived samples compared to the factory-derived samples. Ship-derived eDNA samples were collected only at the surface of each tank as deeper parts of the tanks cannot be sampled, once filled with the catch. The differences observed between the ship and factory eDNA-based estimates can be evaluated when trying to account for different scenarios for mixing of blood water during the discharging. The different mixing scenarios produce comparable results, with more similar ship and factory estimates with increasing mixing. Thus, the most likely scenario is that during landing 3 the total blood water content was mixed during discharge. Following the hypothesis that the full mixing is occurring during the discharge, the blood water sample collected at the end of the discharge could in principle, provide an integrated signal of the overall catch composition. Reducing the eDNA-based assessment to a single last sample would potentially speed up the bycatch assessment analysis, while simultaneously decreasing the cost and increasing time efficiency. However, this hypothesis could not be directly supported with the note of caution that we do not know the "true" bycatch rate and the results therefore should be interpreted with precaution. The eDNA-based fraction of mackerel in the last sample is higher than the eDNA at ship and the eDNA at factory estimates

(eDNA at ship: 804.0 kg, eDNA at factory: 849.343 kg, last sample estimate: 1136.646 kg). Although not big, the differences still lead to somewhat substantial differences in the estimated bycatch biomass. The difference could be caused by a random effect during sampling (i.e. minute variations in the distribution of DNA) or a process of accumulation of mackerel DNA over time. With time, fewer fish remain in the water, hence the fractions in the fisheries process water reflect the DNA-decay scenario more. In the DNA-decay study we observed higher fractions of mackerel DNA compared to the DNA-shedding experiment for the same weight fraction of the species. Thus, the difference between the overall estimate and the estimate from the end-sample might be due to the change in the treatment (DNA-shedding or DNA-decay) to which the samples are subjected. The discrepancy between ship and factory eDNA-based estimates visualizes that there is a need for proper understanding of the whole process from individual hauls, distributions in tanks, and the discharge process that can result in discrepancies between samples collected at different points in the pipeline. Thus, specific Standard Operating Procedures (SOPs) need to be in place to eliminate discrepancies.

3.5.5 Implementation scope

Overall, it is likely that the eDNA-based approach is more time and cost-effective, and more precise and consistent in estimating catch fractions than the currently used methods. Building on the experience from this study, the implementation of the method for routine measurements appears to be achievable within a relatively short period. However, some uncertainties need to be addressed and controlled. First, an optimal sampling scheme needs to be designed that takes into account the full process of mixing fish and water from individual fish hauls to factory discharge. It is of paramount importance that sampling design and sample comparisons are operating on a fully transparent foundation. Secondly, the eDNA to biomass translation needs to be addressed further, including exploration of very low weight fractions, which is the reality for some fisheries and for explaining the difference between the approaches in measuring the DNA content in samples. Additional experiments should also ideally encompass the full enumeration (or very extensive subsampling) of large catches allowing evaluation of the accuracy of the method, which was not feasible for this pilot study. Implementation of the method across pelagic fisheries in Europe would allow a level playing field for pelagic fishermen in Europe and a common framework for control and enforcement. At the same time, the eDNA-based method, if applied for regular bycatch monitoring, would contribute to faster, cheaper, and highly reproducible bycatch estimates ultimately benefitting stock assessment and reducing conflict over bycatch estimation between fisherman and control agencies.

3.6 References

Andruszkiewicz Allan, E., Gordon Zhang, W., Lavery, A. C., & Govindarajan, A. F. (2021). Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. *Environmental DNA*, (3), 492–514. doi: 10.1002/edn3.141

Arnason, R., Hannesson, R., & Schrank, W. E. (2000). Costs of fisheries management: the cases of Iceland, Norway and Newfoundland. *Marine Policy*, *24*(3), 233–243. doi: 10.1016/S0308-597X(99)00029-9

Bakker, J., Wangensteen, O. S., Baillie, C., Buddo, D., Chapman, D. D., Gallagher, A. J., … Mariani, S. (2019). Biodiversity assessment of tropical shelf eukaryotic communities via pelagic eDNA metabarcoding. *Ecology and Evolution*, *9*(24), 14341–14355.

Beng, K. C., & Corlett, R. T. (2020). Applications of environmental DNA (eDNA) in ecology and conservation: opportunities, challenges and prospects. *Biodiversity and Conservation*, *29*(7), 2089–2121. doi: 10.1007/S10531-020-01980-0/FIGURES/2

Booth, H., Arlidge, W. N. S., Squires, D., & Milner-Gulland, E. J. (2021). Bycatch levies could reconcile trade-offs between blue growth and biodiversity conservation. *Nature Ecology & Evolution 2021 5:6*, *5*(6), 715–725. doi: 10.1038/s41559-021-01444-w

Boussarie, G., Bakker, J., Wangensteen, O. S., Mariani, S., Bonnin, L., Juhel, J.-B., … Robbins, W. D. (2018). Environmental DNA illuminates the dark diversity of sharks. *Science Advances*, *4*(5), eaap9661.

Brooks, M. E., Kristensen, K., Van Benthem, K. J., Magnusson, A., Berg, C. W., Nielsen, A., … Bolker, B. M. (2017). glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *The R Journal*, *9*(2), 378–400.

Buxton, A. S., Groombridge, J. J., & Griffiths, R. A. (2018). Seasonal variation in environmental DNA detection in sediment and water samples. *PLOS ONE*, *13*(1), e0191737. doi: 10.1371/JOURNAL.PONE.0191737

Clare, E. L., Economou, C. K., Bennett, F. J., Dyer, C. E., Adams, K., McRobie, B., … Littlefair, J. E. (2022). Measuring biodiversity from DNA in the air. *Current Biology*, *32*(3), 693-700.e5. doi: 10.1016/J.CUB.2021.11.064

Clegg, T. L., Steven, J. K., Geir, B., & Kjell, N. (2021). Applying global best practices for estimating unreported catches in Norwegian fisheries under a discard ban. *Reviews in Fish Biology and Fisheries*, *31*(1), 1–23. doi: 10.1007/s11160-020-09624-w

Cusa, M., St John Glew, K., Trueman, C., Mariani, S., Buckley, L., Neat, F., & Longo, C. (2022). A future for seafood point-of-origin testing using DNA and stable isotope signatures. *Rev Fish Biol Fisheries*, *32*, 597–621. doi: 10.1007/s11160-021-09680-w

del Valle, J. M., & Aguilera, J. M. (1991). Physicochemical characterisation of raw fish and stickwater from fish meal production. *Journal of the Science of Food and Agriculture*, *54*(3), 429–441. doi: 10.1002/JSFA.2740540314/FORMAT/PDF

Dickey-Collas, M., Pastoors, M. A., Van, O. A., Dickey, K., Dickey-Collas, M., Pastoors, M. A., & Van Keeken, O. A. (2007). Precisely wrong or vaguely right: simulations of noisy discard data and trends in fishing effort being included in the stock assessment of North Sea plaice. *ICES Journal of Marine Science*, *64*(9), 1641–1649. doi: 10.1093/ICESJMS/FSM155

European Union. (2009). *Council Regulation (EC) No 1224/2009 of 20 November 2009 establishing a Community control system for ensuring compliance with the rules of the common fisheries policy, amending Regulations (EC) No 847/96, (EC) No 2371/2002, (EC) No 811/2004, (EC) No 768/2.*

Fediajevaite, J., Priestley, V., Arnold, R., & Savolainen, V. (2021). Meta-analysis shows that environmental DNA outperforms traditional surveys, but warrants better reporting standards. *Ecology and Evolution*, *11*(9), 4803–4815. doi: 10.1002/ECE3.7382

Ficetola, G. F., Miaud, C., Pompanon, F., & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biol. Lett*, *4*, 423–425. doi: 10.1098/rsbl.2008.0118

Fiskeristyrelsen. (2021). *Fiskeristyrelsens prøvetagningsplan for vejning af fiskevarer, der landes usorteret til industriformål (Case nr.:19-4000-000459).*

Gilbey, J., Carvalho, G., Castilho, R., Coscia, I., Coulson, M. W., Dahle, G., … Volckaert, F. A. M. (2021). Life in a drop: Sampling environmental DNA for marine fishery management and ecosystem monitoring. *Marine Policy*, *124*, 104331. doi: 10.1016/J.MARPOL.2020.104331

Goldberg, C. S., Sepulveda, A., Ray, A., Baumgardt, J., & Waits, L. P. (2013). Environmental DNA as a new method for early detection of New Zealand mudsnails (Potamopyrgus antipodarum). *Freshwater Science*, *32*(3), 792–800. doi: 10.1899/13-046.1/ASSET/IMAGES/LARGE/I2161-9565-32-3-792-F02.JPEG

Gray, C., & Kennelly, S. (2018). Bycatches of endangered, threatened and protected species in marine fisheries. *Reviews in Fish Biology and Fisheries*, *28*. doi: 10.1007/s11160-018-9520-7

Hansen, Brian Klitgaard, Bekkevold, D., Clausen, L. W., & Nielsen, E. E. (2018). The sceptical optimist: challenges and perspectives for the application of environmental DNA in marine fisheries. *Fish and Fisheries*, *19*(5), 751–768.

Hansen, Brian Klitgaard, Farrant, G. K., Ogden, R., Humble, E., Olafsdottir, G. bjorg, Bekkevold, D., … Nielsen, E. E. (2020)a. From DNA to biomass: opportunities and challenges in species quantification of bulk fisheries products. *ICES Journal of Marine Science*, *77*(7–8), 2557–2566. doi: 10.1093/ICESJMS/FSAA115

Hansen, Brian K., Jacobsen, M. W., Middelboe, A. L., Preston, C. M., Marin, R., Bekkevold, D., … Nielsen, E. E. (2020)b. Remote, autonomous real-time monitoring of environmental DNA from commercial fish. *Scientific Reports*, *10*(1). doi: 10.1038/S41598-020-70206-8

Rodríguez-Ezpeleta, N., Morissette, O., Bean, C. W., Manu, S., Banerjee, P., Lacoursiere-Roussel, A., … Deiner, K. (2021). *Trade-offs between reducing complex terminology and producing accurate interpretations from environmental DNA: Comment on "Environmental DNA: What's behind the term?" by Pawlowski et al THE TOTAL P OOL OF DNA ISOL ATED FROM THE ENVIRONMENT*. doi: 10.1111/mec.15942

Roger, F., Ghanavi, H. R., Danielsson, N., Wahlberg, N., Löndahl, J., Pettersson, L. B., … Clough, Y. (2022). Airborne environmental DNA metabarcoding for the monitoring of terrestrial insects—A proof of concept from the field. *Environmental DNA*, *4*(4), 790–807. doi: 10.1002/EDN3.290

Roungchun, J. B., Tabb, A. M., & Hellberg, R. S. (2022). Identification of tuna species in raw and processed products using DNA mini-barcoding of the mitochondrial control region. *Food Control*, *134*, 108752. doi: 10.1016/J.FOODCONT.2021.108752

Rourke, M. L., Fowler, A. M., Hughes, J. M., Broadhurst, M. K., DiBattista, J. D., Fielder, S., … Furlan, E. M. (2021). Environmental DNA (eDNA) as a tool for assessing fish biomass: A review of approaches and future considerations for resource surveys. *Environmental DNA*. doi: 10.1002/EDN3.185

Rudd, M. B., & Branch, T. A. (2016). *Does unreported catch lead to overfishing?* doi: 10.1111/faf.12181

Russo, T., Maiello, G., Talarico, L., Baillie, C., Colosimo, G., D'Andrea, L., … Mariani, S. (2021). All is fish that comes to the net: metabarcoding for rapid fisheries catch assessment. *Ecological Applications*, *31*(2), e02273. doi: 10.1002/EAP.2273

Ryan, E., Bateman, P., Fernandes, K., van der Heyde, M., & Nevill, P. (2022). eDNA metabarcoding of log hollow sediments and soils highlights the importance of substrate type, frequency of sampling and animal size, for vertebrate species detection. *Environmental DNA*, *4*(4), 940–953. doi: 10.1002/EDN3.306

Salter, I., Joensen, M., Kristiansen, R., Steingrund, P., & Vestergaard, P. (2019). Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. *Communications Biology 2019 2:1*, *2*(1), 1–9. doi: 10.1038/s42003-019-0696-8

Sampels, S. (2014). *The effects of storage and preservation technologies on the quality of fish products: a review*. doi: 10.1111/jfpp.12337

Sassoubre, L. M., Yamahara, K. M., Gardner, L. D., Block, B. A., & Boehm, A. B. (2016). *Quantification of Environmental DNA (eDNA) Shedding and Decay Rates for Three Marine Fish*. doi: 10.1021/acs.est.6b03114

Stoeckle, M. Y., Adolf, J., Charlop-Powers, Z., Dunton, K. J., Hinks, G., & Vanmorter, S. M. (2021). Trawl and eDNA assessment of marine fish diversity, seasonality, and relative abundance in coastal New Jersey, USA. *ICES Journal of Marine Science*, *78*(1), 293–304. doi: 10.1093/ICESJMS/FSAA225

Taberlet, P., Coissac, E., Hajibabaei, M., & Rieseberg, L. H. (2012). Environmantal DNA. *Molecular Ecology*, *21*(21), 1789–1793.

Tenningen, M., Zimmermann, F., & Enberg, K. (2021). Pre-catch and discard mortality in Northeast Atlantic herring and mackerel fisheries: consequences for stock estimates and advice. *ICES Journal of Marine Science*, *78*(7), 2603–2614. doi: 10.1093/icesjms/fsab135

Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P. R., & Rasmussen, M. (2012). Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *PLoS ONE*, *7*(8), 41732. doi: 10.1371/journal.pone.0041732

Thomsen, P. F., Moller, P. R., Sigsgaard, E. E., Knudsen, S. W., Jorgensen, O. A., & Willerslev, E. (2016). Environmental DNA from Seawater Samples Correlate with Trawl Catches of Subarctic, Deepwater Fishes. *PLoS One*, *11*(11), e0165252. doi: 10.1371/journal.pone.0165252

Trenkel, V. M., Huse, G., MacKenzie, B. R., Alvarez, P., Arrizabalaga, H., Castonguay, M., … Speirs, D. C. (2014). Comparative ecology of widely distributed pelagic fish species in the North Atlantic: Implications for modelling climate and fisheries impacts. *Progress in Oceanography*, *129*(PB), 219–243. doi: 10.1016/J.POCEAN.2014.04.030

Wallis, P., & Flaaten, O. (2003). Fisheries management costs: Concepts and studies. In W. Shrank, R. Arnason, & R. Hannesso (Eds.), *The Cost of Fisheries Management* (pp. 211–223). Aldershot, Hampshire, England: Ashgate Publishing.

Wood, S. A., Biessy, L., Latchford, J. L., Zaiko, A., von Ammon, U., Audrezet, F., … Pochon, X. (2020). Release and degradation of environmental DNA and RNA in a marine system. *Science of The Total Environment*, *704*, 135314. doi: 10.1016/J.SCITOTENV.2019.135314

Yates, M. C., Glaser, D. M., Post, J. R., Cristescu, M. E., Fraser, D. J., & Derry, A. M. (2021). The relationship between eDNA particle concentration and organism abundance in nature is strengthened by allometric scaling. *Molecular Ecology*, *30*(13), 3068–3082. doi: 10.1111/MEC.15543

4. eDNA-based bycatch assessment. Too good to be true or still not good enough?

Authors

Paulina Urban[1], Magnus Wulff Jacobsen[1], Dorte Bekkevold[1], Marie Storr-Paulsen[2], Anders Nielsen[3], Reindert Nijland & Einar Eg Nielsen[1]

Affiliations

[1]Section for Marine Living Resources, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark (DTU), Silkeborg, Denmark
[2]Section for Monitoring and Data, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark (DTU), Lyngby, Denmark
[3]Section for Marine Living Resources, National Institute of Aquatic Resources (DTU Aqua), Technical University of Denmark (DTU), Lyngby, Denmark
[4]Marine Animal Ecology Group, Wageningen University, Wageningen, Netherlands

Status

Draft manuscript.
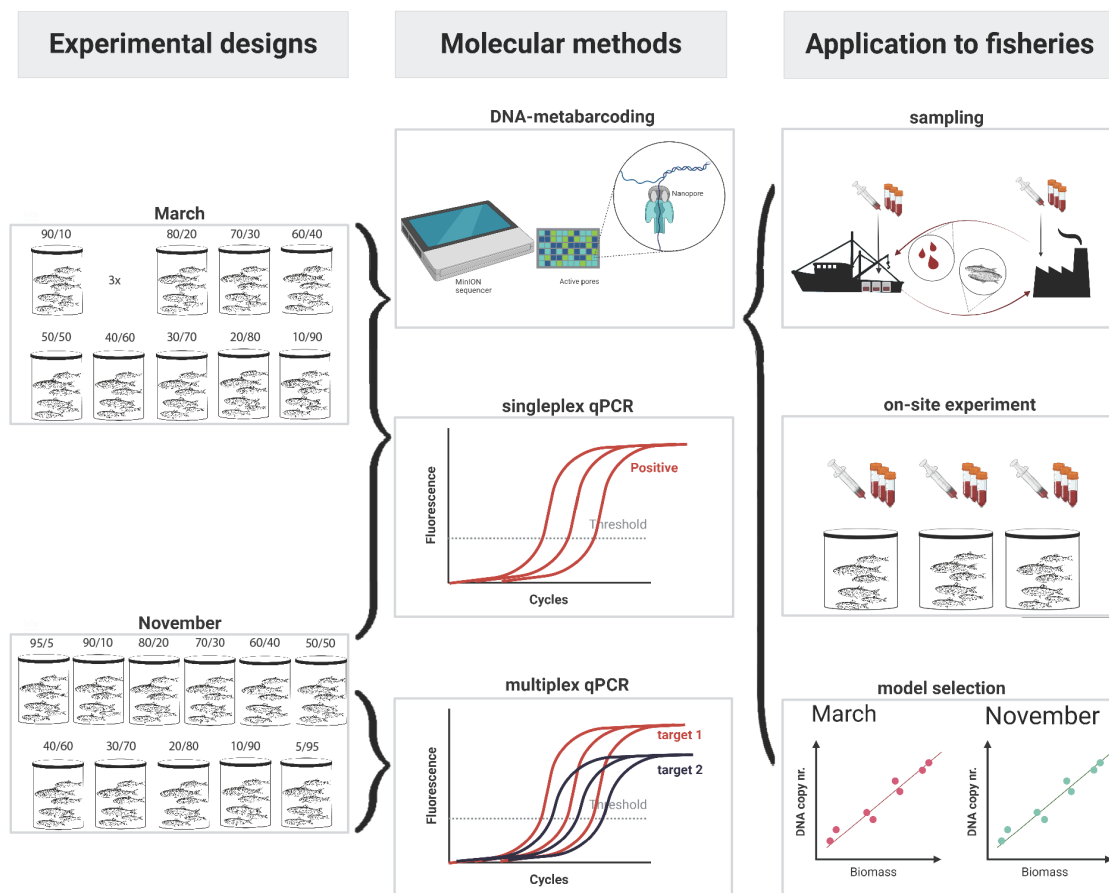
4.1 Abstract

To sustainably meet the growing demand for nutritious food, pelagic fishing is essential, but it is hampered by the lack of proper bycatch estimation methods. On the example of the pelagic sprat fisheries in the Baltic Sea, this study shows a hands-on pipeline for the application of eDNA-based methods for a quantitative assessment of bycatch. First, eDNA-to-biomass relationship models are developed based on repeated experiments measured by qPCR- and sequencing-based methods. Second, eDNA samples are collected directly from fisheries landings. For each landing, appropriate model for biomass estimation is selected according to model fit of on-site test samples performed on the catch. The eDNA-based bycatch biomass was compared to visual assessments, logbook and bucket estimates. Hereafter, repeated experiments show overall extensive comparability in the eDNA-to-biomass model. Estimates of eDNA abundance done with qPCR are most efficient, both in time and reagents costs, moreover provide the most accurate data. Herring biomass estimates vary greatly among methods, with the eDNA-based estimates being the lowest, and most precise out of all, in all landings analysed. The eDNA-based bycatch assessment method has the potential to improve the quality of fisheries data and thus in the long run the sustainable use of these precious marine resources.

4.2 Introduction

Seafood plays a vital role in meeting the growing demand for nutritional rich food and thus it is crucial to food security[1]. The main challenge remains the sustainable harvest of marine resources[2]. Because of its low impact on the ecosystem and high nutritional gains the pelagic fisheries is high on the list of "eco-friendly" solutions[3,4]. As with any fisheries, the biggest threat of a sustainable harvest is bycatch; defined as the accidental intake of non-target species[2,5]. These include species that can be landed, species unprofitable for landing, thus discarded at sea (called "discards"), and lastly charismatic, endangered, or protected species such as many sharks, rays, and sea turtles[5]. Following the increase in public awareness on the issue of bycatch and discards in the early 2000s, more and more focus was put on estimating the composition and quantity of bycaught species[5–7]. Usually the species compositions is determined during observer programs, or when possible data is collected by means of self-reporting done by the fisherman (logbook) [5,7]. Both methods can be biased as they both rely on the fisherman's cooperation (i.e. low coverage of observed vessels can be due to low cooperation of fisherman to take on fisheries observers onboard)[8] and only in a few countries other, fishermen's independent estimates can be gathered[9,10]. Because all the above are based on manual identification of species, estimating bycatch is not only labor intensive and costly, but it is error prone[11]. There are therefore both qualitative and financial incentives to develop new technical solutions for catch composition analysis in pelagic fisheries.

In recent years, environmental DNA (eDNA) became an effective and non-invasive tool for species monitoring in natural environments[12,13]. The basis of the concept is the constant release of species' DNA into their surrounding via the skin, mucus, defecation, and other processes[14,15]. Following this, the species' DNA can be collected from the environment (water, soil, air) and used for species identification. Many applications of the concept to marine management have been proposed ranging from successful monitoring of economically profitable species[16–19], rare or endangered species in the wild[20,21] or in auction houses and markets as means of battling IUU fisheries[22]. Also, quantitative species assessments [23,24], monitoring of catch composition in single hauls[25], or onboard fishing vessels for the quantification of bycatch species in industrial scaled fisheries[26,27] were studied and showed great prospects for future applicability. Yet despite the extensive offer, eDNA-based applications have received little encouragement from governmental bodies and non-governmental fisheries organizations towards implementation.

This study provides a detailed guideline on how to implement the eDNA-based method for estimating bycatch in industrial-scaled pelagic fisheries. Further, the study expands the molecular methodological toolbox for the eDNA estimation of bycatch and provides recommendations towards future application. The specific objectives were to verify i) the universality of the eDNA-to-biomass approach used for the quantitative assessment of bycatch species, through repeated experiments. ii) Test sequencing-based methods along qPCR to pinpoint at most cost-effective and reliable molecular based methods. Lastly, iii) apply the eDNA-based method for bycatch estimation, optimized for the sprat fisheries in the Baltic Sea, to real fisheries samples and compare the estimates to the ones derived from traditionally applied visual assessments (logbook and bucket estimates).

4.3   Results and discussion

Our study contributes to the understanding of the proficiency of eDNA-based methods for a reliable assessment of bycatch quantities in pelagic fisheries. This study was tailored to answer the most pressing questions for decision-makers concerning the reliability, cost efficiency, and hands-on practice of the method on board fishing vessels and factories. Results from the repeated experiments show the robustness of the eDNA-to-biomass relationship in the blood water. On average, herring eDNA fraction differed with 0.047 between blood water samples collected during the November experiment and the March experiment (Figure 4.1**a**) and on average with 0.121 in discharge water (Figure 4.1**b**). Hence, results from the blood water experiments were more comparable between the two seasons analyzed than the results from the discharge water experiments. The systematic differences in eDNA fractions observed in discharge water between the two experiments are likely caused by the changes in the experimental setup, mainly in the temperature used. The susceptibility of eDNA to temperature is well documented[32–35], even for fisheries derived samples[27]. Thus, the most likely is that the lower temperature used in November hampered both the eDNA release (production) and eDNA degradation (decay), which mainly affected the availability of herring DNA in the water, leading to lower DNA-abundances and hence, lower fractions. This was already observed when comparing blood and discharge water herring eDNA fractions, which were generated at different temperatures, for the same experimental units[27]. The sprat fisheries is ongoing in two seasons, summer[28] and winter from November until March. During the winter months, the temperature in the northern hemisphere can vary substantially, i.e. the temperature range tested in the experiments (5-7°C) is a realistic representation of a subset of the possible temperature regimes. For a routine application of the method, the striking similarity in the blood water results indicates that the eDNA-to-biomass relationship established is a general pattern. This pattern, however, needs verification for all possible temperature ranges to ensure the accuracy in estimating weight fractions from discharge water samples.
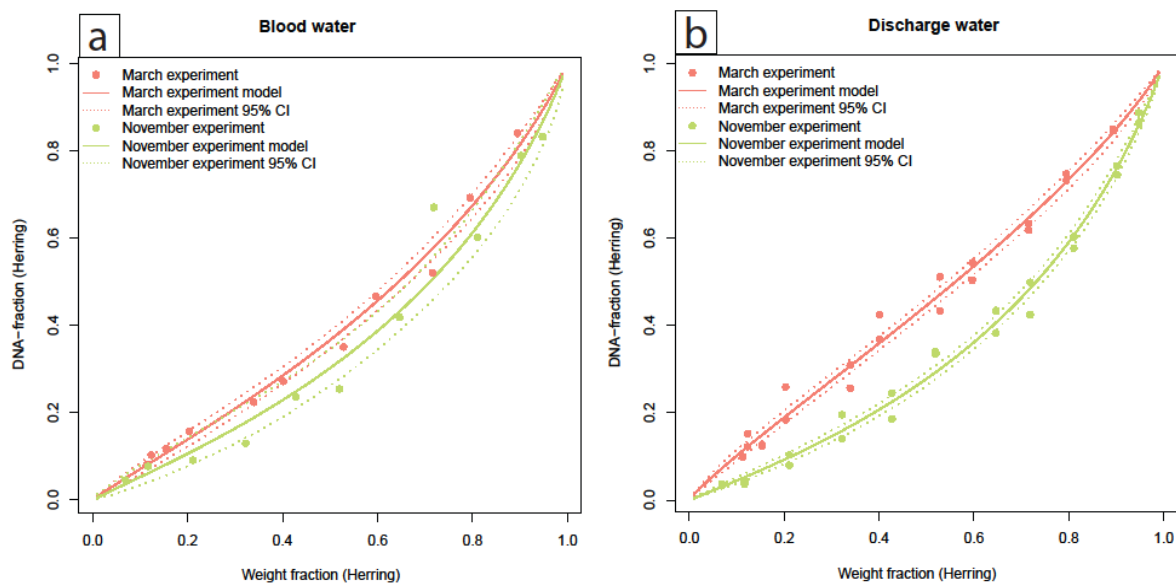
Figure 4.1. Comparison of the repeated experiments to determine the eDNA-to-biomass relationship for blood water (A) and discharge water (B). Both experiments were prepared for fishing seasons relevant to the Baltic Sea sprat fisheries (March and November). In both water typed analyzed herring DNA fraction was underrepresented in November compared to March.

The different molecular methods applied in this study show that for simple catch compositions, such as sprat and herring, the singleplex or multiplex qPCR approach is ready to be used. The eDNA fractions measured using both qPCR approaches followed tightly the weight fraction changes of the experimental units. Overall, both qPCR approaches, singleplex and multiplex, did not differ in the herring eDNA-fraction measured (repeated measures GLM, p = 0.2595). We observed that in blood water samples the measurements were mostly similar at low herring eDNA fractions (Figure 4.2**b**), whereas in discharge water they were most similar at the high herring eDNA fractions (Figure 4.2**d**). Out of the three methods applied for the estimation of the eDNA-to-biomass relationship DNA-metabarcoding proved to be the most difficult. Herring fractions estimated using DNA-metabarcoding were significantly different from both qPCR measures (repeated measures GLM, p < 0.001), with DNA-metabarcoding strongly under-representing herring eDNA fraction. Consequently, in blood water there was no clear, linear relationship between the weight fractions and eDNA fractions estimated, whereas discharge water showed a linear increase (Figure 4.2**c-d**). DNA-metabarcoding replicates of the blood water samples further indicated that there is a huge variation in the fractions estimated for the same samples (Figure 4.2**a**).
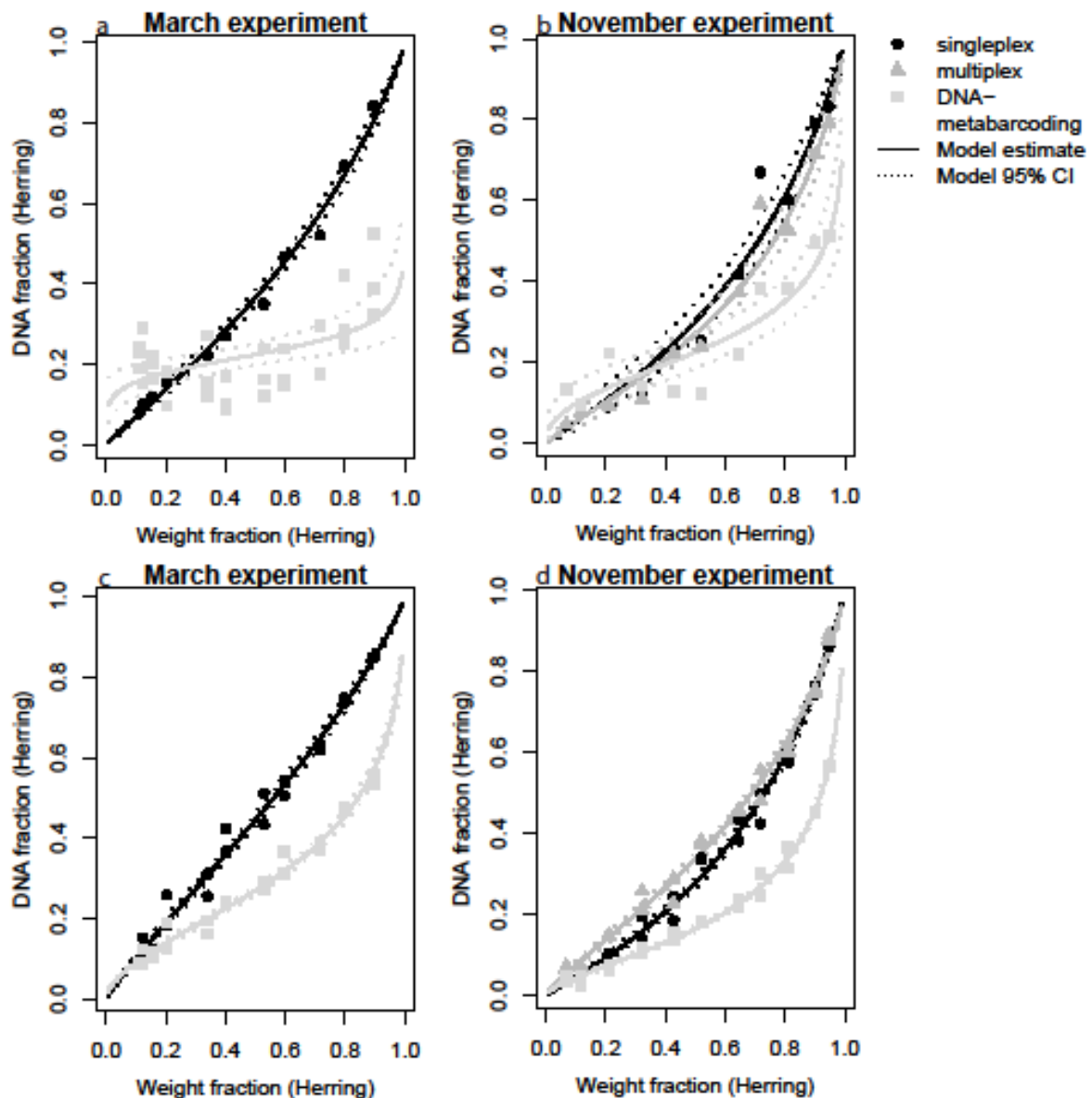
Figure 4.2. Comparison of the three molecular methods applied to the experimental samples. Both qPCR based methods (singleplex and multiplex) can be used interchangeably to derive accurate estimates of DNA fractions. Both methods differ in the cost for analyzing samples, where multiplex proves to be the cheapest option. The DNA-metabarcoding approach showed differences in performance between the two water types analyzed. It appears that reliable DNA fractions can be estimated from discharge water only.

Table 4.1. Price overview for the methods (singleplex and multiplex qPCR, DNA-metabarcoding) used in the manuscript. The price based on the time invested in, as indicated with *, was estimated using average salary (24100 kr) for a lab-technician in Denmark with working hours from 8:00-16:00, Monday to Friday, which resulted in 156.494 DKK per hour. The time estimates per tasks were estimated at our own discretion, with exception of ∘ , with time estimates defined by the supplier (Oxford Nanopore Technologies).

Singleplex

	per plate (96)			per sample
reagents	price (DKK)			price (DKK)
sum	**1866.83**			**19.45**
time	time per plate (h)	price (DKK)	time per sample (h)	price (DKK)
reaction prep.	2.00	312.99	0.17	26.08
PCR running time	3.00	469.48	1.50	234.74
average salary per hour*				
sum	**5.00**	**782.47**	**1.67**	**260.82**
Overall cost		**2649.29**		**280.27**

Multiplex

	per plate (96)		per sample (herring and sprat analysis)	
reagents	price (DKK)			price (DKK)
sum	**1700.75**			**17.72**
time	time per plate (h)	price (DKK)	time per sample (h)	price (DKK)
reaction prep.	1.00	156.49	0.08	13.04
PCR running time	1.50	234.74	1.50	234.74
average salary per hour*				
sum	**2.50**	**391.23**	**1.58**	**247.78**
Overall cost		**2091.98**		**265.50**

DNA-metabarcoding

	per run			per sample
reagents	price (DKK)			price (DKK)
sum	**3202.28**			**35.84**
time				
	time per plate (h)	price (DKK)	time per sample (h)	price (DKK)
PCR (prep)	1.00	156.49	0.08	13.04
PCR running time	2.50	391.23	2.50	391.23
Barcoding ∘	0.25	39.12	0.25	39.12
Library prep	1.25	195.62	1.25	195.62
average salary per hour (156.494 DKK)*				
sum	**5.00**	**782.47**	**4.08**	**639.02**
Overall cost		**3984.75**		**674.85**

It is difficult to pin point why the DNA-metabarcoding approach was successful for one water type only. Even if the board primers used in this study show species specific differences in the specificity of the forward primer (mlCOIintF)[29], with herring DNA having four mismatches in the primer site and sprat only one (Supporting Information 4.2) and thus leading to the observed under-representation, it is unlikely that the lack of specificity could affect one water type more than the other. Although a two-step DNA-metabarcoding approach can be considered to be a source of bias, because of the stochastic amplification process applied twice[36], we are convinced that it did not influence substantially the observed results, moreover it did not contribute to the differences observed between blood and discharge water samples as all samples were pooled in an equimolar manner prior to sequencing on the same run (Supporting Information 4.5). To our knowledge, none of the waters contains PCR inhibitors (following the IPC amplification during qPCR). Of course, for the qPCR and DNA-metabarcoding different master mixes were used, however both were developed to cope with inhibitors, and thus results are expected to be comparable[37,38].

The three molecular approaches used in this study differed in their costs per unit defined as either sample or plate/run (i.e. simultaneous analysis of 96 samples). Overall, the most costly method per sample and per plate appears to be DNA-metabarcoding with 95.7$ (674.85 DKK) per sample and 565.2$ (3984.75 DKK) per plate. This is followed by singleplex qPCR with 39.8$ (280.27 DKK) per sample and 375.8$ (2649.29 DKK) per plate (Table 4.1). The high cost of DNA-metabarcoding result from the high reagents costs, and relative long working time required, which is likely still underestimated, as the estimate does not include the time necessary for bioinformatic analysis of the data. Singleplex and multiplex qPCR, both showed strong linear relationships between the eDNA fractions and weight fractions, thus can be used interchangeably. Here costs for consumables and duration associated with the analysis will determine the most efficient method, which is the multiplex qPCR with 37.6$ (265.5 DKK) per sample and 296.7$ (2091.98 DKK) per plate (Table 4.1). The cost overview was prepared specifically for this study, where only two species were targeted for identification and quantification. This assessment is in accordance with other findings, where qPCR proved to be the cheapest choice for the identification of a limited amount of species in samples[39–42]. An additional, positive "side" effect of multiplexing is that precious eDNA samples are saved, compared to when used in singleplex analyses[43], thus can be used for additional analyses if needed. In case of a more diverse catch, the cost overview is expected to shift substantially. With an increasing number of targets (i.e. species) for the analysis, price and time are expected to increase rapidly. Hence, for eDNA samples from diverse fisheries, DNA-metabarcoding could prove to be the more cost-efficient method[41]. The advantage of DNA-metabarcoding is additionally that it does not require prior knowledge of the species composition[41]. This advantage is particularly crucial in the case of identification and quantification of bycatch species, as their appearance in a catch can be unpredictable, such as the bycatch of charismatic or rare species[5]. However, before DNA-metabarcoding can be applied to fisheries samples, the issues of unspecific amplification and the lack of clear eDNA-to-biomass relationships needs to be resolved.

In terms of the eDNA based method to fisheries, we show the usefulness of a detailed, full-spectrum model developed for each fishery and fishing season, and highlight that on a landing-to-landing base adequate model selection can be archived through a simple, on-site test sample prepared from the unloaded catch (Figure 4.3). Accordingly, the model derived from the March experiment predicted the weight fractions of the "on-site" test sample in landing 1 and landing 3 best (Figure 4.3). In landing 2, both models overestimated the herring weight fractions, in landing 4 the true weight fraction was between the two model estimates (Figure 4.3). The difficulty in selecting the appropriate model for landing 2 was very likely caused by the very mature stage of the catch (18 days old) compared to other landings landed in half the time on average. Landing 2, unlike other landings and the experimental

setup, was not kept in seawater during the fishing process (hence no blood water was generated). We speculate that a strong decomposition of the fish following both, the duration and storage of the catch, skewed the eDNA-to-biomass relationship substantially. In total, it appears that the model derived from the March experiment performed better in the estimation of weight fractions for all landings than the model derived from the November experiment (Figure 4.3, Supporting Information 4.3), and thus it was chosen for the estimation of weight fractions from discharge water samples collected during the landing. Model selection for blood water collected from the ships is straightforward as both models provide very comparable results (Supporting Information 4.4). The fishery samples were collected mid January to mid February, hence we decided to apply the model based on the March experiment as it seems to be the most relevant for the time of sampling.
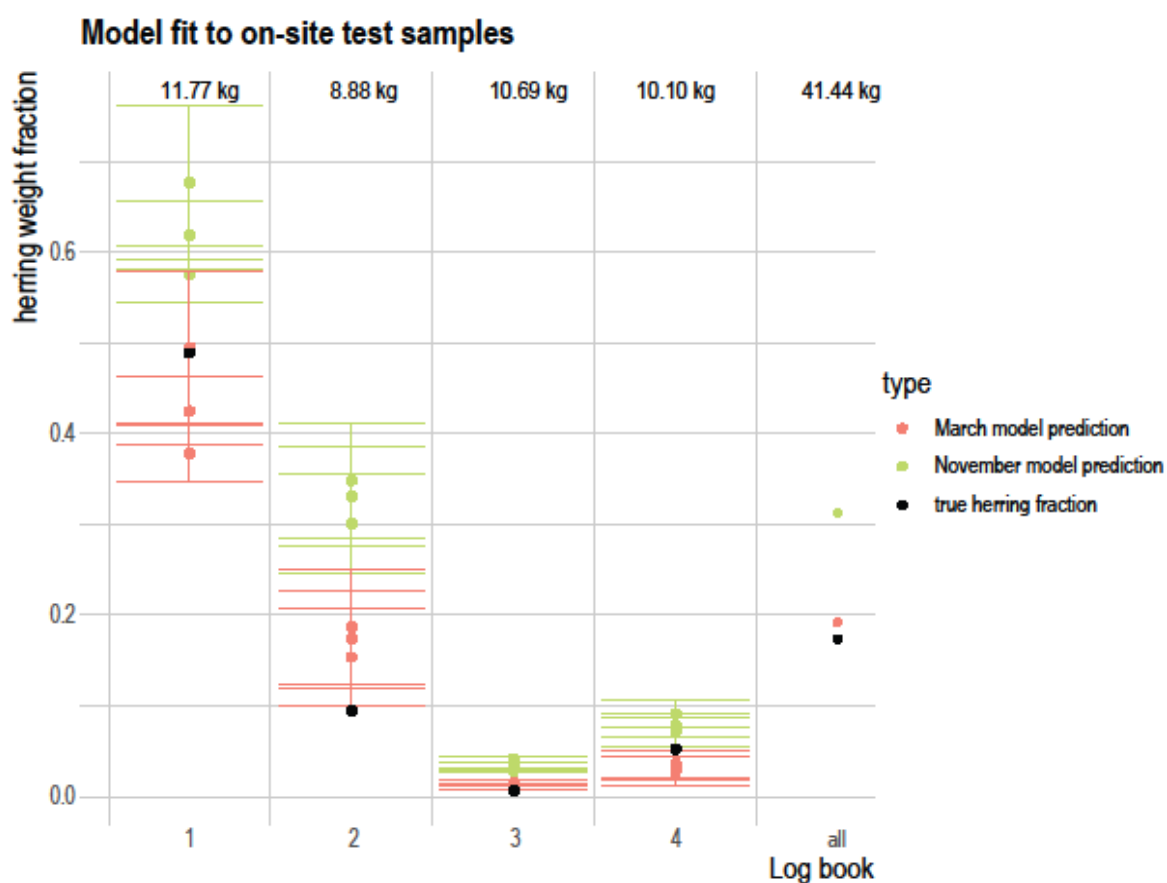
Figure 4.3. Shows the fit of each model March (salmon) and the November model (green), to on-site test samples collected at each landing and compares the predicted weight fraction to the true herring fraction found in the total on-site test sample (black). Each on-site test sample was collected in 3 replicates (each between 2-4 kg of the catch), that were first rinsed with freshwater and then sampled after 10 min, 2h and 4h. Each point in the graph represents the mean fraction of all 3 replicates for each time per landing with a standard deviation. The weight numbers on top show the total amount of catch used for the on-site test sample.

In all the landings analysed, the eDNA-based assessment lead to the lowest herring biomass reported and the highest precision in the estimates out of all methods applied (Figure 4.4, Table 4.2). The only exception here is the blood water samples analysed in landing 4. Since the true total composition is unknown, the accuracy of each of the methods can not be assessed directly. Overall in landing 1, the genetic estimates differ the most to the bycatch estimates reported in the logbook and by the fisheries control (Figure 4.4, Table 4.2). The genetic based estimates are more similar to one another (difference between blood and discharge water is 30 t), then the visual method for the assessment: bucket

estimates and logbook estimates differ by 150 t, bucket and fisheries control values by 52 t (Table 4.2). Comparisons to the logbook directly should be made carefully as two different logbook values were reported for this landing. In landing 2, difference between the genetic and the bucket estimate was 163 t (Table 4.2) and the difference to the logbook was 231 t. The visual methods showed greater comparability to one another as seen in landing 1 (difference between bucket and logbook estimates is 68 t), however the standard error of both is higher (Table 4.2).

Table 4.2. Overview of estimates of bycatch per method for the four landings investigated in this study. Estimates from logbook, fisheries control and bucket are derived following the visual estimation of bycatch from subsamples. For each method, we provide the end estimate of bycatch in kilograms (kg) of herring and as fraction of the total catch. Sample size for logbook assumed to be one estimate for haul, i.e. sample size equals amount of hauls.

| Lan-ding nr.	Method	Herring bycatch estimate (kg)	Herring bycatch standard error (kg)	Herring bycatch estimate (fraction)	Sample size
1	logbook	622059.9	63786.4	0.471	12
1	logbook_corr	545254	NA	0.400	NA
1	fisheries control	523892.7	23599.2	0.385	63
1	bucket	472294.4	57564.5	0.348	15
1	Blood water (eDNA)	404349.5	44647.7	0.297	5 (á 3 rep.)
1	Discharge water (eDNA)	374351.3	26874.8	0.275	8 (á 3 rep.)
2	logbook	744875	114250.4	0.492	18
2	bucket	676864.1	76759.3	0.447	15
2	Blood water (eDNA)	NA	NA	NA	NA
2	Discharge water (eDNA)	513988.3	26874.8	0.339	10 (á 3 rep.)
3	logbook	558857.1	208106.3	0.388	6
3	bucket	306446.3	117645.1	0.213	15
3	Blood water (eDNA)	529790.3	58692.6	0.368	8 (á 3 rep.)
3	Discharge water (eDNA)	237658.5	42563.9	0.165	8 (á 3 rep.)
4	logbook	546891	133263.7	0.441	10
4	bucket	604850	88174.1	0.488	15
4	Blood water (eDNA)	761963.7	109783.9	0.614	6 (á 3 rep.)
4	Discharge water (eDNA)	215405.6	30617.1	0.174	8 (á 3 rep.)
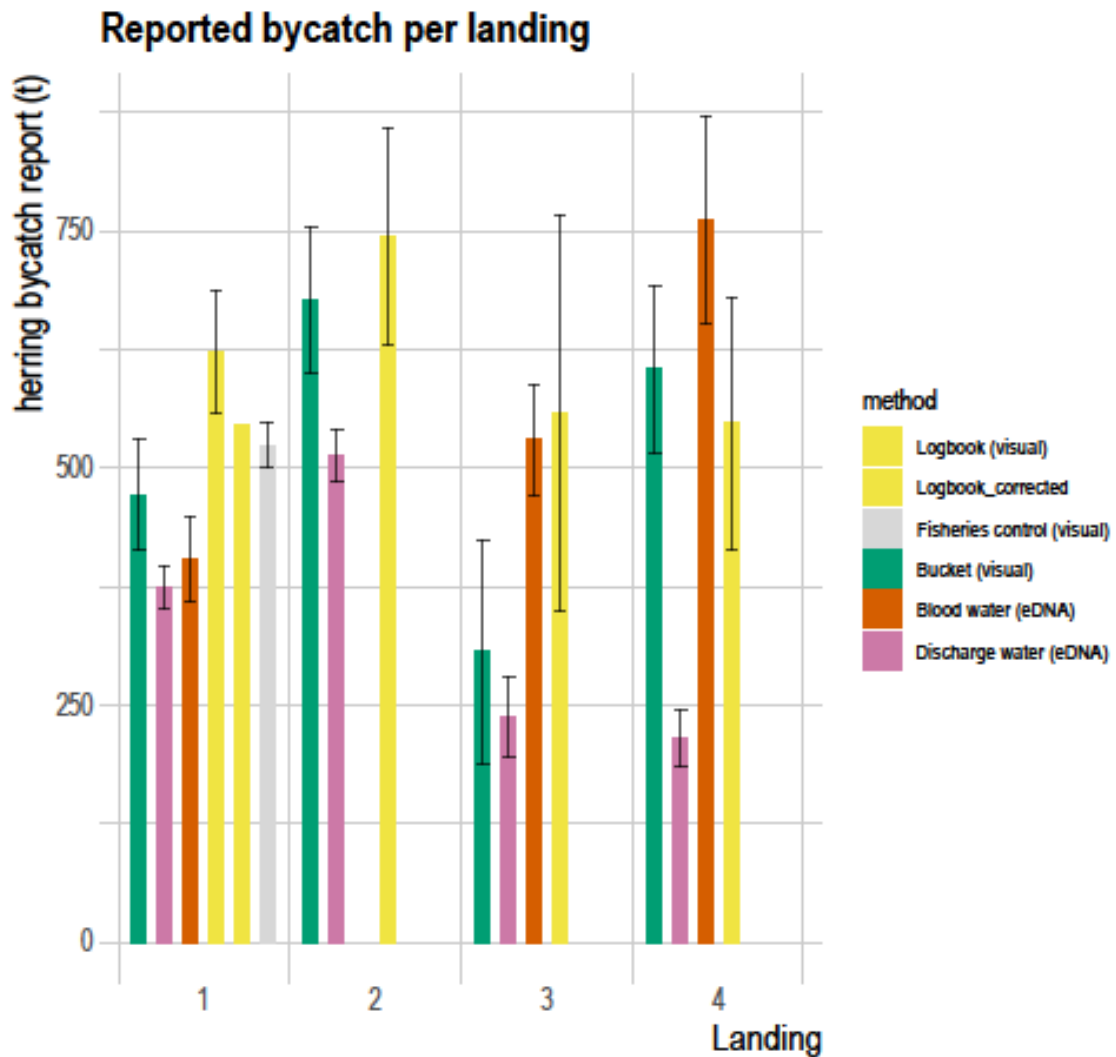
Figure 4.4. Overview of the herring bycatch estimated for each landing assessed using different methods. The catch from landing 1 provides the greatest amount of estimates. Besides of the shared estimates, hence the genetic ship and factory estimates, the logbook and the bucket estimates we obtained estimates form the fisheries control agency that inspected the catch from landing 1. The logbook data from landing 1 reported two different values, both are highlighted in this graph. For landing 2 no genetic samples of the blood water from the ship could be obtained, hence only one genetic based estimate is provided. The error bars on the bar chars represent the standard error of the estimated bycatch rates per method.

In landing 3 and 4 the genetic estimates based on blood and discharge water show quite deviating results (Figure 4.4). The difference between the blood water and the discharge water estimates in landing 3 is 292.13 T and in landing 4 it is 546.56 T (Table 4.2). At both landings, the blood water eDNA estimates were not collected at the very end of the fishing, but soon before the last catch was made (Figure 4.4). The discharge water eDNA estimate in landing 3 compares well to bucket estimates (69 t difference), blood water eDNA on the contrary compares better with the logbook estimate (29 t difference). In landing 4 the blood water eDNA estimate compares better with both visual estimates (logbook and bucket) then with the discharge water estimates. The discrepancy between the

logbook and genetic ship fraction for landing 3 and 4 could very likely be due to the fact that at the time point of the genetic sampling the whole catch was not made. Also, the distribution of bycatch rich hauls and bycatch poor hauls in separate tanks (Figure 4.5, Supporting Information 4.10) could play a role. If the size of the tanks that hold the bycatch rich or pool fractions would be known, then the genetic values could be corrected, as it has been shown that accounting for mixing during the unloading process and the size of the catch inside each tank improves the bycatch end estimates[26]. However, due to lack of the necessary information it was not possible to account for it.
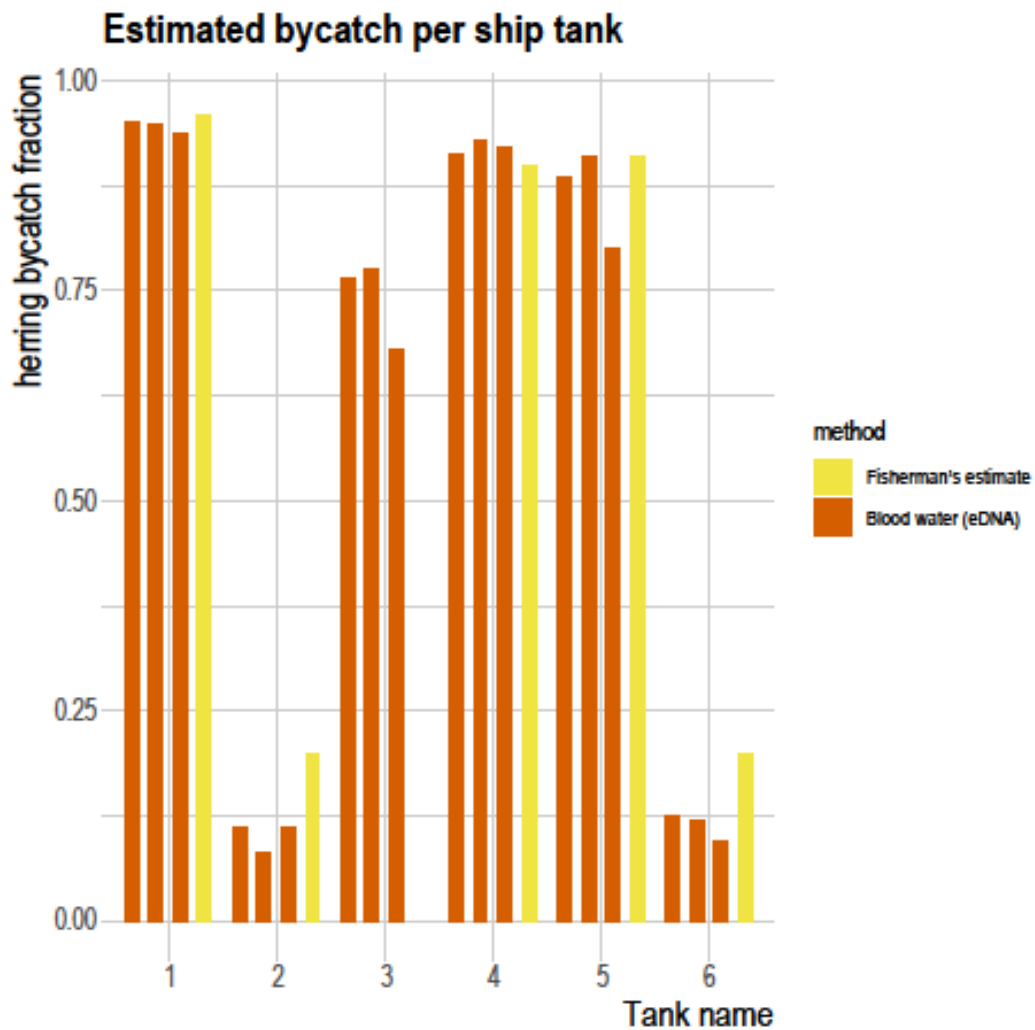
Figure 4.5. Detailed overview of the herring fractions estimated for each tank holding fish in landing 4. The genetic ship estimates (orange) are compared to additional fisherman's estimates. For all tanks the estimates show substantial overlap.

Table 4.3. Comparison of two experiments performed simulating the Baltic Sea sprat fisheries and aimed at establishing the eDNA-to-biomass relationship. Overall, the experiments show great similarities in the targeted mock samples, catchment area, and experimental facility. The main difference is the seasons studied and the temperature profiles (same temperature in blood water experiments, however the November- experiment was shortly interrupted by a power outage, slightly lower temperatures during the discharge water experiment in November).

	March experiment	November experiment
Targeted mock samples	90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90	95/5 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, 5/95
Season	March	November
Seawater take up	015°19'367 E 54°48'730 N	016°33'584 E 55°49'695 N
Seawater salinity	~ 10 PSU	~ 9 PSU
Catch place fish	015°35'111 E 54°36'775 N Bornholm Basin (South of Bornholm)	016°28'131 E 55°51'892 N Bornholm Basin (North of Bornholm, close to Öland)
Blood water experiment duration	8	7 (power cut on day 6, increase in temp.)
Blood water experiment place	RV DANA	RV DANA
Discharge water experiment duration	18h	18h
Discharge water experimental condition	6-7 °C, ~ 0 PSU	5-6 °C, ~ 0 PSU
Discharge water experiment place	10 min away from the ship landing place	2h away from the ship landing place
Fish size	Large size difference between herring and sprat	Small size difference
Methods for DNA analysis	Singleplex qPCR, DNA-metabarcoding	Singleplex qPCR, Multiplex qPCR, DNA-metabarcoding

Even though the overall composition of the total catch is unknown, the evaluation of the genetic method's accuracy and precision can be done partially when comparing the fisherman's weight fraction estimates and the eDNA derived weight estimates for the same tanks in landing 4 (Figure 4.5, Supporting Information 4.9). The similarity between the fisherman's estimation and the genetic method is striking. Not least due to the overall high precision when comparing the three genetic replicates collected from each tank. The similarity in per tank estimates of both methods unfortunately does not translate directly to a similar end estimate of herring bycatch, which is caused by the lack of the fisherman's report for the tank 3.

The consolidated knowledge of the eDNA-to-biomass relationship and the new understanding gained through the replication of the eDNA-to-biomass experiment and the application of other molecular

tools enhance the level of trust for the accuracy and precision of the eDNA-based method. This method is ready to be used on simple mix compositions, where the eDNA fractions are derived from quantitative PCR methods. The challenge associated with the usage of DNA-metabarcoding in these samples is a fruitful area for future work necessary to be able to apply the eDNA-based bycatch estimation beyond catches with few bycatch species. With the straightforward understanding of the system already, the method's application in parallel to the traditional ways of estimating bycatch in fisheries would be most desired to further ground-truth and familiarize the stakeholders with the new method. In the long run, improved estimates of bycatch quantities gained using the eDNA-based approach would improve the quality of fisheries derived products, through improved traceability of the product composition, quality of assessment models which ensure exploitation within biological sound limits and thus extensively contribute to a sustainable pelagic fishery.

4.4 Material and Methods

4.4.1 General description of the fishery process

Industrial pelagic catches commonly constitute more than 1000 tons (Nøttestad et al., 2016). To maintain fish quality the catch is kept in pre-cooled natural seawater in onboard tanks before landing (del Valle & Aguilera, 1991). This pre-cool seawater is referred to as "blood water". Before transferring the catch from the boat to the processing factory on land the blood water is drained and freshwater used for the transfer of the catch. In contrast to the blood water, this freshwater (hereafter discharge water) is in contact with the catch for a relative short time (2-10 h). In order to comply with the Landing Obligation, the species composition of each sprat landing is estimated and reported by a logbook and in some countries also by the bucket method[28]. Both methods rely on the subsampling of the catch at different intervals, the identification and quantification of the species from the subsamples[26]. Additionally during the landing process the total catch can be inspected by fisheries control that inspects the catch following the same principle as the bucket method and logbook.

4.4.2 Experiment: weight to eDNA relationship

We used data from two experiments on "mock" fishery samples prepared to mimic the Baltic Sea sprat fishery. The mock samples consisted different mixtures of sprat and herring prepared during the Baltic International Trawl Survey (BITS) on the research vessel DANA in March 2021[27] and in November 2021 (Table 4.3). The experiments were conducted at almost exacts same conditions. Table 4.3 presents a number of similarities and some differences between the experimental set ups.

Each experimental unit in each experiment was subject to two different treatments 1) blood water, 2) discharge water. The blood water experiment was set up while at sea and blood water was sampled upon arrival at the harbor after the end of the cruise. The sampling consisted of collecting 45 ml of blood water into sterile falcon tubes (Sarstedt, 50 ml), using a sterile syringe (Codan™, 60 ml). The falcon tubes were immediately frozen at -20 °C and kept until DNA-extraction. The experimental units were then transported to the lab, blood water and fish were separated using a mosquito net (mesh size: 1.4 x 1.6 mm) to avoid larger tissue particles. After draining, the fish were returned to the experimental containers double wrapped with new plastic bags (Cater Line, Freezer bags, 40 L). To simulate the discharge process, 2 L of freshwater (tap water) was added to each of experimental container and stirred manually to ensure mixing. Plastic bags were closed to prevent possible cross-contamination and the containers were stored at 5-7 °C for the subsequent 18h to simulate the natural discharge process from ship to factory. The discharge water was sampled at two different time points: 2h, and 18h. All discharge samples were collected in 3 ml tubes (Sarstedt, 57x15.3 mm) using a sterile syringe (Injekt®, 20 ml). All units were stirred at regular intervals and before sampling, to ensure full mixed samples. After collection, samples were frozen at -20 °C until DNA-extraction.

4.4.3   eDNA sampling of the pelagic sprat fishery "On-site" test samples

In order to decide which model to use to most precisely translate the eDNA fractions measured in samples to weight fractions, at each landing of sprat we collected "on-site" test samples. Each test sample consisted of three replicates of the catch (2-4 kg of catch), which were rinsed with freshwater three times before starting the experiment. The experiment started with pouring freshwater on top of the catch-subsample in defined proportion (30% of water for 70% of catch, i.e. approx. 300 ml of freshwater for 1kg of subsampled catch). The mixture was thoroughly mixed and the discharge water consequently sampled at 10min, 2h and 4h. The discharge water was sampled in triplicates, each replicate (up to 45 ml) was collected into a sterile 50 ml falcon tube (Sarstedt, Screw cap tube, 50 ml) using a sterile 60 ml syringe (Codan™). All samples were kept on ice during sampling and frozen at -20 °C immediately after the end of the landing, until DNA-extraction. After eDNA sampling the fish were visually identified and weight to determine the weight fraction in each replicate of the "on-site" test sample.

4.4.4   Sampling the fisheries

Blood and discharge water samples were collected from four different landings of the sprat fishery from January 2021 until February 2021. All catches were harvested from different locations in the Baltic Sea (Supporting Information 4.7). Each sampling started with collecting blood water from each holding tank of the ship. Hereby three replicates (one sample = three replicates) from each holding tank were collected. Once the landing facility stared with the discharge (landing of the catch from the ship to a land-based factory) we started with sampling the discharge water, in triplicates at regular intervals (every 200 T). We sampled up to 50 ml of blood or discharge water into a sterile 50 ml falcon tube (Sarstedt, Screw cap tube, 50 ml) using a sterile 60 ml syringe (Codan™). All samples were kept on ice during sampling and frozen at -20C immediately after the end of the landing. Logbook information and the bucket method estimates for each landing were recorded.

4.4.5   DNA-extraction

Before extraction, the samples were centrifuged at 3700 rpm for 30s to minimize the chance of extracting tissue particles present in water. 1 ml of water was used for the extraction of DNA with the Omega Bio-tek E.Z.N.A. Tissue DNA kit (Omega Bio-tek, USA) following an adjusted version of their standard "tissue DNA protocol", i.e., using a 2.5x volume of buffers and solutions to adjust for the large sample volume. Samples were eluted in 50 µl pre-heated elution buffer, and stored at -20°C.

4.5   Genetic Analyses

4.5.1   Singleplex species-specific qPCR

Species-specific sprat[27] and herring[19] qPCR assays targeting the cytochrome b sequence of the mitochondrial DNA (mtDNA) were used for DNA quantification. Both assays were tested and validated in vitro in relation to assay optimization (primer and probe concentration adjustment), specificity (testing assay performance on closely related, co-occurring species and sensitivity with determination of LOD (Limit Of Detection) and LOQ (Limit Of Quantification)[27]. All samples were analysed in duplicates on the StepOne Real-Time PCR System (Life Technologies, USA) with triplicate negative controls and triplicated standard curve ranging from 3 x 106 to 3 x 100 copies/reaction in each run. Total volume of each reaction was 10 µl with 3 µl of sample, 4 µl TaqMan™ Environmental Master Mix 2.0 (Thermo Fisher Scientific), assay-specific volumes of primers and probes to obtain optimal reaction conditions[27] and 1.2 µl TaqMan™ Exogenous Internal Positive Control Reagents (Thermo Fisher Scientific) to monitor potential inhibition. The qPCR consisted of 5°C for 5 min and 95°C for 10 min followed by 50 cycles at 95°C for 30 s and 60°C for 1 min. Species-specific estimates of DNA copy numbers were then used to calculate herring and sprat fractions i.e. herring DNA copy number to total DNA copy number (sum of herring and sprat DNA copy) ('DNA-based fractions').

4.5.2 Multiplex species-specific qPCR

For the multiplexing qPCR the herring probe was modified TAMRA-dye to separate its fluorescence signal from the FAM-dyed sprat probe. An internal positive control (hereafter IPC) dyed with VIC was used in each reaction to monitor inhibition. We used the VetMAX Xeno Internal Positive Control containing BHQ-3 quencher (Applied Biosystems). The PCR settings for reagents and thermo cycler set up were the same as in the singleplex reaction (see above). Multiplexing was performed only on the November experimental samples (Table 4.3).

4.5.3 DNA-metabarcoding

The DNA-metabarcoding approach followed a 2-step PCR process. The first PCR amplified the Leray fragment (forward, mlCOIintF: GGWACWGGWTGAACWGTWTAYCCYCC[29], reverse, jgHCO2198: TANACYTCNGGRTGNCCRAARAAYCA)[30]. Each sample was PCR amplified using 10µL 2x Phire Tissue Direct PCR Master Mix (ThermoFisher Scientific, USA), 0.5µL of each primer (10nM), 2 µL template DNA and 7 µL DNA free water. The termocycler settings consisted of initial denaturation at 98°C for 180s, 35 cycles of 98°C for 10s, 50°C for 10s, 72°C for 20s followed by a final extension at 72°C for 180s. The second PCR was used to attach a unique sample-tag to each sample that would allow sample pooling and sequencing on the same run. The second PCR was performed using the PCR barcoding kit 96 (PCB-096) (Oxford Nanopore Technologies Ltd., UK). We followed the manufacturer's protocol, hence each sample was PCR amplified in total volume of 15µL containing 12.5µL LongAmp® master mix (New England BioLabs®), 0.5µL PCR barcode primer and 1µL amplicon (from the first PCR) and 11µL of DNA-free water. The cycling conditions consisted of initial denaturation at 95°C for 180s, 15 cycles of 95°C for 15s, 62°C for 15s, 65°C for 90s, and final extension at 65°C for 180s. Three replicates of the blood water samples collected from the March experiment were amplified and sequence separately to assess the effect of PCR amplification on the results. All PCR products were visually inspected on a 1% agarose gel. Sequencing was performed on a MinION Mk1C using R.10 flow cells and sequencing ligation kit SQK-LSK-112 (Oxford Nanopore Technologies Ltd., UK). All samples were pooled in equimolar rations prior to the library preparation step. All samples were run for 8h, with a total amount of reads of 1.4M for the first library (96 barcodes), and 2.5 M for the second library (36 barcodes). Raw reads were basecalled in Guppy (Version 6.1.1, Oxford Nanopore Technologies Ltd., UK), using super accuracy (SUP) mode. The performance of the runs (i.e. pore activity, pore availability, sequence length distribution) was visually inspected using Nanoplot (https://github.com/wdecoster/NanoPlot). The raw sequences were filtered to for lengths between 340 and 380 base pairs (bp) using decona (version 1.3, https://github.com/Saskia-Oosterbroek/decona). The FASTQ filtered files were then processed using Geneious Prime Software (Version 2021.2, Kearse et al. 2012). For each barcode, the sequences were classified against whole mtDNA genomes of herring and sprat. We chose a sequence overlap identity at min. 80% of 340 bp, from which 85% similarity was needed for sequence identification at species, 82% similarity for genus and 80% similarity at family level was selected. The DNA-metabarcoding approach was applied to experimental samples from both, March and November experiment (Table 4.3).

The accuracy of the DNA-metabarcoding approach was tested on eleven mock samples of sprat/herring amplicons (95/5, 90/10, 80/20, 70/30, 60/40, 50/50, 40/60, 30/70, 20/80, 10/90, 5/95) (Supporting Information 4.5).

4.5.4 Cost overview

An overview of the costs for reagents and manual labor for each of the molecular methods applied in this manuscript was created (Table 4.1). The overview consists only of the costs associated with the analysis itself, hence the costs for eDNA sampling and eDNA extraction are not considered. The estimation of manual labor costs was done by estimating the extent of time necessary per tasks first, and

then calculating the price per estimated time, based on the average salary (24100 kr) for a lab-technician in Denmark with working hours from 8:00-16:00, Monday to Friday. The extent of each task was estimated at our own discretion, with the exception of the DNA-metabarcoding steps performed with time estimates provided by the supplier (Oxford Nanopore Technologies).

4.5.5 Data analysis

Each experimental data served for establishing an eDNA-to-biomass model (one model per experiment and one model per method). For that generalized linear mixed models (GLM) built using the package *glmmTMB* 1.0.2.9 (Brooks et al., 2017) were used. The DNA quantities estimated from sprat and herring were converted into fractions; hence the genetic observations are continuous numbers between 0 and 1, naturally described by a beta distribution:

$O_i \sim$ Beta(μ_i, ϕ) independent where:

logit (μ_i) = $\alpha+\beta$ logit(true weight fraction*i*)

The model parameter ϕ is a precision parameter scaling the variance of O_i as $Var(O_i) = \mu_i(1 - \mu_i)/(1 + \phi)$. The logit-scale genetic fractions (O_i) are described as a linear function of the logit transformed true weight-based fraction.

To decide for the most suitable herring DNA fraction to biomass model the fit of the model estimates was compared to true measurements of fractions from simple on-site experiments performed on the catch-subsamples.

4.6   References

1.   FAO. The State of the Worlds Fisheries and Aquaculture 2022. Towards Blue Transformation. https://doi.org/10.4060/cc0461en (2022) doi:10.4060/cc0461en.

2.   Booth, H., Arlidge, W. N. S., Squires, D. & Milner-Gulland, E. J. Bycatch levies could reconcile trade-offs between blue growth and biodiversity conservation. *Nat. Ecol. Evol. 2021 56* **5**, 715–725 (2021).

3.   Bianchi, M. *et al.* Assessing seafood nutritional diversity together with climate impacts informs more comprehensive dietary advice. *Commun. Earth Environ. 2022 31* **3**, 1–12 (2022).

4.   Gephart, J. A. *et al.* Environmental performance of blue foods. *360 | Nat. |* **597**, (2021).

5.   Pérez Roda, M. A. et al. A third assessment of global marine fisheries discards. FAO Fisheries and Aquaculture Technical Paper vol. 633 (2019).

6.   Borges, L. The evolution of a discard policy in Europe. *Fish Fish.* **16**, 534–540 (2015).

7.   Quirijns, F. & Pastoors, M. *Discard Atlas of North Sea fisheries*. http://www.seafish.org/media/1337320/discardatlas_northsea_demersalfisheries_2014.pdf (2014).

8.   Benoît, H. P. & Allard, J. Can the data from at-sea observer surveys be used to make general inferences about catch composition and discards? *Can. J. Fish. Aquat. Sci.* **66**, 2025–2039 (2009).

9.   Fiskeristyrelsen. Fiskeristyrelsens prøvetagningsplan for vejning af fiskevarer, der landes usorteret til industriformål (Case nr.:19-4000-000459). (2021).

10. Faunce, C. H. & Barbeaux, S. J. The frequency and quantity of Alaskan groundfish catcher-vessel landings made with and without an observer. *ICES J. Mar. Sci.* **68**, 1757–1763 (2011).

11. Fischer, J. Fish identification tools for biodiversity and fisheries assessments. Review and guidance for decision-markers. *FAO Fish. Aquac.* 107 pp. (2013).

12. Thomsen, P. F., Kielgast, J., Iversen, L. L., Møller, P. R. & Rasmussen, M. Detection of a Diverse Marine Fish Fauna Using Environmental DNA from Seawater Samples. *PLoS One* **7**, 41732 (2012).

13. Boussarie, G. *et al.* Environmental DNA illuminates the dark diversity of sharks. *Sci. Adv.* **4**, eaap9661 (2018).

14. Taberlet, P., Coissac, E., Hajibabaei, M. & Rieseberg, L. H. Environmantal DNA. *Mol. Ecol.* **21**, 1789–1793 (2012).

15. Rodríguez-Ezpeleta, N. *et al.* Trade-offs between reducing complex terminology and producing accurate interpretations from environmental DNA: Comment on 'Environmental DNA: What's behind the term?' by Pawlowski et al THE TOTAL P OOL OF DNA ISOL ATED FROM THE ENVIRONMENT. (2021) doi:10.1111/mec.15942.

16. Canals, O., Mendibil, I., Santos, M., Irigoien, X. & Rodríguez-Ezpeleta, N. Vertical stratification of environmental DNA in the open ocean captures ecological patterns and behavior of deep-sea fishes. *Limnol. Oceanogr. Lett.* **6**, 339–347 (2021).

17. Thomsen, P. F. *et al.* Environmental DNA from Seawater Samples Correlate with Trawl Catches of Subarctic, Deepwater Fishes. *PLoS One* **11**, e0165252 (2016).

18. Stoeckle, M. Y. *et al.* Trawl and eDNA assessment of marine fish diversity, seasonality, and relative abundance in coastal New Jersey, USA. *ICES J. Mar. Sci.* **78**, 293–304 (2021).

19. Knudsen, S. W. *et al.* Species-specific detection and quantification of environmental DNA from marine fishes in the Baltic Sea. *J. Exp. Mar. Bio. Ecol.* **510**, 31–45 (2019).

20. Truelove, N., Andruszkiewicz, E. & Block, B. A rapid environmental DNA method for detecting white sharks in the open ocean. *Methods Ecol. Evol.* **10**, 1128–1135 (2019).

21. Nester, G. M. *et al.* Characterizing the distribution of the critically endangered estuarine pipefish (Syngnathus watermeyeri) across its range using environmental DNA. *Environ. DNA* **5**, 132–145 (2023).

22. Richards, J. L. *et al.* Development of an eDNA-based survey method for urban fish markets. *Methods Ecol. Evol.* **13**, 1568–1580 (2022).

23. Salter, I., Joensen, M., Kristiansen, R., Steingrund, P. & Vestergaard, P. Environmental DNA concentrations are correlated with regional biomass of Atlantic cod in oceanic waters. *Commun. Biol. 2019 21* **2**, 1–9 (2019).

24. Shelton, A. O. *et al.* Environmental DNA provides quantitative estimates of Pacific hake abundance and distribution in the open ocean. (2022) doi:10.1098/rspb.2021.2613.

25. Russo, T. *et al.* All is fish that comes to the net: metabarcoding for rapid fisheries catch assessment. *Ecol. Appl.* **31**, e02273 (2021).

26. Urban, P. *et al.* Using eDNA to estimate biomass of bycatch in pelagic fisheries. *Environ. DNA* (2022) doi:10.1002/edn3.377.

27. Urban, P. *et al.* Scaling from eDNA to biomass: controlling allometric relationships imporves precision in bycatch estimation. *ICES J. Mar. Sci.* **0**, 1–13 (2023).

28. Lassen, H. Industrial Fisheries in the Baltic Sea. (2011).

29. Leray, M. *et al.* A new versatile primer set targeting a short fragment of the mitochondrial COI region for metabarcoding metazoan diversity: application for characterizing coral reef fish gut contents. *Front. Zool.* **10**, 34 (2013).

30. Geller, J., Meyer, C., Parker, M. & Hawk, H. Redesign of PCR primers for mitochondrial cytochrome c oxidase subunit I for marine invertebrates and application in all-taxa biotic surveys. *Mol. Ecol. Resour.* **13**, 851–861 (2013).

31. Brooks, M. E. *et al.* glmmTMB Balances Speed and Flexibility Among Packages for Zero-inflated Generalized Linear Mixed Modeling. *R J.* **9**, 378–400 (2017).

32. Andruszkiewicz Allan, E., Gordon Zhang, W., Lavery, A. C. & Govindarajan, A. F. Environmental DNA shedding and decay rates from diverse animal forms and thermal regimes. *Environ. DNA* 492–514 (2021) doi:10.1002/edn3.141.

33. Jo, T. & Minamoto, T. Complex interactions between environmental DNA (eDNA) state and water chemistries on eDNA persistence suggested by meta- analyses. *Mol. Ecol. Resour.* 1490–1503 (2021).

34. Saito, T. & Doi, H. A Model and Simulation of the Influence of Temperature and Amplicon Length on Environmental DNA Degradation Rates: A Meta-Analysis Approach. *Front. Ecol. Evol.* **9**, 139 (2021).

35. Lamb, P. D., Fonseca, V. G., Maxwell, D. L. & Nnanatu, C. C. Systematic review and meta-analysis: Water type and temperature affect environmental DNA decay. *Mol. Ecol. Resour.* (2022) doi:10.1111/1755-0998.13627.

36. Shelton, A. O. *et al.* Toward quantitative metabarcoding. *Ecology* (2022) doi:10.1002/ECY.3906.

37. Biosystems™, A. TaqMan™ Environmental Master Mix 2.0. https://www.thermofisher.com/order/catalog/product/4396838.

38. Scientific™, T. Phire Tissue Direct PCR Master Mix. https://www.thermofisher.com/order/catalog/product/F170S.

39. Harper, L. R. *et al.* Needle in a haystack? A comparison of eDNA metabarcoding and targeted qPCR for detection of the great crested newt (Triturus cristatus). *6330 | Ecol. Evol.* **8**, 12 (2018).

40. Wilcox, T. M. *et al.* Parallel, targeted analysis of environmental samples via high-throughput quantitative PCR. *Environ. DNA* **2**, 544–553 (2020).

41. Bylemans, J., Gleeson, D. M., Duncan, R. P., Hardy, C. M. & Furlan, E. M. A performance evaluation of targeted eDNA and eDNA metabarcoding analyses for freshwater fishes. *Environ. DNA* **1**, 402–414 (2019).

42. Westfall, K. M., Therriault, T. W. & Abbott, C. L. Targeted next-generation sequencing of environmental DNA improves detection of invasive European green crab (Carcinus maenas). *Environ. DNA* **4**, 440–452 (2022).

43. Tsuji, S., Takahara, T., Doi, H., Shibata, N. & Yamanaka, H. The detection of aquatic macroorganisms using environmental DNA analysis—A review of methods for collection, extraction, and detection. *Environ. DNA* **1**, 99–108 (2019).

4.7   Supporting information

Supporting Information 4.1. Overview of standard curve values of the singleplex and multiplex qPCR for the repeated November experiment (B and C) and for the sprat fishery data (A).

A) Sprat fishery (singleplex)

target species	R2	Eff%
Sprat	0.998	102.35
Herring	0.996	94.62
Sprat	0.997	92.49
Herring	0.999	97.19
Sprat	0.999	97.65
Herring	0.996	90.04
Sprat	0.998	97.50
Herring	0.997	91.34
Sprat	0.993	100.12
Herring	0.998	95.91
Sprat	0.996	95.42
Herring	0.997	93.54
sprat	0.997	90.42
herring	0.995	89.78
herring	0.994	93.89
sprat	0.996	93.24

B) Repeated November experiment (singleplex)

target species	R2	Eff%
herring	0.998	95.92
sprat	0.998	95.35
herring	0.996	92.90

C) Repeated November experiment (multiplex)

target species	R2	Eff%
sprat	0.997	94.39
herring	0.998	90.53
sprat	0.997	91.12
herring	0.996	99.22

Supporting Information 4.2. DNA-metabarcoding primer mismatch in sprat and herring mtDNA. **Forward primer is the mlCOlintF with 1 mismatch for sprat and 4 mismatches in herring, the reverse primer jgHCO2198 matches both species perfectly.**

Forward primer mlCOlintF (GGWACWGGWTGAACWGTWTAYCCYCC)
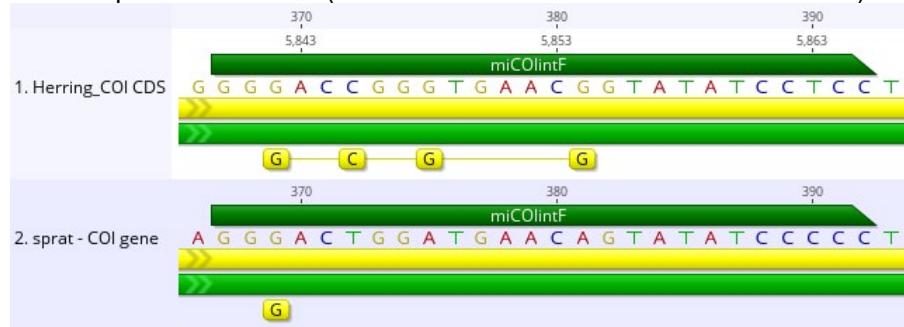
Reverse primer jgHCO2198 (TANACYTCNGGRTGNCCRAARAAYCA)
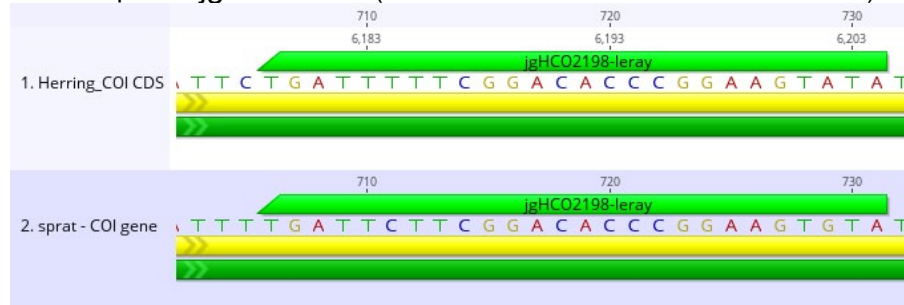
Supporting Information 4.3. The accuracy in herring biomass prediction using the November experiment and March experiment on eDNA estimates derived from the on-site test samples collected at factories (hence discharge water).

	Variance	Bias of the estimator	Root mean square error
March experiment prediction	0.031337	0.000209	0.076097
November experiment prediction	0.031337	0.121018	0.159227

Supporting Information 4.4. Estimated vs. True measured fraction for the November (A) and the March (B) experiment based on the singleplex qPCR. For the estimation in A the model developed based on March sample was used, for B the one based on November samples. In both cases, the true and estimated fractions show very comparable results.
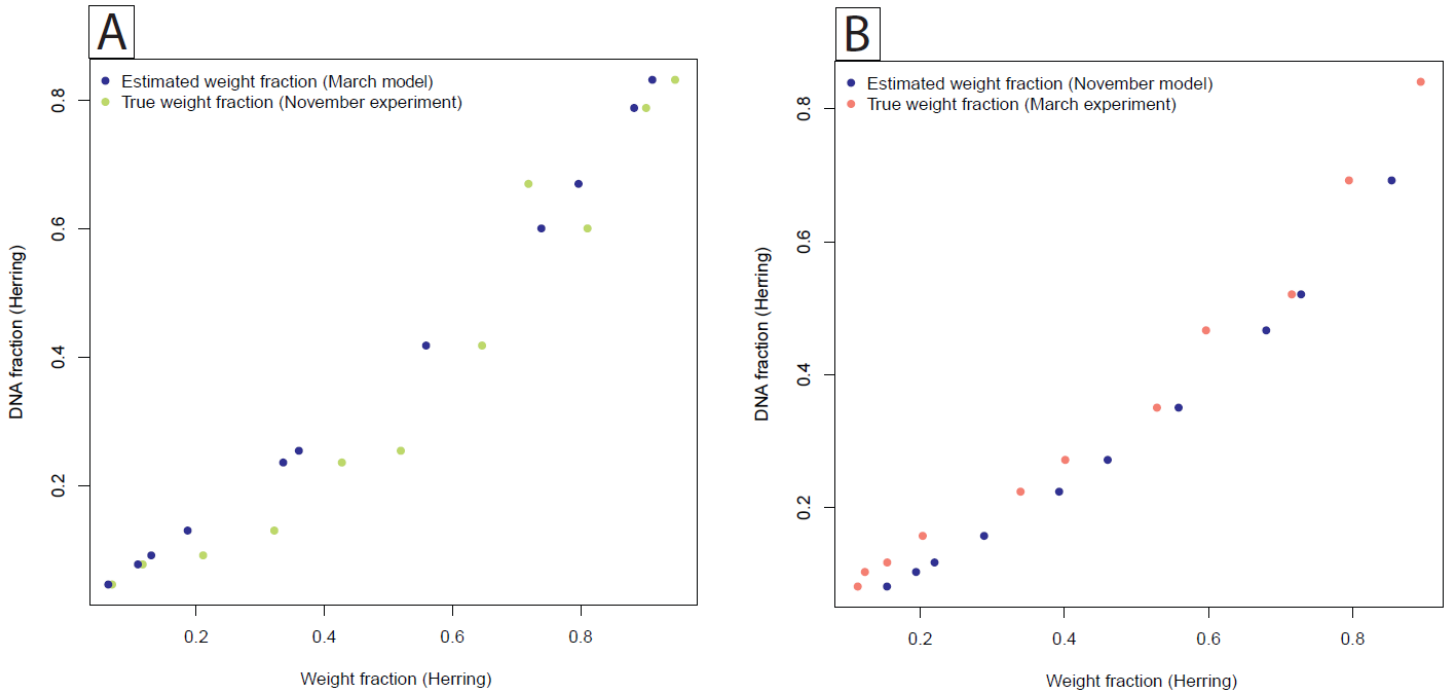
Supporting Information 4.5. Testing accuracy of the two-step DNA-metabarcoding approach. Eleven sprat-herring-amplicon mixtures with targeted herring proportions were prepared from sprat and herring amplicons (derived from the first PCR). These mixtures underwent the second PCR step, the barcoding PCR, in the same way as the experimental samples. The identification of reads was performed in Geniouse (as described in material and methods). Estimated proportions from the sequencing run show a strong linear relationship to the targeted herring proportions (open circles). The solid line indicates the 1:1 straight line.
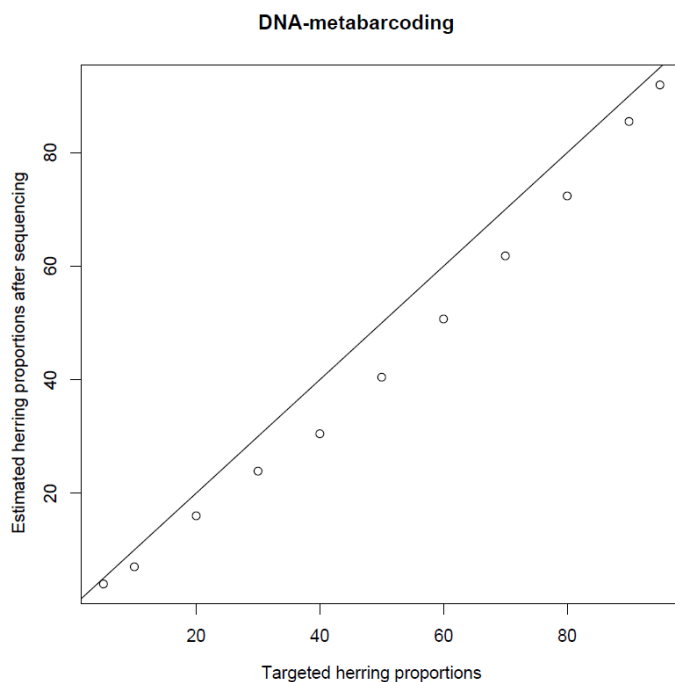
DNA-metabarcoding

Supporting Information 4.6. DNA-metabarcoding results from the seawater blank samples collected from each experimental unit before starting the experiment.The seawater blanks were processed along the blood and discharge water samples. During the pooling of samples for library preparation all samples, including blanks, were pooled in equimolar concentrations.
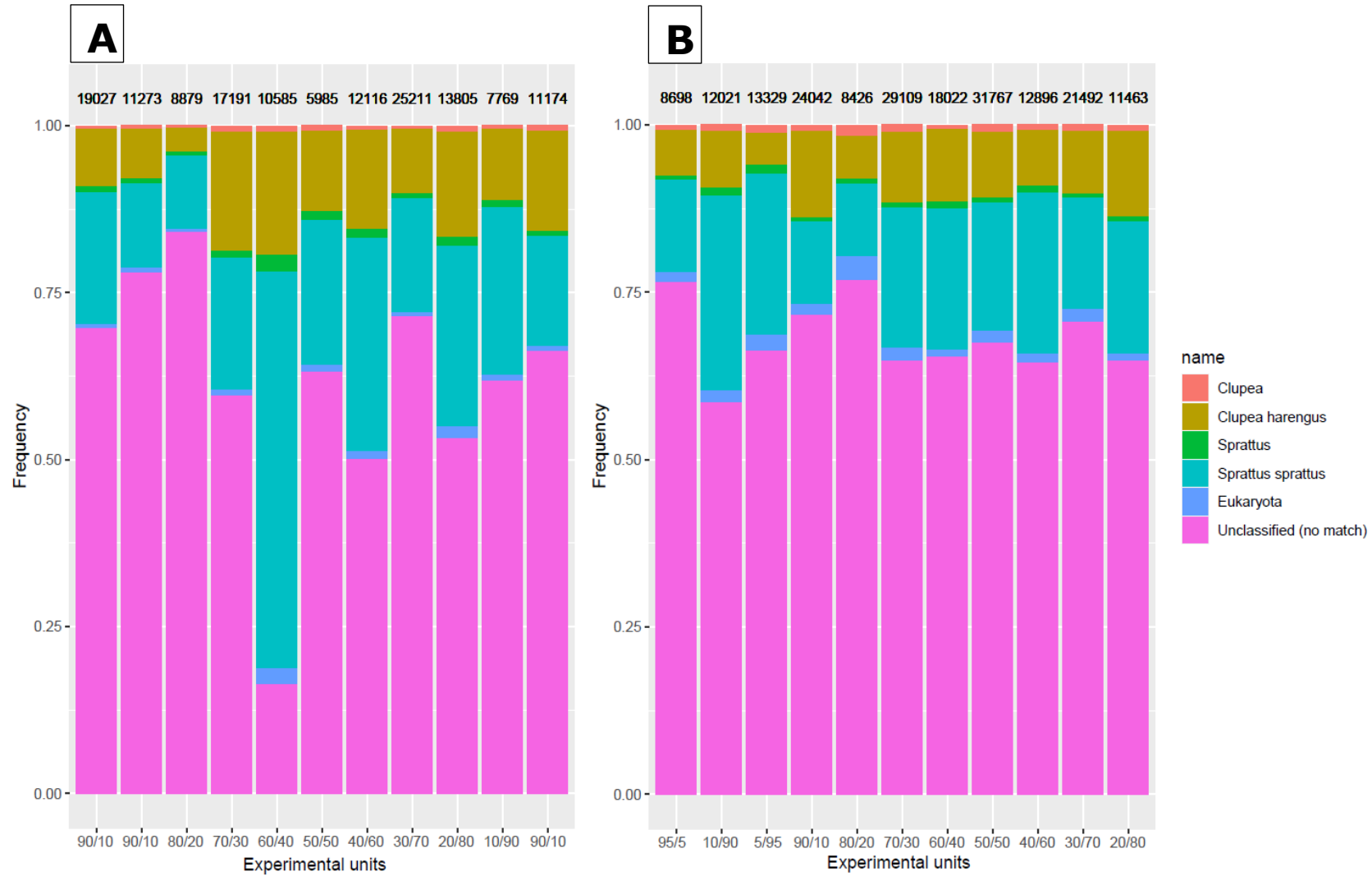
Supporting Information 4.7. Geographical distribution of the 4 total catches (landings) of the Baltic Sea sprat fisheries investigated in this study. All catches were made in the winter fishing season for the fishery (between January and beginning of March).
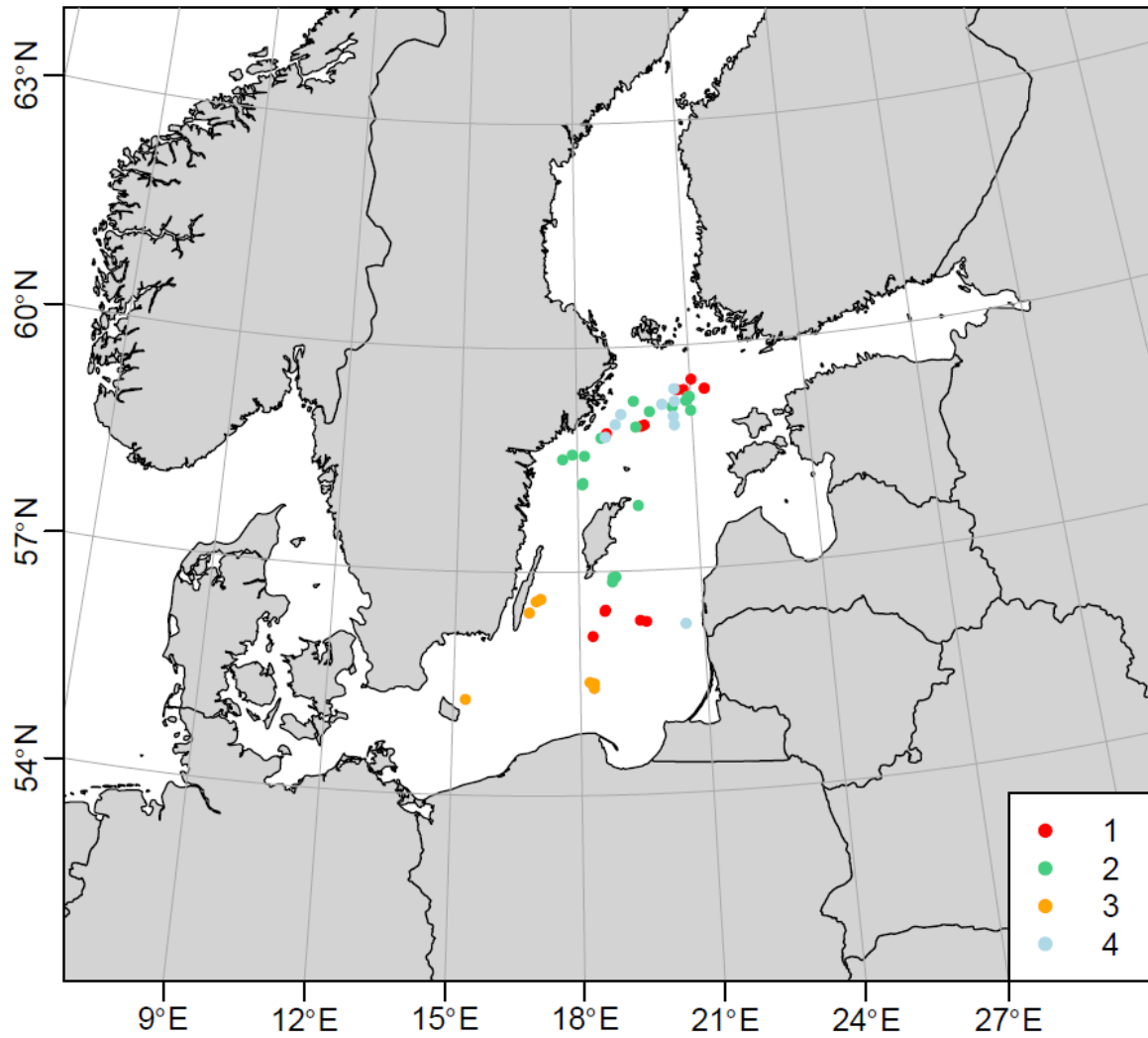
Supporting Information 4.8. Distribution of herring fraction throughout four different sprat landings (1-4). The herring fractions are represented as eDNA based fractions (pink) and fractions derived from subsamples collected for the bucket method.
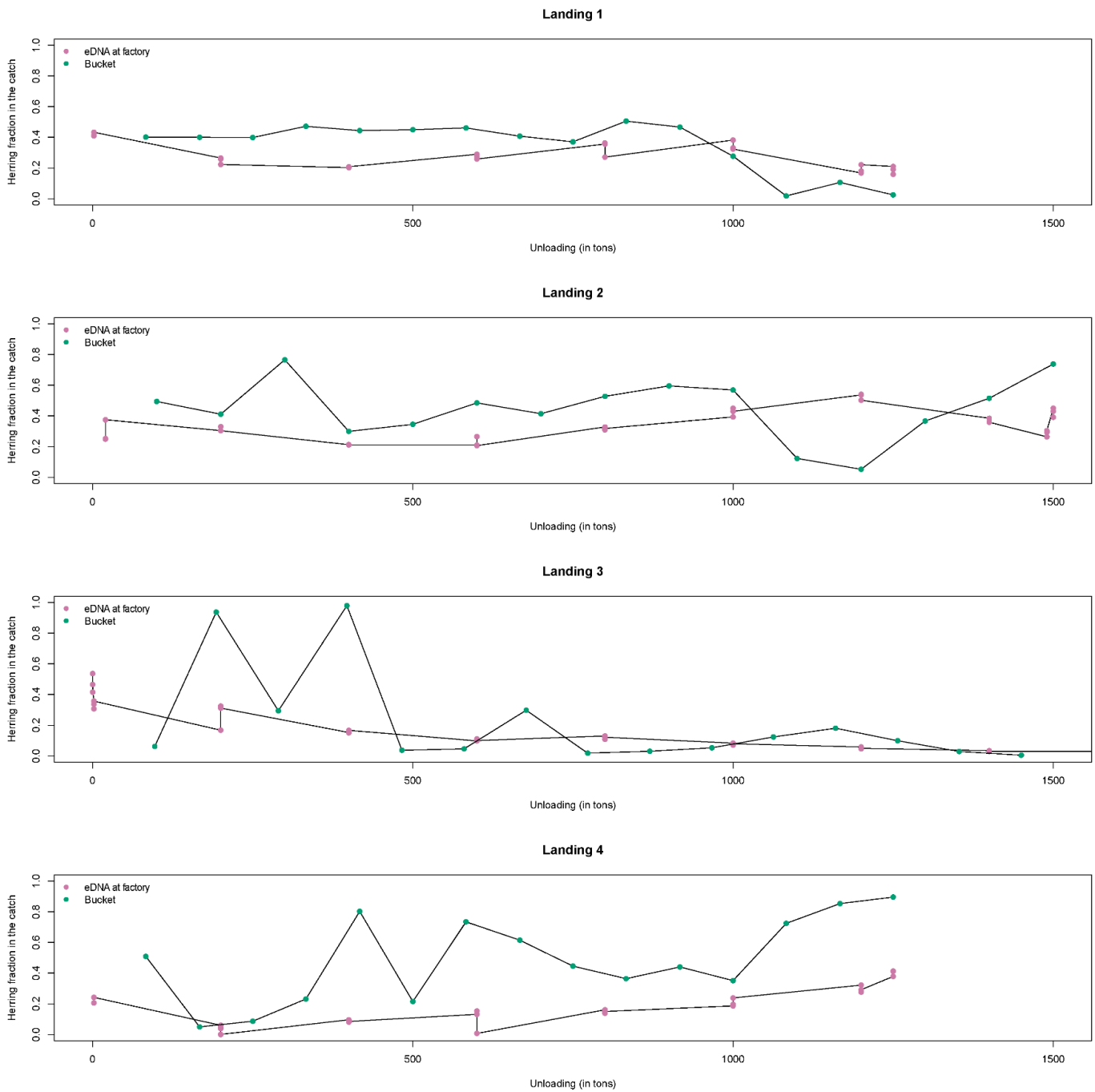
Supporting Information 4.9. Relationship between DNA-based herring fractions derived from both experiments and allometrically scaled weights.
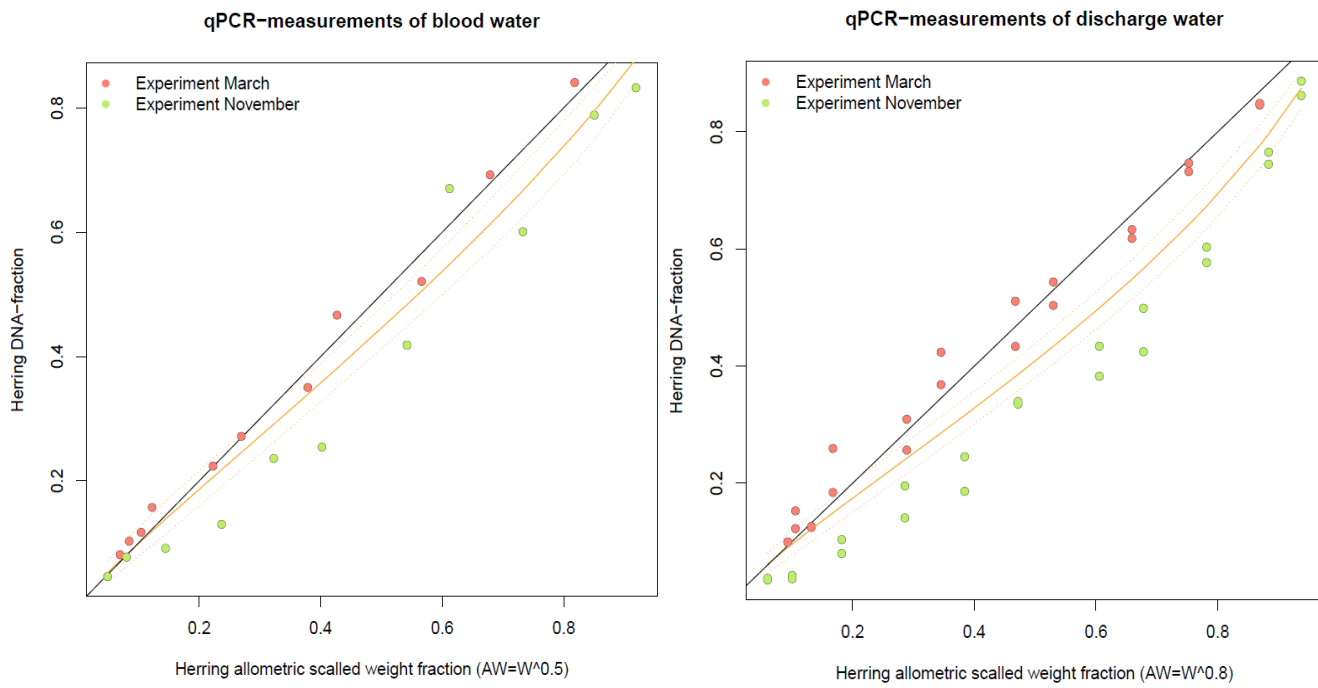
Supporting Information 4.7. Herring bycatch fractions estimated using blood water collected from ship tanks. From each tank (i.e. tank name 1-8) three replicates were collected. No blood water was collected during landing 2.

a Estimated bycatch per ship tank landing 1
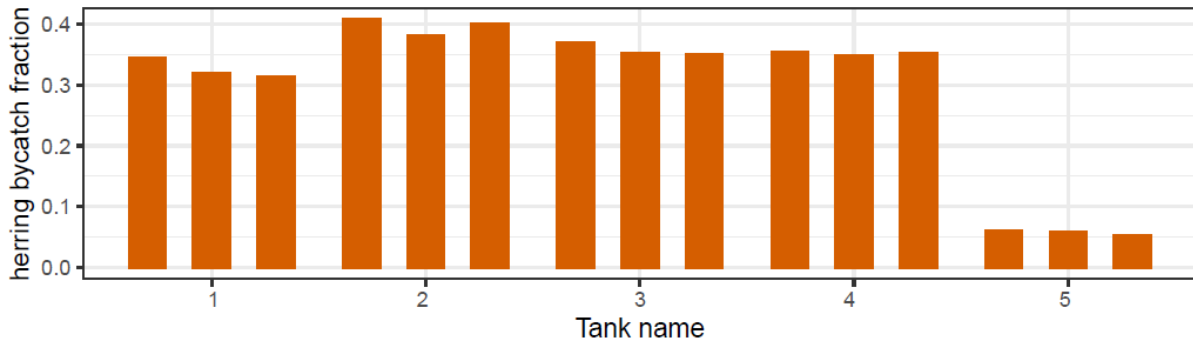
b Estimated bycatch per ship tank landing 3
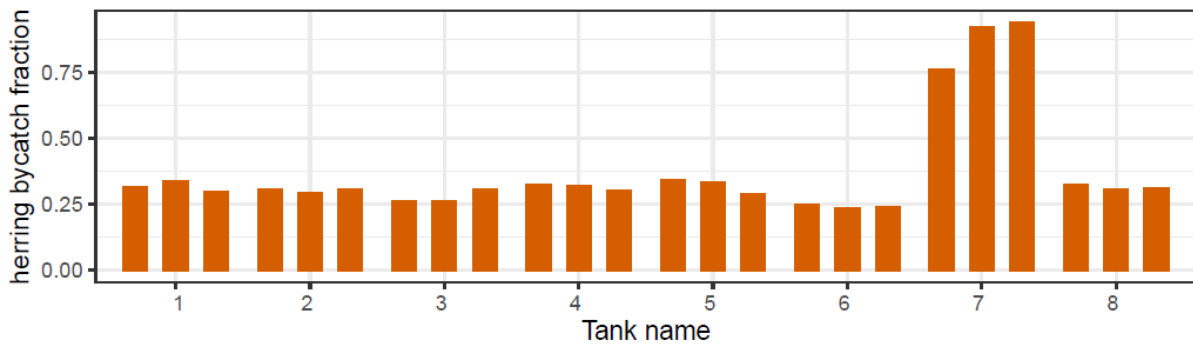
c Estimated bycatch per ship tank landing 4
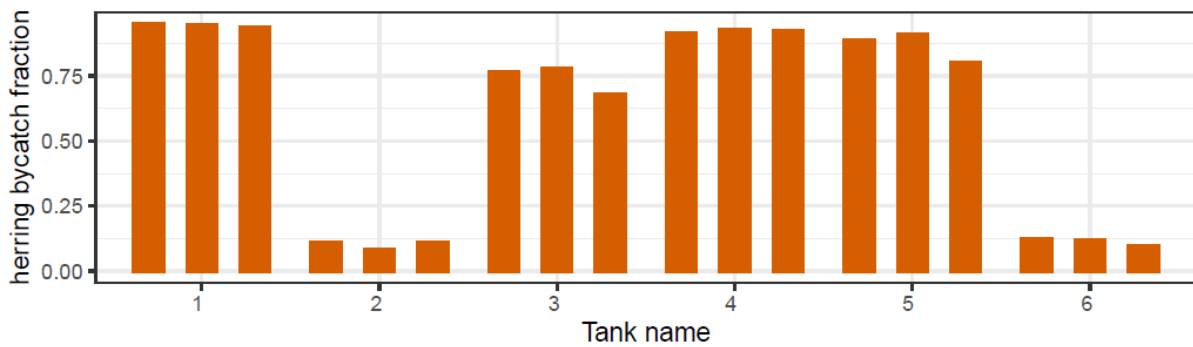
5. A study of the potential use of DNA from catch water to assess biodiversity of fishing catches in the consumption fisheries

Authors

Sara Maggini[2], Magnus W. Jacobsen[1], Jos Kielgast[1], Paulina Urban[1], Garry Carvalho[2], Alexander Papadopulos[2] & Einar E. Nielsen[1]

Affiliations.

[1]Section for Marine Living Resources, DTU Aqua, Technical University of Denmark, Vejlsøvej 39, 8600 Silkeborg, Denmark
[2]Molecular Ecology and Evolution Bangor, School of Natural Sciences, Bangor University, Environment Centre Wales, Bangor, UK

Status.

Draft manuscript

5.1 Introduction

The consumption fishery targets a wide array of fishery targets (including bony fish, elasmobranch and invertebrates) and the fishing hauls often include both targeted catch and bycatch species. While information about the landed fraction is easily accessible though the logbooks, information about the unwanted fraction of the catch, which normally is discarded, is limited. However, precise information about the total catch composition is important for ensuring precise monitoring of the fisheries and for accurate assessment of the ecological impact on species, including non-targeted biodiversity. While taxonomic identification has historically been performed by visual inspections, it is often time consuming and can easily lead to misidentification without the expertise from leading taxonomic experts.

In the last decade species identification through DNA analysis has gained considerable momentum as a way to identifying marine species (Ovenden et al. 2003). Until recently, the method relied on analysing DNA extracted from tissue collected from single specimen samples (Herbert et al. 2003; Ivanova et al. 2007). However, with the advance of so-called Next Generation Sequencing (NGS) methods it is now possible to identify species composition in mixed samples, which has led to new possibilities in fishery related biodiversity assessment (Jacobsen et al. 2019). As such, a completely new scientific field has emerged within the last decade relying on the analysis of DNA from environmental samples (so-called eDNA) for aquatic biodiversity monitoring (Hansen et al. 2018). Normally eDNA is defined as the genetic material, sampled from the environment outside the living hosts. According to this definition the DNA is typically from faces, urine, skin cells, hair, dead and degraded organisms (e.g. Thomsen & Willerslev 2015) but may also represent whole organisms present in the environmental sample like microorganisms, bacteria and algae (Pawlowski, 2020). Depending on DNA-concentration eDNA can be extracted directly from the water or up concentrated by filtrating water through a micropore filter to retain and concentrate eDNA before extraction (Hansen et al. 2018).

In parallel with the new field of eDNA, a growing number of studies have also investigated the use of analysing species composition in fisheries samples. These studies comprise a wide array of samples spanning from processes food (e.g. canned fish and surimi) to total fisheries samples of water draining from the net codend to fish silage (Heylar et al. 2017; Huxley-Jones et al. 2012; Pepe et al. 2007; Russo et al. 2021). Samples collected directly from fishing storage tanks or water used for pumping fish into fish factories has shown great promise for assessing catch and bycatch in the pelagic fisheries (Urban et al. 2022, 2023). In general, these studies support the prospect of using DNA for analysing species composition from complex samples including taxonomic diverse groups of organisms spanning a large part of the animal kingdom (e.g. Stats et al. 2017). The most used method for analysing complex samples is metabarcoding. Metabarcoding combines DNA based identification and NGS. It relies on the use of so-called primers (short DNA oligoes), which allows amplification of a specific target region across species groups (e.g. fish) in environmental or other mixed species samples, which subsequently can be sequenced (Jacobsen et al. 2019). One shortcoming of the method is that the sequencing instruments normally used are very expensive and hence only found in special dedicated molecular laboratories, which lead to a significant time lag from sampling to results. However, a new generation of small (pocket size) portable sequencers like the MinION from Oxford Nanopore Technologies (https://nanoporetech.com/) are now available. Such instruments are much cheaper than the traditional large laboratory based sequencing platforms, which combined with their small size, makes it possible to use them under none laboratory condition (e.g. landing facilities). Here they present a potential for *in-situ* identification and quantification of species composition in mixed fisheries samples (Jacobsen et al. 2019). In this study, we assessed for the potential for using the MinION sequencing platform for identifying catch composition of fisheries samples collected in the consumption fishery by analysing so-called "catch water" collected from a water tank use for storing the total catch before sorting it. We had two overall research questions: 1) Can eDNA from storage water be used

accurately identify species in the consumption fishery and does proportions mimic the actually proportion in the landings? 2) Does eDNA provide a means for identification of rare and vulnerable species, or other species, which are not normally reported by the fishermen?

5.2 Methods

5.2.1 Sample collection of catch water

Water samples were collected from a catch storage tank onboard of a commercial bottom trawler (mesh size 90 mm) fishing in the Skagerrak (ICES subdivision 3a) in January 2021. The samples originated from 10 different fishing hauls targeting either Norway lobster *(Nephrops norvegicus)* or roundfish, such as Atlantic cod (*Gadus morhua*). Based on the target species, we refer to these samples as *Nephrops* and roundfish samples. Notably, the *Nephrops* fishery is at shallower depth (average mean depth= -80.37m, max= -172.78m, min= -54.01m) and during daytime, while roundfish fishery occurred at deeper depth (average mean depth= -237.74m, max=-305.89m, min= -183.38m) and at night. For each haul, three water samples were collected in 50 ml centrifuge sterile tube from a storage tank in which the whole catch was stored before being sorted, hence referred as *catch water*. The samples were stored at -20°C until further processing. For each fishing haul we noted down all the species being sorted during the first ten minutes. These observations were limited to species in the landing obligation. Rare and protected species part of each fishing haul were also noted down when possible.
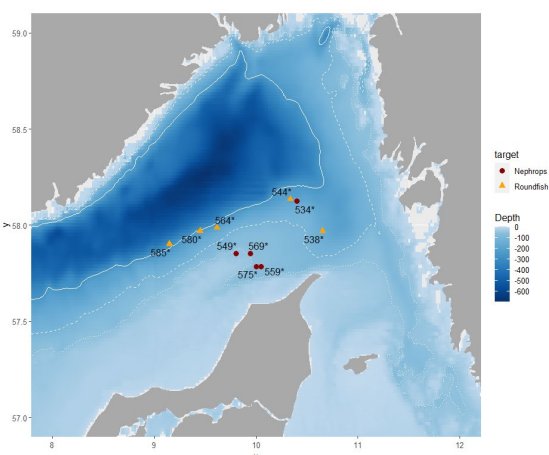
Figure 5.1. Sampling locations in the Skagerrak. *Nephrops* samples are indicated by red circles while roundfish samples by yellow triangle. For more details on each fishing haul see Supplementary information.

5.2.2 Catch water extraction

Extraction of eDNA from the catch water was performed in a dedicated clean laboratory facility at DTU Aqua (Technical University of Denmark, Silkeborg, Denmark). We analysed one sample per haul, and for four hauls – two for each target fishery – three sample replicates (three replicates from one collected water sample) were analysed to investigate the reproducibility of the species identification. After thawing the samples at room temperature for 4 hours, a total of 20 mL of catch water was pressed through a 0.22 µm Sterivex filter (SVGPL10RC, Sigma-Aldrich, St. Louis, MO, USA) using a sterile 60 mL disposable syringe (Becton, Dickinson and Company, Franklin lakes, NJ, USA) to retain eDNA and cells. We followed a modified version of Spens et al. 2017 extraction protocol for eDNA extraction from filters, using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The final elution volume was adjusted to 60 µl of Buffer AE and samples were stored at -20°C. Each extraction batch included a blank sample to test for exogenous DNA contamination through the used reagents or from the laboratory. Surfaces and laboratory equipment were cleaned with bleach solution, distilled water and 70% ethanol before and after samples processing in each batch. The eDNA concentration was measured for all samples using a Qubit fluorometer and the Qubit dsDNA high sensitivity kit (ThermoFisher Scientific, Waltham, MA, USA).

5.2.3 Primers

We used four different primer sets, targeting short (MiFish and COI) and long regions (*fish-2kb* and *metazoan-2kb*) of the mitochondrion genome in fish, elasmobranchs and more broadly metazoan, including: Mifish (targeting 170 bp ca. of the 12S gene in bony fish); Fish_12S-16S-ONT (here referred as *fish-2kb*, targeting a long region of the 12S-16S genes in fish and elasmobranchs (Doorenspleet et al. 2021)); *Metazoan-2kb* (targeting a 2 kb region in fish, elasmobranchs and hagfish, although originally intended for the broad Metazoan taxa); Leray-XT (amplifying ~313 bp of the *cytochrome c oxidase subunit I* in metazoan Wangensteen et al. 2018). The *Metazoan-2kb* was made from two different primers. The forward primer was designed in Machida et al. (2012) 5'-GTGCCAGCHNHHGCGGTYA-3', while the reverse in Kelly et al. (2016) 16s_Metazoa_rev 5'-CCGGTCTGAACTCAGATCAYGT-3'.

Table 5.1. Information on regions and taxa targeted by the primer used in this study.

Primer	Sequences	Target gene	Length (bp)	Target taxa group	Reference
MiFish	f: 5'-GTCGGTAAAACTCGTGCCAGC-3' r: 5'-CATAGTGGGGTATCTAATCCCAG-TTTG-3'	12S	~170	Bony fish	Miya et al. (2015)
Leray-XT	f: mlCOIintF-XT 5'-GGWACWRGWT-GRACWITITAYCCYCC-3' r: jgHCO2198 5'-TAIACYTCIGGRT-GICCRAARAAYCA-3'	COI	~313	Metazoan	Wangensteen et al. (2018) Geller et al. (2013)
Fish_2kb	f: 5'-**TGGG**ATTAGATACCCYACTATGC-3' r: 5'-GATTGCGCTGTTATCCCTAG-3'	12S-16S	~2000	Bony fish & Chondrichthyes	Doorenspleet et al. (2021)
Meta-zoan_2kb	f: 5'GTGCCAGCHNHHGCGGTYA-3' r: 16s_Metazoa_rev CCGGTCTGAACTCAGATCAYGT	12S-16S	~2400	Bony fish & Chondrichthyes & myxine	Kelly et al. (2016) Machida et al. (2012)

5.2.4 PCR amplification and library building

For the COI, the final PCR reactions were performed in 20 µL reaction volumes containing 10 µL of AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µl of each forward and reverse primers (5µM), 0.16 µL BSA (20 µg/ µL), 3.84 µl of water and 4 µl of eDNA. The PCR reactions were run with an initial denaturation step of 10 minutes at 95°C, followed by 40 cycles of 94°C for 1 min, annealing at 45°C for 1 min, followed by 1 min extension at 72°C, and a final extension at 72°C for 5 min after the 40 cycles. Analogously for MiFish, the reaction included the same reagent in the same volumes as for COI but with a higher concentration of each forward and reverse (10µM) primers. The PCR run consisted of 10 minutes at 95°C, followed by 40 cycles of 95°C for 30 sec, 60°C for 30 sec, 30 sec extension at 72°C, followed by a final extension at 72°C for 5 min.

For the long region markers (*metazoan-2kb* and *fish*-2kb), the final PCR reactions were performed in 20 µL reaction volumes containing 10 µL of the Platinum SuperFi II DNA Polymerase–High-Fidelity master mix (ThermoFisher Scientific, Waltham, MA, USA) for long range PCR amplification, 1 µl of each forward and reverse primers (10µM), 4 µl of water and 4 µl of eDNA. The PCRs were run with an initial denaturation at °C for 1 min, 40 cycles of 94°C for 1 min, 58.6°C for 2 minutes and 72°C for 1 min, and a final extension at 72°C for 5 min.

All PCR runs included a negative control. After the amplification, 2 μl of amplicons were run on an electrophoresis agarose gel (2% for short and 0.7% long regions markers, respectively). After visualising successful amplification, the three PCR replicates were pooled.

The four primer sets were also tested on a mock community containing 12 different marine species, including Actinopterygii (represented by five orders), elasmobranch (two orders), myxines (1), cephalopods (1), bivalve (1), and malacostraca (2 families) (Table 5.S1). DNA extracts of tissue samples were pooled so all species contained the same initial DNA concentration (12ng/μl). PCR amplifications were carried out as described for the catch water samples, with the only difference that 2 μl of final DNA was used instead of 4 μl. The library preparation followed the same protocol as described below for the eDNA samples.

The pooled eDNA extracts were cleaned using Agencourt AMPure XP magnetic beads in accordance with manufacturer's specifications, and DNA concentration assessed using Qubit fluorometer and the Qubit dsDNA high sensitivity kit (ThermoFisher Scientific, Waltham, MA, USA). Next, the amplified regions (MiFish, COI, *fish-2kb* and *metazoan-2kb*) were mixed in equimolar ratios for each of the 18 samples individually, and 100 fmol of the pooled amplicons mix was used for the library preparation. A library was prepared for each sample separately, using the Nanopore Amplicons by Ligation (SQK-LSK110) sequencing kit, according to manufacturer's instruction. Sequencing was performed on the MinION Mk1C from Oxford Nanopore using one Flongle flow cell (FLO-FLG001) for each of the 18 samples. Following the protocol recommendation, 20 fmol of the final library was loaded onto the flow cell and the sequencing run started, enabling live base calling using Guppy, selecting the High accuracy (HAC) model. The base called reads were then exported for further analysis.

5.2.5   Bioinformatic analysis of Nanopore reads

We used a custom pipeline for pre-processing the reads including quality and length filtering followed by primer removal. For the taxonomical assignment, we used MetONTIIME (https://github.com/MaestSi/MetONTIIME), a metabarcoding pipeline for analysing ONT data in QIIME2 framework (Maestri et al. 2021).

The pre-processing pipeline was written specifically for this study, in which each sequencing run consists of a combination of four amplicons of an individual eDNA sample. The pre-processing pipeline involves quality and length filtering of the reads using NanoFilt (De Coster et al. 2018), and primers and adapter removal using Cutadapt 2.4. Only reads for which both forward and reverse primers were located were retained, while untrimmed reads were discarded.

The MetONTIIME analysis started from the filtered sample and marker specific fastq files and was run using BLAST to assign all processed sequence reads to lowest taxonomic level. For BLAST, we set the maximum number of hits to 3, the minimum alignment identity threshold to 0.94 and minimum query coverage to 0.8 and 0.2 for short and long regions, respectively.

We used a local reference database comprising COI, 12S and 16S genes, as well as whole mitochondrion genome sequences of Norway lobster and North Sea fish species i.e., teleost, elasmobranch and myxine. A list of North Sea fish species was downloaded from FishBase and consisted of 196 species, including teleost, agnates and Chondrophytes. The database was built by merging MIDORI2_UNIQ_NUC_GB252 CO1, lrRNA and srRNA databases (http://www.reference-midori.info/download.php), and filtering for North Sea fish species. Whole mitochondrion genome sequences of North Sea fish species and Norway lobster were downloaded from the NCBI and added to the MIDORI2 database. Statistical analyses were conducted in R version 4.2.2 (R Core Team, 2022).

Alpha and Beta diversity were calculated in phyloseq (McMurdie & Holmes 2013) and vegan (version 2.6-4) R packages, PCoAs were built using the Jaccard distance of dissimilarities. Permutational Multivariate Analysis of Variance (PERMANOVA) were performed using the *adonis2* function in vegan with 9999 permutations, to test for differences in species detected among samples and evaluate the effect of sampling replicate (fishing haul), target fishery and mean depth.

5.2.6 Filtering of reads

The output of the taxonomic assignment was imported into R. We restricted the analyses to species level, since the aim was to test the potential of using eDNA to estimate catch and bycatch composition. We applied several filters to account for potential miss assignments because of the high error rate of the MINION sequencer. These filters were based on observations from the mock analysis. First, we applied a general filter, retaining only species detected with at least 3 reads.

We further defined primer-specific abundance thresholds, chosen based on the mock analysis results, to filter out false positive species.

A successive filter was applied for highly similar species. In the mock we detected some highly similar species (identity ≥ 98%) to the one put in the mock sample although they were not included in the mock sample. Interestingly, the proportion of reads of these miss assignments were always negligible compared to the true species in the mock (range 0.04%-3.7%; mean=0.83±1.15). Hence, for each eDNA sample, highly similar species represented by less than 2% of the total reads for the group were discarded from successive analyses.

5.3 Results

5.3.1 Catch water analysis

Overall, we sequenced 18 samples collected from 10 different fishing hauls, including three sample replicates in four hauls. Nanopore sequencing generated 239539-1704608 sequences per library (average=631164, SD= ±388556).

Across the 18 samples and the combined primer sets, 52 unique species were detected, representing 45 genera, 28 families, 17 orders and 4 classes (Actinopterygii, Myxine, Chondrichthyes, Malacostraca). For the individual samples, the number of species ranged from 17 to 37 (mean=25.5, SD=4.72). Considering the two target fisheries separately, roundfish samples included on average 22.44 (SD=2.74) species, while *Nephrops* samples on average included 26.05 species (SD=5.54). Accordingly, the number of species detected in Norway lobster fishery samples is significantly higher compared to Roundfish fishery samples (Wilcoxon signed-rank test, p < 0.05). No significant difference was found in number of species reported as observed onboard (information obtained by observation of first ten minutes of catch sorting by the scientific crew and species landed) between the two target fisheries (Wilcoxon signed-rank test, p > 0.05). All the species in the mock sample were detected (Fig. 5.S1) (Number of detected species for each individual primer sets can be found in the appendix Fig. 5.S2 and Fig. 5.S3).

Several of the species found using the eDNA method are listed on the IUCN red list. Among the species found across samples, *M. dypterygia*, *E. spinax*, *H. hippoglossus* and *C. monstrosa* are listed as Vulnerable species in the IUCN Red List. Here, on average 2.53 (SD=0.88) vulnerable species were found across samples. While, near threatened species —*Raja clavata or Cyclopterus lumpus*—were found in 12 samples (For full overview of all fishery species observed by the fishermen or scientific observers and all species detected by eDNA, see Appendix Fig. 5.S4).

## 5.3.2 Variation of sampling replicates

Analysis of sampling variation was carried out for a total of four different hauls: two samples from the *Nephrops* fishery (sample 549 and 569) and two samples from the Roundfish fishery (538 and 544). Alpha diversity between sampling replicates were highly similar for most replicates (Fig. 5.2) and most of the species found in a sample was also detected in the replicates (as showed by the Venn'diagrams (Figure 5.S5). The PCoA plot based on the Jaccard distance of dissimilarities showed replicates from the same fishing haul clustered together. Moreover, the PERMANOVA identified significant difference among fishing haul (p<0.0001). Additionally, fishing haul (sample replicate) in the PERMANOVA model explained the 85% of the variation (p-value < 0.0001), indicating that the intra-variability within each haul is lower than the inter-variability among samples from different hauls. Hence, there is a significant effect of the haul and the diversity found across replicates within the same fishing haul is consistent.
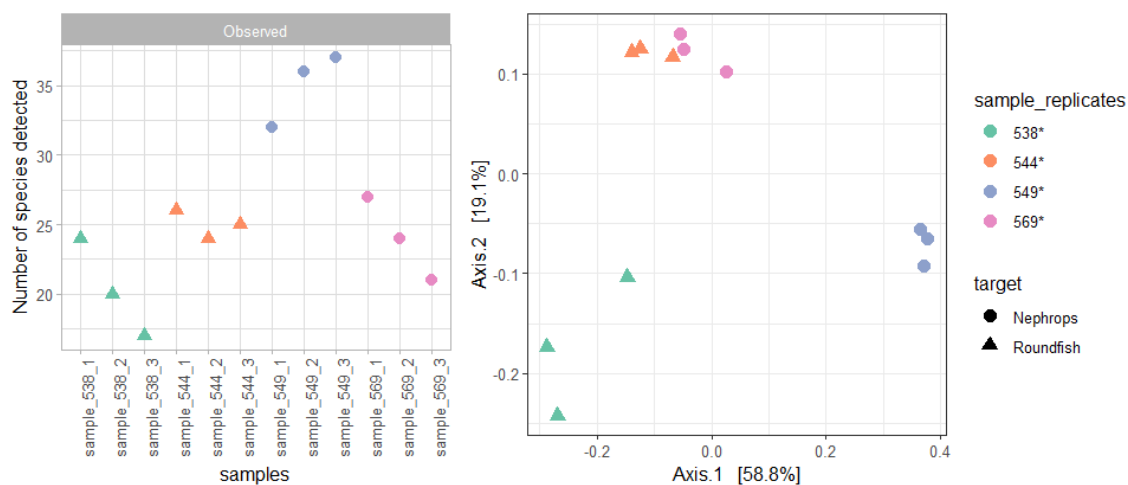
Figure 5.2. Alpha diversity (number of species detected in each sample replicates and on the right beta diversity, PCoA of Jaccard distance of dissimilarities.

5.3.3 Comparison between eDNA and observations

On average the eDNA analysis detected 82.45% of the species reported as observed onboard across samples (SD=11.74). Remarkably, in *Nephrops* samples on average the 86.58% (SD=12.45) of the species reported as observed onboard was also detected in the eDNA while in roundfish samples on average 78.33% of the species was also detected (SD=9.98) (Fig. 5.3). More of the species observed onboard were detected in the *Nephrops* samples than roundfish fishery samples (Wilcoxon rank sum test p-value< 0.005). The species that were not detected by the eDNA method were in all cases limited to non-landed species that represented a low biomass compared to the total catch (Fig. 5.S4).
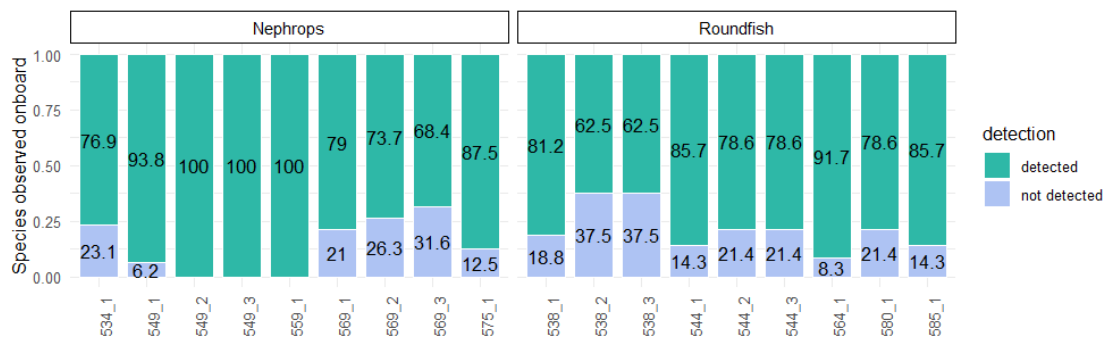
Figure 5.3. Proportions of species observed onboard the fishing vessel that were also detected in the eDNA (green) or not detected in the eDNA (light blue).

The eDNA method also found a larger number of species that was not observed visually in the fishing hauls. In fact, on average 45.50% (±8.74) of the species were only detected by eDNA, ranging between 29.6 to 54.3% (7-21 species across samples, see supplementary material for number of species). The proportion of species detected using eDNA and reported as observed onboard ranged between 32.3% to 50% (mean= 44.20, SD=4.41). Lastly, on average 12.36% of the species observed onboard were not detected by the eDNA analysis (range 3–26.1%, SD=6.90) (Fig. 5.4).
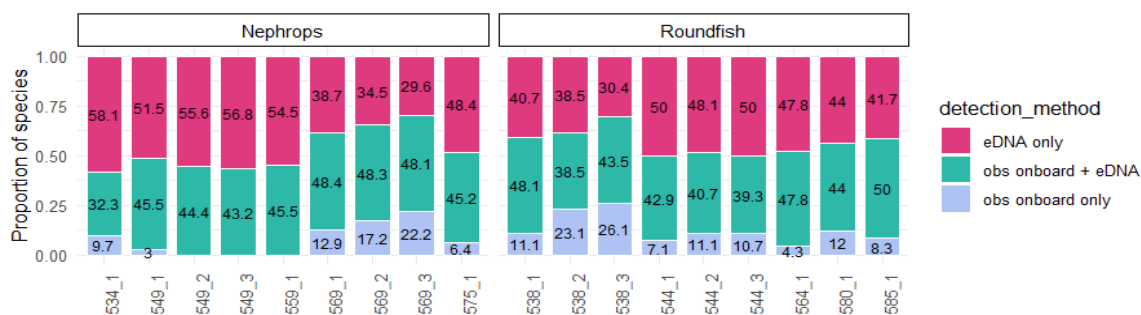
Figure 5.4. Proportion of species that were observed onboard and at the same time detected in the eDNA (light blue), observed onboard only (hence not detected in the eDNA) (dark blue) and species detected only in the eDNA (magenta).

5.3.4   Comparison of beta diversity among samples

Analysis of beta diversity showed an almost identical pattern for both eDNA and visual identified species. For both analyses, the ordination plots (PCoA) based on the Jaccard distance of dissimilarities showed two main clusters. The clusters matched the two fisheries with the exception of one *Nephrops* sample (sample 534), which clustered closer to the roundfish samples. Importantly, sample 534 was collected at greater depth (-172 m) than the other *Nephrops* samples (average -80m) and matched in depth more the samples collected during the round fishery. In the PERMANOVA, there was a significant difference between the two target fisheries -*Nephrops* and roundfish- explaining the 33% of the variance (PERMANOVA p-value<0.05). However, mean depth explained the 48% of the variance (PERMANOVA p-value<0.05).
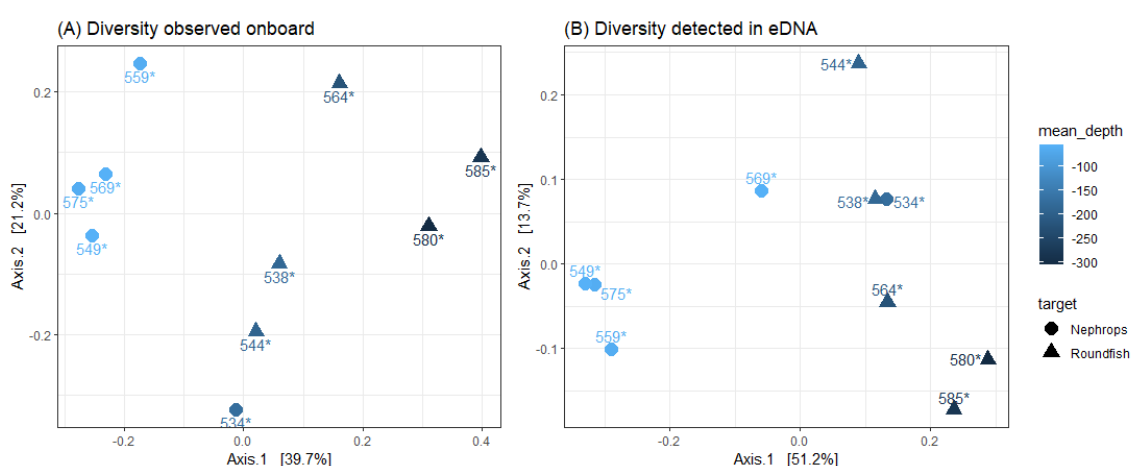
Figure 5.5. PCoA based on the Jaccard distance of dissimilarities using (A) species observed onboard (including landed species) and (B) species detected in the eDNA (for samples with replicates a consensus was used). Samples are coloured by mean depth. Nephrops samples are represented by circles while roundfish samples by triangles.

5.4 Discussion

5.4.1 Primers and species coverage

In this study, we combined results from four different primer sets to ensure broad species detection. It is possible to use fewer primer sets to reduce laboratory time and costs. However, such approach may restrict the number of species that can be detected, since different primer sets have different capacities to identify species from specific taxonomic groups. For example, the MiFish primer is developed for fish detection, while COI allows broader biodiversity assessment (Miya et al. 2015; Wangensteen et al. 2018). The different primer sets used in this study showed variable species coverage. This was apparent from the analysis of the mock community where COI detected the most species compared to the three other primer sets. The exact number of species depended on the database. When using the NS database COI found 9 of the 12 species in the mock sample but all 12 species when using the broader Midori COI database (not restricted for NS species). Analysis of catch water showed that the COI and MiFish primers, targeting shorter mtDNA regions, identified more species than the Fish-2kb and Metazoan-2Kb primers that target a longer part of the mitogenome. This finding suggests that the eDNA in catch water is already degraded. On another possibility is that some of the DNA actually comes from other sources (i.e. not from the catch itself). Such source could be from eDNA from the water itself. However, a larger bias due to detection of species from eDNA is not supported here given the results of the PCoA analyses where species observed visually and by eDNA show very similar patterns.

5.4.2 Sampling variation

Sampling variation was assessed through the analysis of extraction replicates from the same water samples. This analysis showed that sampling replicates show a comparable number of species, which in most cases are shared between replicates. While this supports the overall approach used here, where we based our analyses on 20 mL of catch water, it also shows the potential need for sample optimization, as sample replicates also contains unique species. Hence, future studies should include further assessment of the optimal sampling strategy. Such strategy may include the use of larger sampling volumes or more sampling replicates to reduce the number of potential false-negatives. The result from this study is nevertheless encouraging and supports the potential for using eDNA for future monitoring of species composition in the consumption fishery.

5.4.3 Qualitative assessment of fishing catches

The eDNA approach detected most of the species identified through visual observations from the individual fishing hauls. The species that were not detected by the eDNA method were in all cases limited to non-landed species that represented a low biomass compared to the total catch. This was expected given that rare species are only anticipated to contribute with limited DNA to the catch water, which decrease the chance of them being detected.

Interestingly, the eDNA method found a significant number of fisheries species (limited to fish, elasmobranch and Norwegian lobster in the study), which were not reported as observed. This results is likely explained partly by that the visual observations do not constitute full species lists as observations were limited to the first 10 min of the sorting of the catch and the occasional reporting of unqoutated and rare and protected when observed. As such, we find it likely that many of the species identified by eDNA actually have been present in the individual fishing hauls. This is also supported by the analysis of beta diversity, which show identical patterns between visual identifications and eDNA observations.

Overall, the DNA based method identified more species than the visual approach. Moreover, the DNA based method identified seven species of sharks and rays and provided means for identifying differences in overarching biodiversity indices between cod and Norway lobster fisheries. However, as for the test samples, the quantitative DNA/weight relationships were weak.

5.4.4   Evaluation of the MinION

Overall, the study shows that portable sequencing instruments like the MinION can be used to identify relevant species caught as part of the consumption fishery. This instrument show a higher sequencing error rate compared to its larger commercial benchtop equivalents but is much cheaper (both to purchase, maintain and use) than these platforms. The obtained results from this study show that sequencing errors do not hinder precise species identification. Further, the size of the MinION makes it possible to use it on board fishing vessel or landing facilities, where it can be implemented to reduce the potential time lag between sampling and DNA based result that would normally be expected, when analysing samples in dedicated molecular facilities. However, the application of the method still includes the use of special equipment like PCR machines and centrifuges. While such machinery also exist in portable versions, purchasing such equipment add to the upfront expenses. Hence, successful implementation of the method might, at least initially, include collection of samples on board the vessels and subsequent DNA analysis at dedicated or mobile laboratory facilities. This should decrease the cost of purchasing the needed laboratory equipment and further allow sample processing by highly trained technical personnel, thereby reducing handling time and error.

5.4.5   Conclusion

This study supports the possibility to analysis fishing catches based on storage catch collected from storage tanks. Such analyses can be used for catch, bycatch and unwanted catch assessment, but may prove particularly valuable for identification of non-target species, which are rarely being reported by the industry. Information about these species may increase our understanding about species distribution and bycatch rates, which is important for managing the fisheries.

The DNA method has potential for species monitoring (including PETS) and development of biodiversity indices for the complex demersal fishery. For large and less complex pelagic catches the methods has a large and immediate potential for implementation and will provide a significant improvement regarding, precision, accuracy, speed and cost efficiency.

5.5   References

De Coster W, D'Hert S, Schultz DT, Cruts M, Van Broeckhoven C (2018) NanoPack: Visualizing and processing long-read sequencing data. Bioinformatics 34:2666–2669.

Doorenspleet K, Jansen L, Oosterbroek S, Bos O, Kamermans P, Janse M, Wurz E, Murk A, Nijland R (2021) High resolution species detection: accurate long read eDNA metabarcoding of North Sea fish using Oxford Nanopore sequencing. bioaRxiv:1–31.

Jacobsen M.W., Hansen B.K., Nielsen E.E. (2019) Possible Uses of Genetic Methods in Fisheries Under the EU Landing Obligation. In: Uhlmann S., Ulrich C., Kennelly S. (eds) The European Landing Obligation. Springer, Cham. https://doi.org/10.1007/978-3-030-03308-8_20

Hansen, B. K., Bekkevold, D., Clausen, L. W., & Nielsen, E. E. (2018). The sceptical optimist: challenges and perspectives for the application of environmental DNA in marine fishes, Fish and Fisheries, 19, 751-768. https://doi.org/10.1111/faf.12286

Hebert, P.D.N., Ratnasingham, S., deWaard, J.R. (2003). Barcoding animal life: Cytochrome c oxidase subunit 1 divergences among closely related species. Proceedings of the Royal Society B-Biological Sciences, 270, S96S99. https://doi.org/10.1098/rsbl.2003.0025.

Helyar, S.J., Lloyd, H.A., de Bruyn, M., Leake, J., Bennett, N., Carcalho, G.R. (2014). Fish product mislabelling: Failings of traceability in the production chain and implications for illegal, unreported and unregulated (IUU) fishing. PLoS One, 9, https://doi.org/10.1371/journal.pone. 0098691.

Huxley-Jones, E., Shaw, J.L.A., Fletcher, C., Parnell, J., Watts, P.C. (2012). Use of DNA barcoding to reveal species composition of convenience seafo

Ivanova, N.V., Zemlak, T.S., Hanner, R.H., Hebert, P.D.N. (2007). Universal primer cocktails for fish DNA barcoding. Molecular Ecology Notes, 7, 544–548. https://doi.org/10.1111/j.1471-8286.2007.01748.x

Kelly RP, O'Donnell JL, Lowell NC, Shelton AO, Samhouri JF, Hennessey SM, Feist BE, Williams GD (2016) Genetic signatures of ecological diversity along an urbanization gradient. PeerJ.

Machida RJ, Kweskin M, Knowlton N (2012) PCR primers for metazoan mitochondrial 12S ribosomal DNA sequences. PLoS One 7:1–6.

Maestri S (2021) Development of novel bioinformatic pipelines for MinION-based DNA barcoding. Università degli Studi di Verona, Verona, Italy

McMurdie PJ, Holmes S (2013) Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One 8.

Miya M, Sato Y, Fukunaga T, Sado T, Poulsen JY, Sato K, Minamoto T, Yamamoto S, Yamanaka H, Araki H, Kondoh M, Iwasaki W (2015) MiFish, a set of universal PCR primers for metabarcoding environmental DNA from fishes: Detection of more than 230 subtropical marine species. R Soc Open Sci 2.

Oksanen J, Simpson G, Blanchet F, Kindt R, Legendre P, Minchin P, O'Hara R, Solymos P, Stevens M, Szoecs E, Wagner H, Barbour M, Bedward M, Bolker B, Borcard D, Carvalho G, Chirico M, De Caceres M, Durand S, Evangelista H, FitzJohn R, Friendly M, Furneaux B, Hannigan G, Hill M, Lahti L, McGlinn D, Ouellette M, Ribeiro Cunha E, Smith T, Stier A, Ter Braak C, Weedon J (2022). vegan: Community Ecology Package. R package version 2.6-4, <https://CRAN.R-project.org/package=vegan>.

Ovenden, J.R., Berry, O., Welch, D.J., Buckworth, R.C., Dichmont, C.M. (2015). Ocean's eleven: A critical evaluation of the role of population, evolutionary and molecular genetics in the management of wild fisheries. Fish and Fisheries, 16, 125–159. https://doi.org/10.1111/faf. 12052.

Pepe, T., Trotta, M., Di Marco, I., Anastasio, A., Bautista, J.M., Cortesi, M.L. (2007). Species identification in surimi-based products. Journal of Agricultural and Food Chemistry, 55, 36813685. https://doi.org/10.1021/jf063321o

Russo, T., Maiello, G., Talarico, L., Baillie, C., Colosimo, G., D'Andrea, L., Di Maio, F., Fiorentino, F., Franceschini, S., Garofalo, G., Scannella, D., Cataudella, S., and Mariani, S. (2021). All is fish that comes to the net: metabarcoding for rapid fisheries catch assessment. Ecological Applications 31(2):e02273. 10.1002/eap.2273

Spens, J., Evans, A. R., Halfmaerten, D., Knudsen, S. W., Sengupta, M. E., Mak, S. S. T, Sigsgaard, E. W. & Hellström, M. 2017. Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter, Methods in Ecology and Evolution, 8: 635-45. https://doi.org/10.1111/2041-210X.12683

Stat, M., Huggett, M.J., Bernasconi, R. *et al.* (2017) Ecosystem biomonitoring with eDNA: metabarcoding across the tree of life in a tropical marine environment. *Sci Rep,* **7,** 12240. https://doi.org/10.1038/s41598-017-12501-5

Thomsen, P. F., & Willerslev, E. 2015. Environmental DNA—an emerging tool in conservation for monitoring past and present biodiversity, *Biological Conservation*, 183, 4-18. https://doi.org/10.1016/j.biocon.2014.11.019

Urban P., Bekkevold D., Degel H., Hansen B. K., Jacobsen M. W., Nielsen A., Nielsen E. E., Scaling from eDNA to biomass: controlling allometric relationships improves precision in bycatch estimation, *ICES Journal of Marine Science*, 2023;,fsad027, https://doi.org/10.1093/icesjms/fsad027

Urban, P., Bekkevold, D., Hansen, B. K., Jacobsen, M. W., Nielsen, A., & Nielsen, E. E. (2022). Using eDNA to estimate biomass of bycatch in pelagic fisheries. *Environmental DNA*, 00, 1– 16. https://doi.org/10.1002/edn3.377

Wangensteen O.S., Palacín C., Guardiola M., Turon X. (2018). DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. *PeerJ*. 2018;6:e4705. doi:10.7717/peerj.4705

5.6 Appendix

Table 5.S1. Species added in the mock community sample in equal equimolar concentration (12ng/µl). Fish species are from different orders to cover fish biodiversity.

| Species	Phylum	Class	Order	Family
Gadus morhua	Chordata	Actinopteri	Gadiformes	Gadidae
Clupea harengus	Chordata	Actinopteri	Clupeiformes	Clupeidae
Scophthalmus maximus	Chordata	Actinopteri	Pleuronectiformes	Scophthalmidae
Lophius piscatorius	Chordata	Actinopteri	Lophiiformes	Lophiidae
Mullus surmuletus	Chordata	Actinopteri	Syngnathiformes	Mullidae
Raja clavata	Chordata	Chondrichthyes	Rajiformes	Rajidae
Scyliorhinus canicula	Chordata	Chondrichthyes	Carcharhiniformes	Scyliorhinidae
Myxine glutinosa	Chordata	Myxini	Myxini	Myxiniformes
Eledone cirrhosa	Mollusca	Cephalopoda	Octopoda	Eledonidae
Ostrea edulis	Mollusca	Bivalvia	Ostreida	Ostreidae
Nephrops norvegicus	Arthropoda	Malacostraca	Decapoda	Nephropidae
Liocarcinus depurator	Arthropoda	Malacostraca	Decapoda	Polybiidae

Figure 5.S1. Species analysis of the the mock community sample, primer and reference database specified in the x-axes. The number of reads is reported for each species detected. Specie indicated with ** are species added in the mock sample. The NS abbreviation indicates the North Sea reference database, while Midori indicates the COI Midori database. The results were initially filtered to include only species with at least 3 reads (reads ≥3).
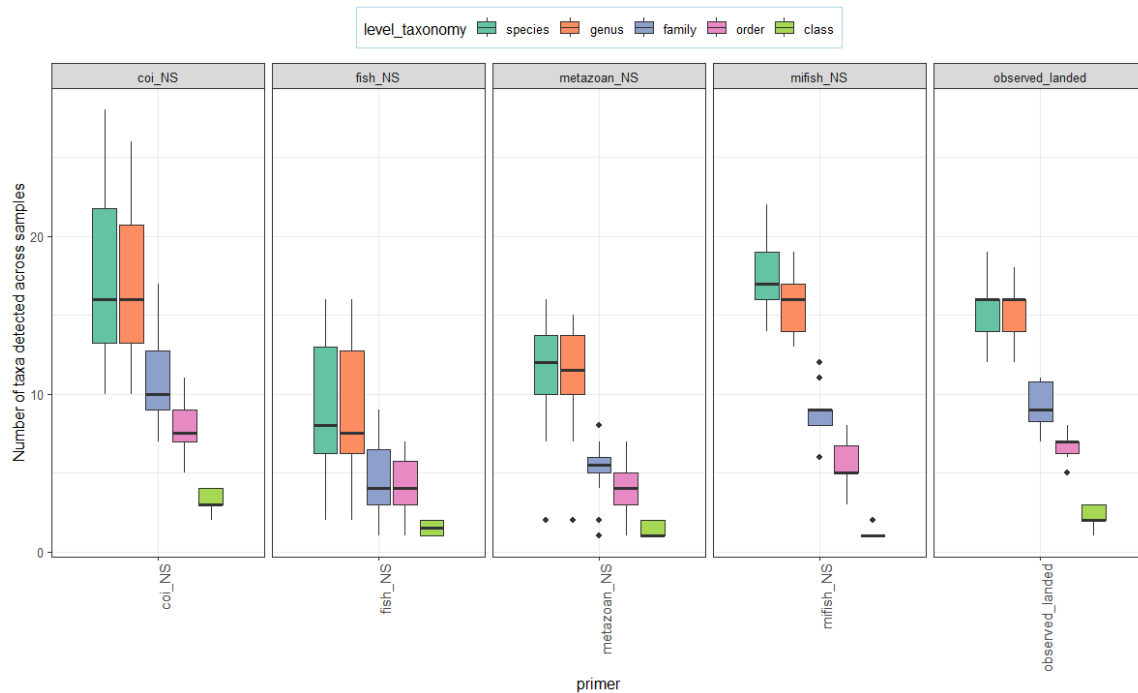
Figure 5.S2. Taxa detected across different taxonomical levels by the four primer sets. From left to right: COI, *fish-2kb*, *metazoan*, MiFish markers and observation onboard.
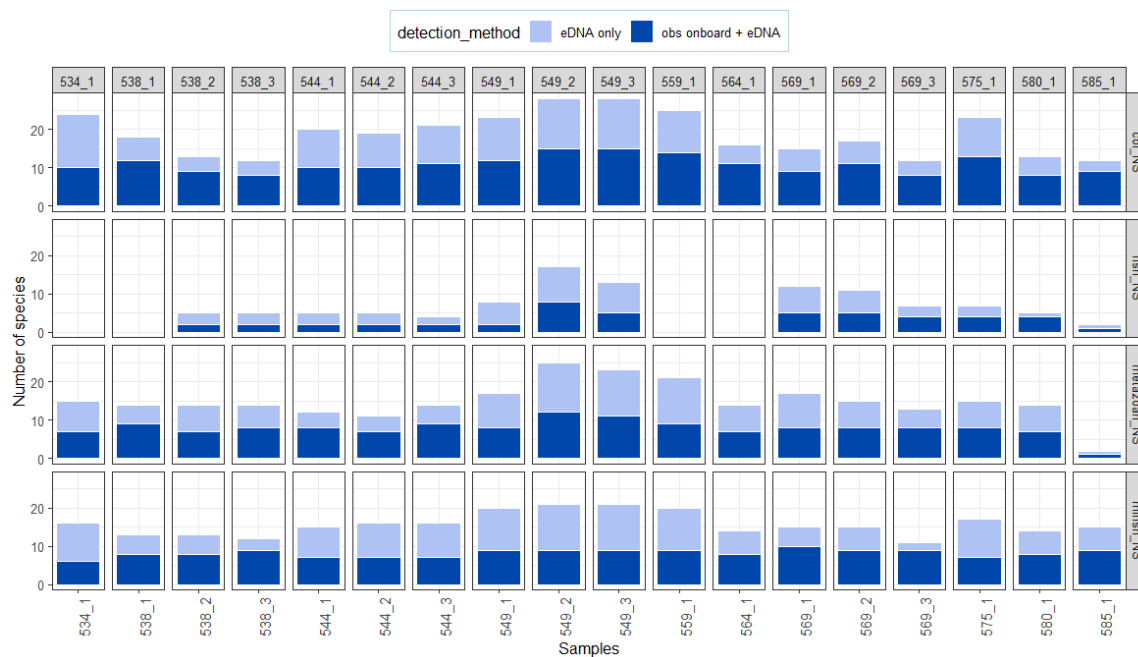
Figure 5.S3. Total number of species detected in the eDNA per sample (x-axis) for each primer set. Dark blue color represents the fraction of species that were observed onboard and in light blue the species that were not observed on board (hence, detected only in the eDNA).
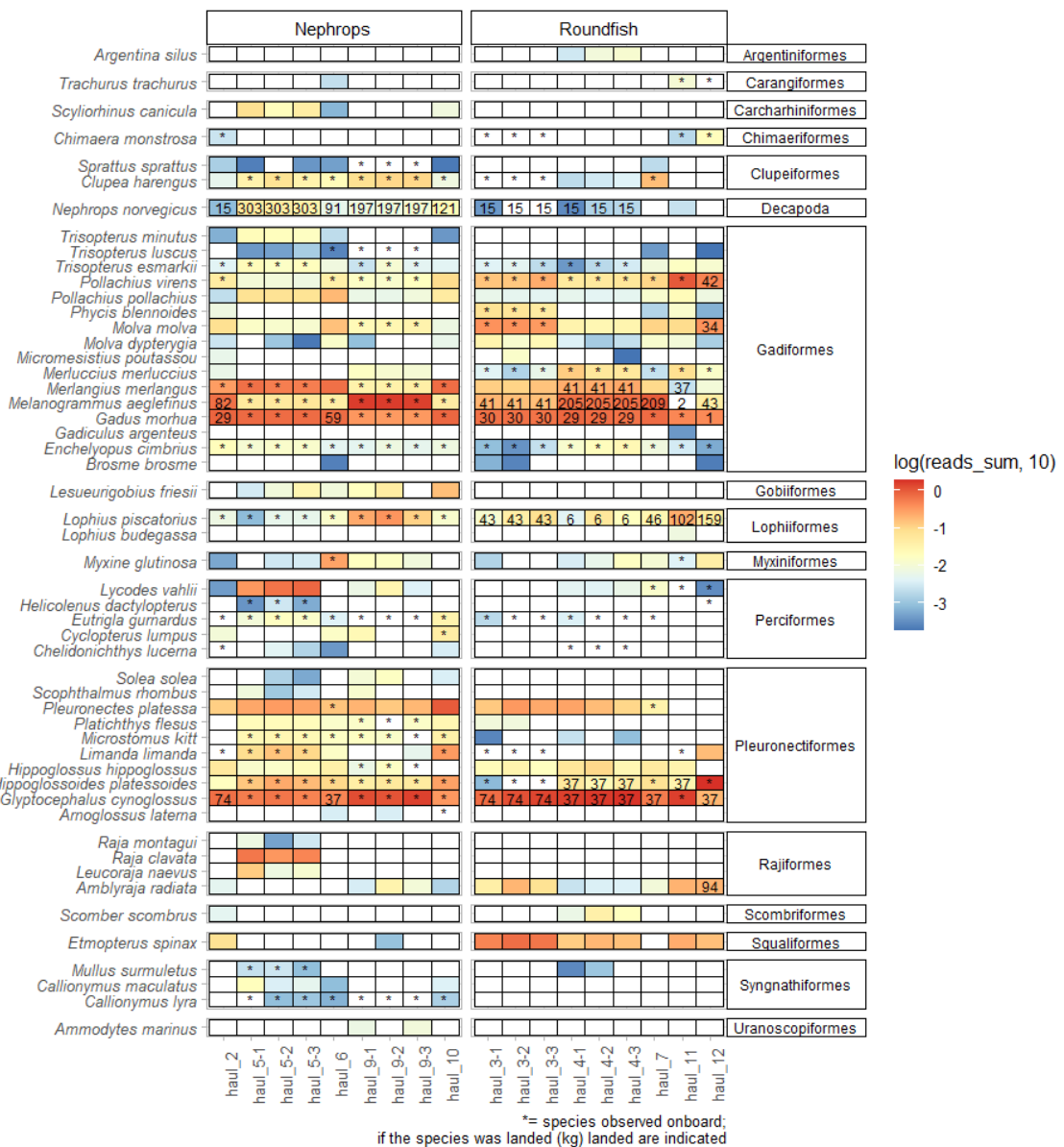
Figure 5.S4. Full overview of all observed fishery species (bony fishes, elasmobranch and Norwegian lobster). Number of sequences are depicted by colors and species observed by the scientific observer are shown by asterisks. Numbers inside the cells represent kilo of landed specimens.
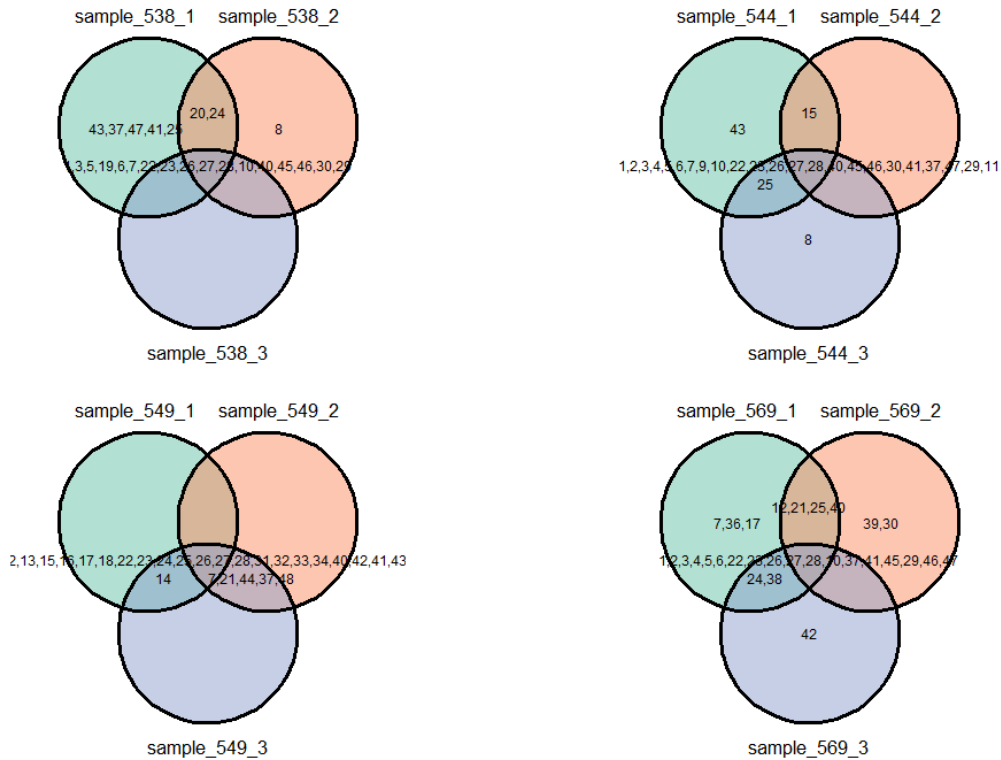
Figure 5.S5. Venn's diagram of species detected across three samples replicates and observed on board (white). 1=Gadus morhua, 2=Clupea harengus, 3=Molva molva, 4=Lycodes vahlii, 5=Enchelyopus cimbrius, 6=Trisopterus esmarkii, 7=Molva dypterygia, 8=Micromesistius poutassou, 9=Scomber scombrus, 10=Merluccius merluccius, 11=Argentina silus, 12=Lesueurigobius friesii, 13=Trisopterus minutus, 14=Sprattus sprattus, 15=Mullus surmuletus, 16=Trisopterus luscus, 17=Scophthalmus rhombus, 18=Helicolenus dactylopterus, 19=Phycis blennoides, 20=Brosme brosme, 21=Solea solea, 22=Glyptocephalus cynoglossus, 23=Pleuronectes platessa, 24=Platichthys flesus, 25=Microstomus kitt, 26=Lophius piscatorius, 27=Pollachius virens, 28=Pollachius pollachius, 29=Amblyraja radiata, 30=Etmopterus spinax, 31=Leucoraja naevus, 32=Raja clavata, 33=Scyliorhinus canicula, 34=Raja montagui, 35=Callionymus maculatus, 36=Cyclopterus lumpus, 37=Myxine glutinosa, 38=Ammodytes marinus, 39=Arnoglossus laterna, 40=Hippoglossus hippoglossus, 41=Hippoglossoides platessoides, 42=Limanda limanda, 43=Eutrigla gurnardus, 44=Chelidonichthys lucerna, 45=Melanogrammus aeglefinus, 46=Merlangius merlangus, 47=Nephrops norvegicus, 48=Callionymus lyra.

6. Assessment of the species composition on unwanted fishing catches through DNA analysis of storage water

Authors

Jos Kielgast[1], Magnus W. Jacobsen[1], Paulina Urban[1], Sara Maggini[2], Dorte Bekkebold[1] & Einar E. Nielsen[1]

Affiliations

[1]Section for Marine Living Resources, DTU Aqua, Technical University of Denmark, Vejlsøvej 39, 8600 Silkeborg, Denmark
[2]Molecular Ecology and Evolution Bangor, School of Natural Sciences, Bangor University, Environment Centre Wales, Bangor, UK

Status
Draft manuscript

6.1 Introduction

The landing obligation, which was introduced in connection with the revision of the common fisheries policy in 2013 and implemented in 2015-2019, stipulates that all quota fish species caught in commercial fisheries must be brought ashore and written off against the quota. Undersized fish must be taken home, may not be used for consumption, but must be entered separately in the logbook. This effectively means that a large number of species must be sorted (e.g. 28 for the North Sea) and this means that in the consumption fishery you have to set aside personnel resources for the task. At the same time, knowledge of species composition in unwanted catches can be relatively uncertain, as it depends on the crew's knowledge of species, as in certain cases may be limited outside specific target species for specific fisheries.

Due to this, there is a need for new methods that are capable of fast, safe and high precision assessment of the composition of unwanted catches commercial fishing. The methods must reduce the degree of manual work with sorting the catches (to reduce costs and time) but also reduce risk of misidentifications. One potential method is DNA-based identification. DNA-analysis has emerged as a powerful tool for marine species identification, also of complex samples where DNA from several species is mixed together (Jacobsen et al. 2019). One such example is so-called environmental DNA (eDNA). eDNA typically refers to genetic material sampled from the environment outside living hosts, such as feces, urine, skin cells, hair, dead or degraded organisms (e.g. Thomsen & Willerslev 2015). Depending on the DNA concentration, eDNA can be extracted directly from the water or up-concentrated by filtering water through a micropore filter to retain and concentrate eDNA before extraction (Hansen et al. 2018). Based on the eDNA approach (analyzing multiple species from a water sample), an increasing number of studies have explored the use of DNA analysis for assessing species composition in fisheries samples. Samples include processed food (e.g. canned fish and surimi) and fisheries samples of water draining from the net codend to fish silage (Heylar et al. 2017; Huxley-Jones et al. 2012; Pepe et al. 2007; Russo et al. 2021). Samples collected from fishing storage tanks or water used for pumping fish into fish factories also show great promise for assessing catch and bycatch in pelagic fisheries (Urban et al. 2022, 2023).

Here we investigate the potential use of storage water to identify and quantify unwanted catch representing either 'discard' (undersized quotated specimens) or samples with maximum diversity (mainly none quotated species, including some vulnerable species). This is done by analysing mock community samples and comparing the species' weight proportions in the mock with the observed DNA proportions from samples of storage water.

6.2 Methods

6.2.1 Sampling area

All samples were collected onboard a commercial bottom trawler (mesh size 90 mm) fishing in the Skagerrak (ICES subdivision 3a) in January 2021. The samples originated from 10 different fishing hauls targeting either Norway lobster *(Nephrops norvegicus)* or roundfish, such as Atlantic cod (*Gadus morhua*). Based on the target species, we refer to these samples as *Nephrops* and roundfish samples. Notably, the *Nephrops* fishery is at shallower depth (average mean depth= -80.37m, max= -172.78m, min= -54.01m) and during daytime, while roundfish fishery occurred at deeper depth (average mean depth= -237.74m, max=-305.89m, min= -183.38m) and at night.
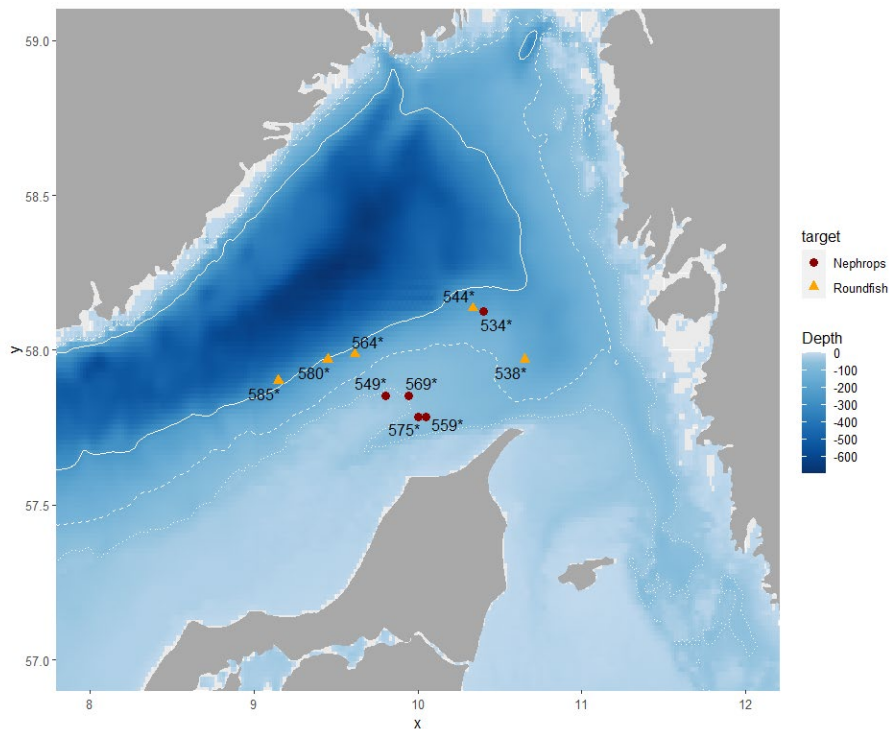
Figure 6.1. Sampling locations in the Skagerrak. *Nephrops* samples are indicated by red circles while roundfish samples by yellow triangle. For more details on each fishing haul see Supplementary information.

6.2.2 Experimental setup and sample collection

For each haul, two experiments where carried out to investigate the potential for utilizing complex DNA in water samples to quantify species in the total catch. Experiment one focused on quantifying the composition of species discarded under the landing obligation in a representative fraction of the total catch. This was done by collecting all discard from the first 10 minutes on the conveyer belt when processing each haul. All individuals were identified to species, weighed, total length measured. If necessary, fin clip samples were collected for later molecular based identification (barcoding). Then all specimens were placed in an experimental container double wrapped with two plastic bags (black trash bags, 40L). Natural seawater was poured into the container in a ratio of 30% seawater to 70% of fish based on weight. After one hour, three water samples were collected in sterile 50 ml centrifuge tubes. The second experiment examined a smaller fraction of the total catch aiming to maximise species diversity rather than focussing on reflecting a representative fraction of the catch. This was done by collecting a single individual of each species from each haul. The setup and sampling was identical to that of the first experiment.

6.2.3 DNA extraction

Extraction of eDNA from the water samples was performed in a dedicated clean laboratory facility at DTU Aqua (Technical University of Denmark, Silkeborg, Denmark). We analysed three samples per haul and 5 hauls from each target fishery. After thawing the samples at room temperature, a total of 20 mL of catch water was pressed through a 0.22 µm Sterivex filter (SVGPL10RC, Sigma-Aldrich, St. Louis, MO, USA) using a sterile 60 mL disposable syringe (Becton, Dickinson and Company, Franklin lakes, NJ, USA) to retain eDNA and cells. We followed a modified version of Spens et al. 2017 extraction protocol for eDNA extraction from filters, using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The final elution volume was adjusted to 200 µl of Buffer AE and samples were stored at -20°C. Each extraction batch included a blank sample to test for exogenous DNA contamination through the used reagents or from the laboratory. Surfaces and laboratory equipment were cleaned with bleach solution, distilled water and 70% ethanol before and after samples processing in each batch. The eDNA concentration was measured for all samples using a Qubit fluorometer and the Qubit dsDNA high sensitivity kit (ThermoFisher Scientific, Waltham, MA, USA).

6.2.4 DNA metabarcoding

DNA samples were processed in 4 technical replicates using well characterized generic primers Leray-XT (Wangensteen et al. 2018) amplifying ~313 bp of the cytochrome c oxidase subunit I in metazoans. (Forward primer: mlCOIintF-XT 5'-GGWACWRGWTGRACWITITAYCCYCC-3' Reverse primer: jgHCO2198 5'-TAIACYTCIGGRTGICCRAARAAYCA-3' Each sample was run with primers containing unique identifier barcodes that enable pooling of amplicons in a single sequencing run. Each barcode was 7 bp long and had a minimum of 3 mismatches to other barcodes. Due to the relative high error rate introduced during MinION sequencing, barcodes were applied on both forward and reverse primers to ensure low risk of misidentification. PCR reactions were performed in 20 µL reaction volumes containing 10 µL of AmpliTaq Gold Master Mix (Applied Biosystems, Foster City, CA, USA), 1 µl of each forward and reverse primers (5µM), 0.16 µL BSA (20 µg/ µL), 3.84 µl of water and 4 µl of eDNA. The PCR reactions were run with an initial denaturation step of 10 minutes at 95°C, followed by 40 cycles of 94°C for 1 min, annealing at 45°C for 1 min, followed by 1 min extension at 72°C, and a final extension at 72°C for 5 min after the 40 cycles. All PCR runs included a negative control. After the amplification, 2 µl of amplicons were verified by electrophoresis on an 2% agarose gel. The four PCR replicates were pooled and cleaned using Agencourt AMPure XP magnetic beads in accordance with manufacturer's specifications. DNA concentration assessed using Qubit fluorometer and the Qubit dsDNA high sensitivity kit (ThermoFisher Scientific, Waltham, MA, USA). Next, the amplified DNA was mixed in equimolar ratios for each of the samples individually, and 100 fmol of the pooled amplicons was used for the library preparation. A library was prepared for each sample separately, using the Nanopore Amplicons by Ligation (SQK-LSK110) sequencing kit, according to manufacturer's instruction. Sequencing was performed on the MinION Mk1C from Oxford Nanopore using flow cell (R9.4.1). Following the protocol recommendation, 20 fmol of the final library was loaded onto the flow cell and the sequencing run started, enabling live base calling using Guppy, selecting the High accuracy (HAC) model. The base called reads were then exported for further analysis.

6.2.5 Bioinformatic analysis of Nanopore reads

Analysis of the sequenced DNA fragments was conducted using different bioinformatics software. First, all sequences were filtered for size and quality using NanoFilt (https://github.com/wdecoster/nanofilt) to exclude all none target sequences and ensure high quality data. Specifically we excluded all sequences <345 and >500 base pairs and included only reads with a quality score of ≥10 for the downstream analysis. Reads representing the individual samples were sorted accordingly to their unique DNA barcodes using the software cutadapt (https://cutadapt.readthedocs.io/en/stable/)allowing a maximum of one base mismatch. Given the barcodes

shows a minimum three mismatches from each other this ensured correct identification of the bar-coded samples. Subsequently, all sequences were analysed again using cutadapt to remove the Leray-XT primer sequence. NGSpeciesID (https://github.com/ksahlin/NGSpeciesID) was used to collapse and count highly similar reads into consensus sequences that then could be matched against a reference nucleotide sequence database (https://www.ncbi.nlm.nih.gov/) to identify and quantify species specific DNA in each samples (The general bioinformatics pipeline can found on github https://github.com/Srm18crx/reads_pipeline).

6.2.6   Catch water analysis

Overall, we analyzed replicate samples from 19 different mock communities. The mock samples represented 'discard' samples made from unwanted catch (undersized and not quotated specimens) (N = 10) and samples with maximal biodiversity including PETS (protected, endangered and threatened species) (N = 9), which were collected and sampled on board a fishing vessel targeting either *Nephrops* or roundfish.

The mock diversity samples included between 10-14 different species (average 11.66). The mock discard samples contained 9-12 species (average 10.44) per sampled from the Nephrops fishery and 8-10 species (average 8.6) collected during the roundfish fishery. The number of identified species was approximately the same for the eDNA samples with 9-14 species (average 10.48) for the diversity samples, but higher for the eDNA discard samples with 9-18 species (average 14.4) and 8-15 species (average 11.07) from the *Nephrops* and roundfish fishery. The eDNA method detected the majority of the species in the mock community samples, but it was considerably lower for the diversity samples. Here, the DNA method detected 41.7-72.7% of the species (average 55.4%) in the individual analysed replicates. When combining results from all replicates the number increased to 46.1-72.7% (average 59.6%) positive detections per sample. For the discard samples, the DNA method showed a higher detection rate with 44.4-83.3% of the species (average 61.1%) detected in the individual analysed replicates from the Nephrops fishery and 50-100% of the species (average 73.4%%) from the roundfish fishery. When combining results from all replicates the numbers were 44.4-83.3% (average 62.9%).and 62.5-100% (average 81%), respectively. Venn diagrams showing the number of shared species are shown in Figure 6.2-4 below. For an overview of the specific species in each mock sample and the species detected in the individual DNA samples see Figure 6.S1-S3 in the Appendix.

DNA analysis of control samples (representing 'clean' storage tank water without fishing catch) and PCR negatives showed low levels of back ground contamination. However, read counts were generally very low supporting, which supported a neglectable level of back ground contamination.
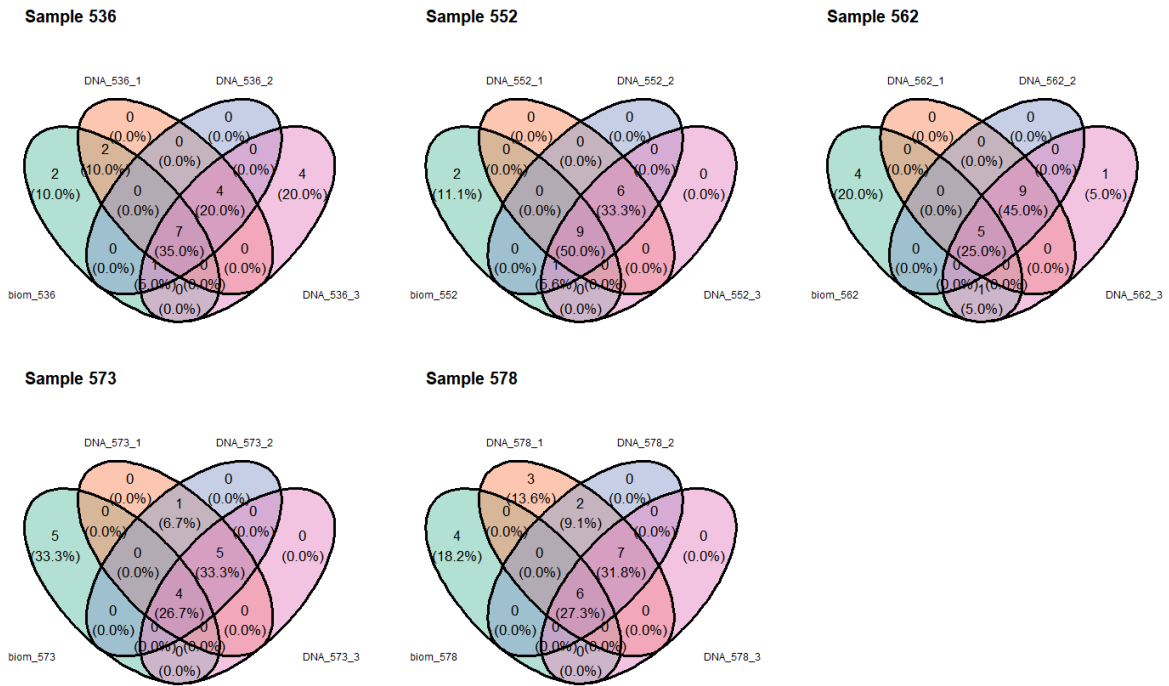
Figure 6.2. Venn diagrams showing the number of shared species between mock and eDNA samples representing discard samples collected from the Nephrops fishery.
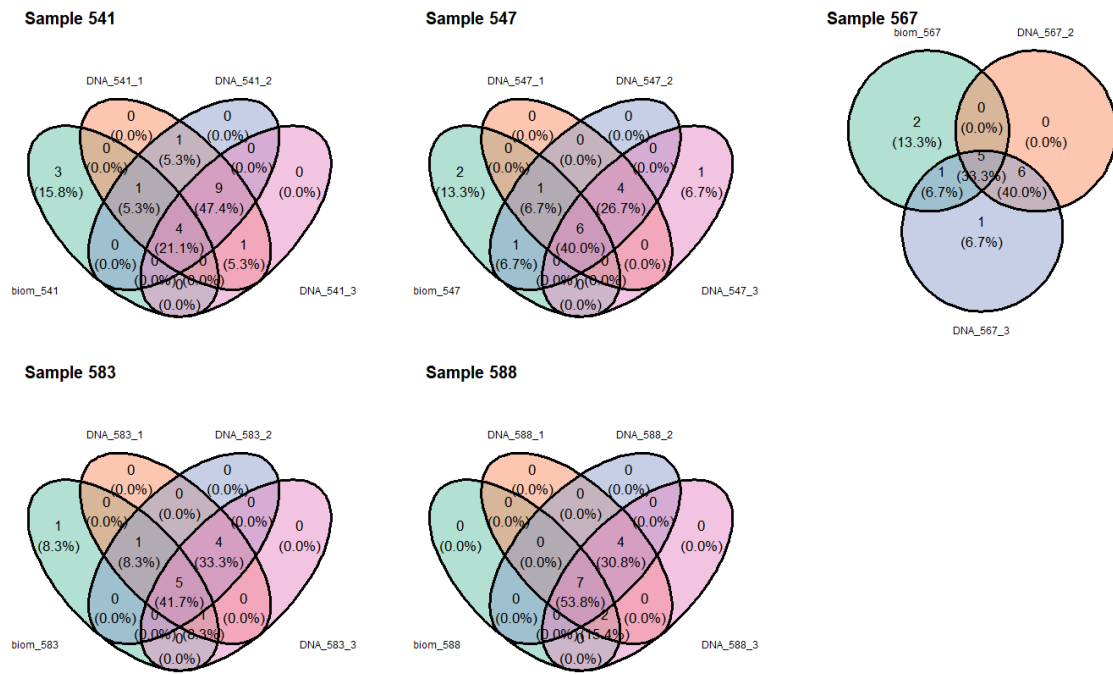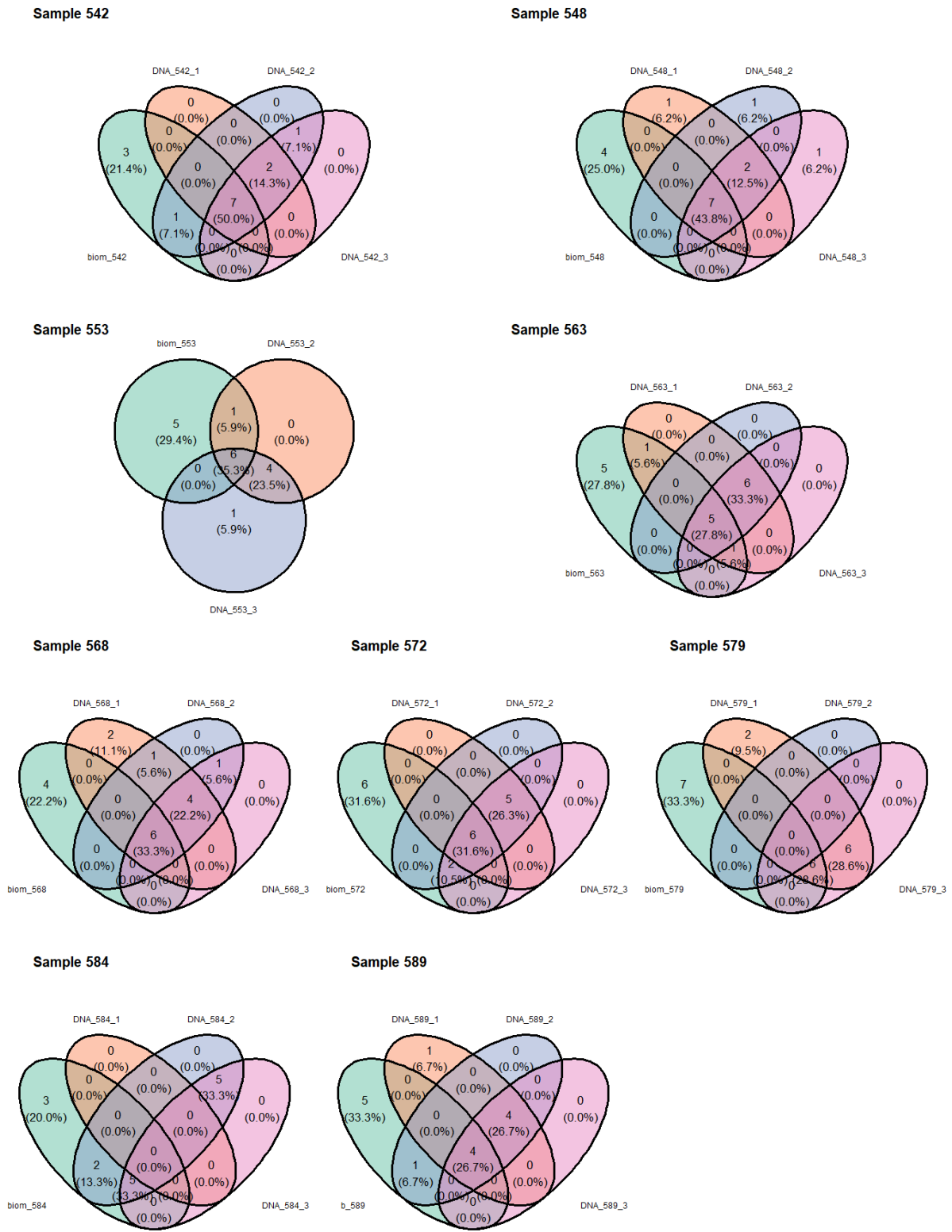

Figure 6.3. Venn diagrams showing the number of shared species between mock and eDNA samples representing discard samples collected from the Nephrops fishery.

Figure 6.4. Venn diagrams showing the number of shared species between mock and eDNA samples representing discard samples collected from the Nephrops fishery.

6.2.7 Species proportions and comparisons between mock and DNA samples

Sampling variation was generally low as most sampling replicates showed very similar DNA profiles in terms of identified species and their relative DNA proportions (Fig. 6.4 and Fig. 6.5). However, the

proportions in the mock sample (calculated using the weight) did not match the proportions analysed from the eDNA samples.
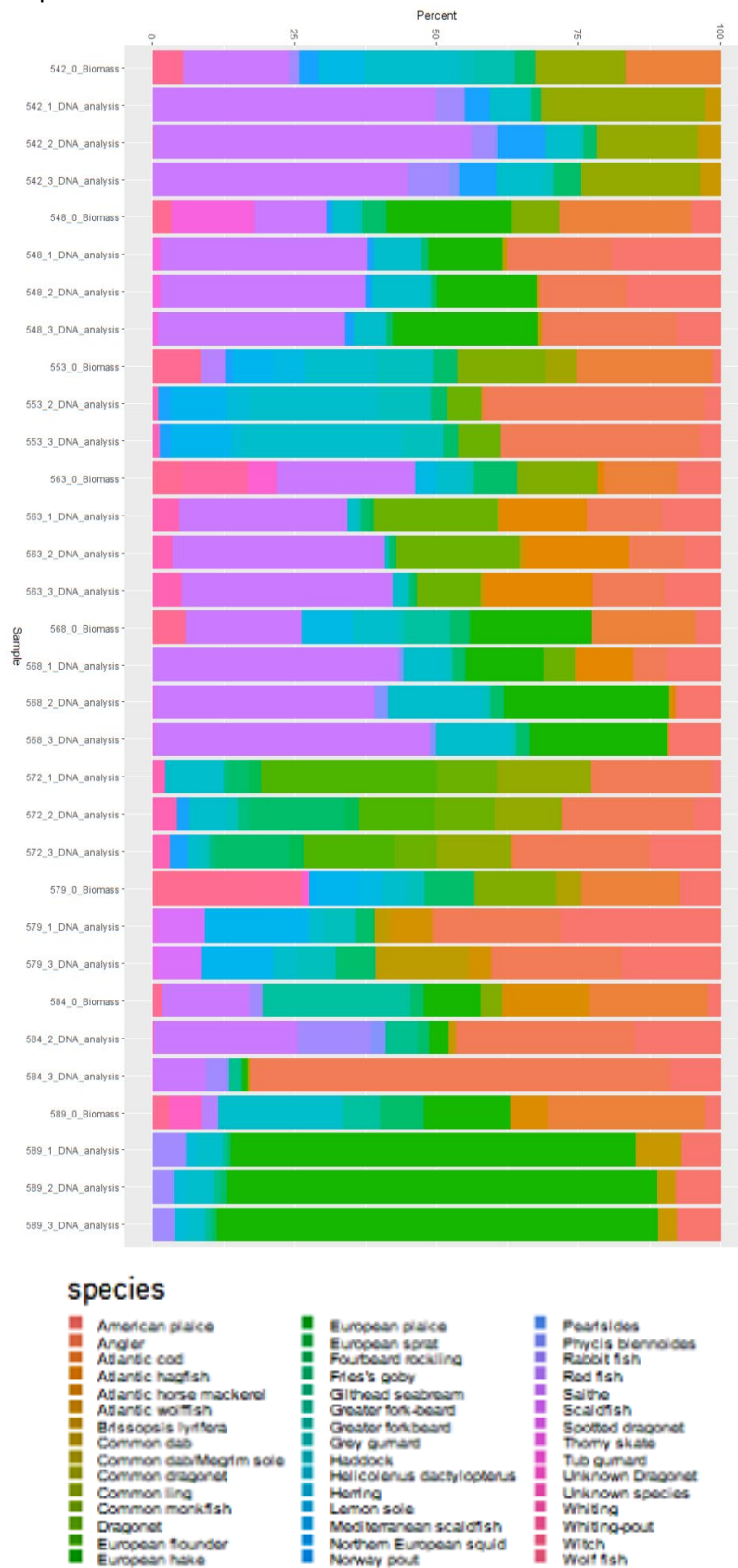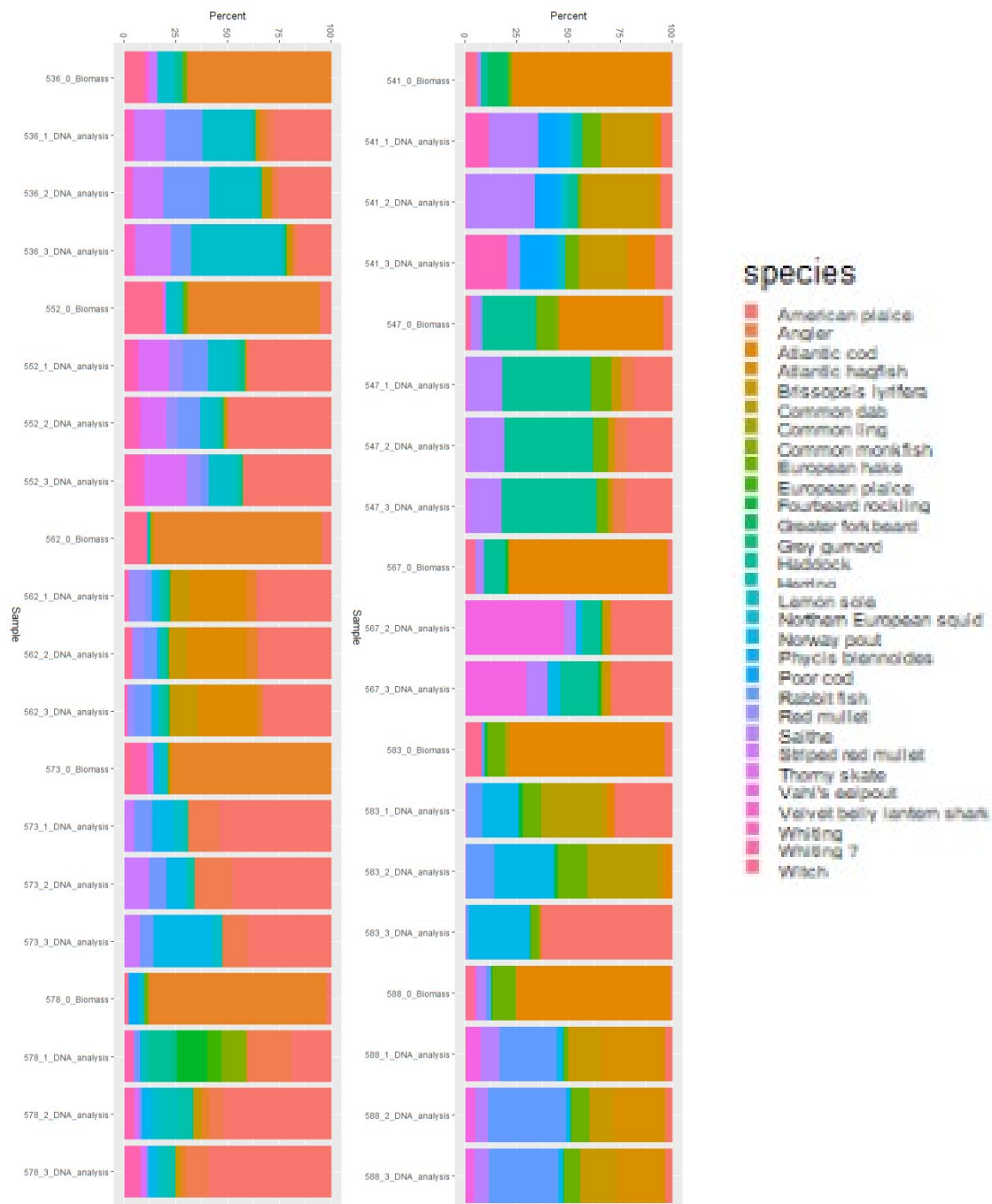

Figure 6.5. Histogram showing the species proportions in the mock (biomass) and eDNA samples (DNA_analysis) observed in the diversity samples. Mock samples are matched with the representative

Figure 6.6. Histogram showing the species proportions in the mock (biomass) and eDNA samples (DNA_analysis) observed in the discard samples. The plot on the top represents samples collected during the Roundfish fishery and the plot in the bottom represents samples collected during the Nephrops fishery. Mock samples are matched with the representative eDNA samples in the plot and represented by a sampling number. Species names and species colour codes can be found in the legend to the right.

6.3 Discussion

The DNA analyses of the complex samples from the demersal fisheries (lobster and roundfish) showed a high potential for describing species composition and biodiversity in the samples. The DNA signal was very similar across replicate samples, which supports the use of small water volumes to

analyse fishing catches. However, there was a relatively weak correlation between weight and DNA proportions. This might be a result of species differences in DNA excretion and/or primer bias leading to biased DNA proportions. Hence, it likely difficult to obtain more than a semi-quantitative estimate of the catch composition.

6.4   References

Jacobsen M.W., Hansen B.K., Nielsen E.E. (2019) Possible Uses of Genetic Methods in Fisheries Under the EU Landing Obligation. In: Uhlmann S., Ulrich C., Kennelly S. (eds) The European Landing Obligation. Springer, Cham. https://doi.org/10.1007/978-3-030-03308-8_20

Helyar, S.J., Lloyd, H.A., de Bruyn, M., Leake, J., Bennett, N., Carcalho, G.R. (2014). Fish product mislabelling: Failings of traceability in the production chain and implications for illegal, unreported and unregulated (IUU) fishing. PLoS One, 9, https://doi.org/10.1371/journal.pone. 0098691.

Pepe, T., Trotta, M., Di Marco, I., Anastasio, A., Bautista, J.M., Cortesi, M.L. (2007). Species identification in surimi-based products. Journal of Agricultural and Food Chemistry, 55, 36813685. https://doi.org/10.1021/jf063321o

Russo, T., Maiello, G., Talarico, L., Baillie, C., Colosimo, G., D'Andrea, L., Di Maio, F., Fiorentino, F., Franceschini, S., Garofalo, G., Scannella, D., Cataudella, S., and Mariani, S. (2021). All is fish that comes to the net: metabarcoding for rapid fisheries catch assessment. Ecological Applications 31(2):e02273. 10.1002/eap.2273

Urban P., Bekkevold D., Degel H., Hansen B. K., Jacobsen M. W., Nielsen A., Nielsen E. E., Scaling from eDNA to biomass: controlling allometric relationships improves precision in bycatch estimation, *ICES Journal of Marine Science*, 2023;,fsad027, https://doi.org/10.1093/icesjms/fsad027

Urban, P., Bekkevold, D., Hansen, B. K., Jacobsen, M. W., Nielsen, A., & Nielsen, E. E. (2022). Using eDNA to estimate biomass of bycatch in pelagic fisheries. *Environmental DNA*, 00, 1– 16. https://doi.org/10.1002/edn3.377

Wangensteen O.S., Palacín C., Guardiola M., Turon X. (2018). DNA metabarcoding of littoral hard-bottom communities: high diversity and database gaps revealed by two molecular markers. *PeerJ*. 2018;6:e4705. doi:10.7717/peerj.4705
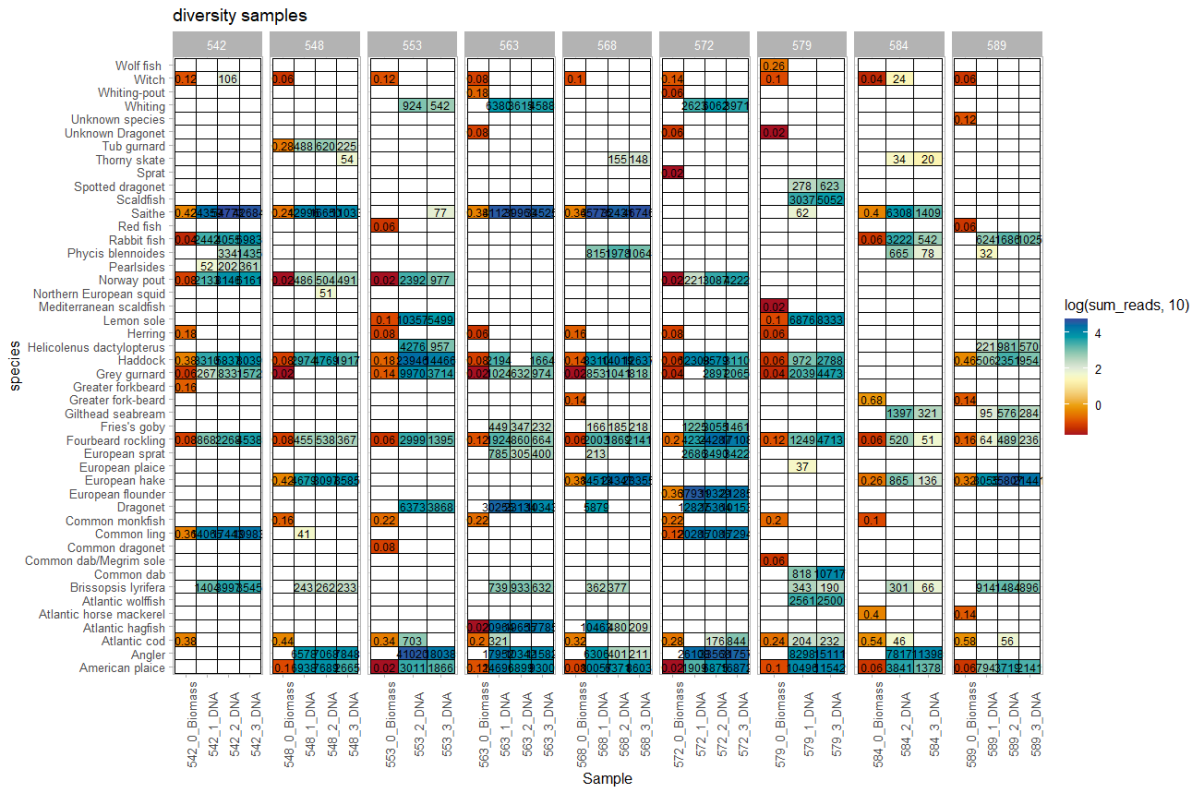
6.5 Appendix
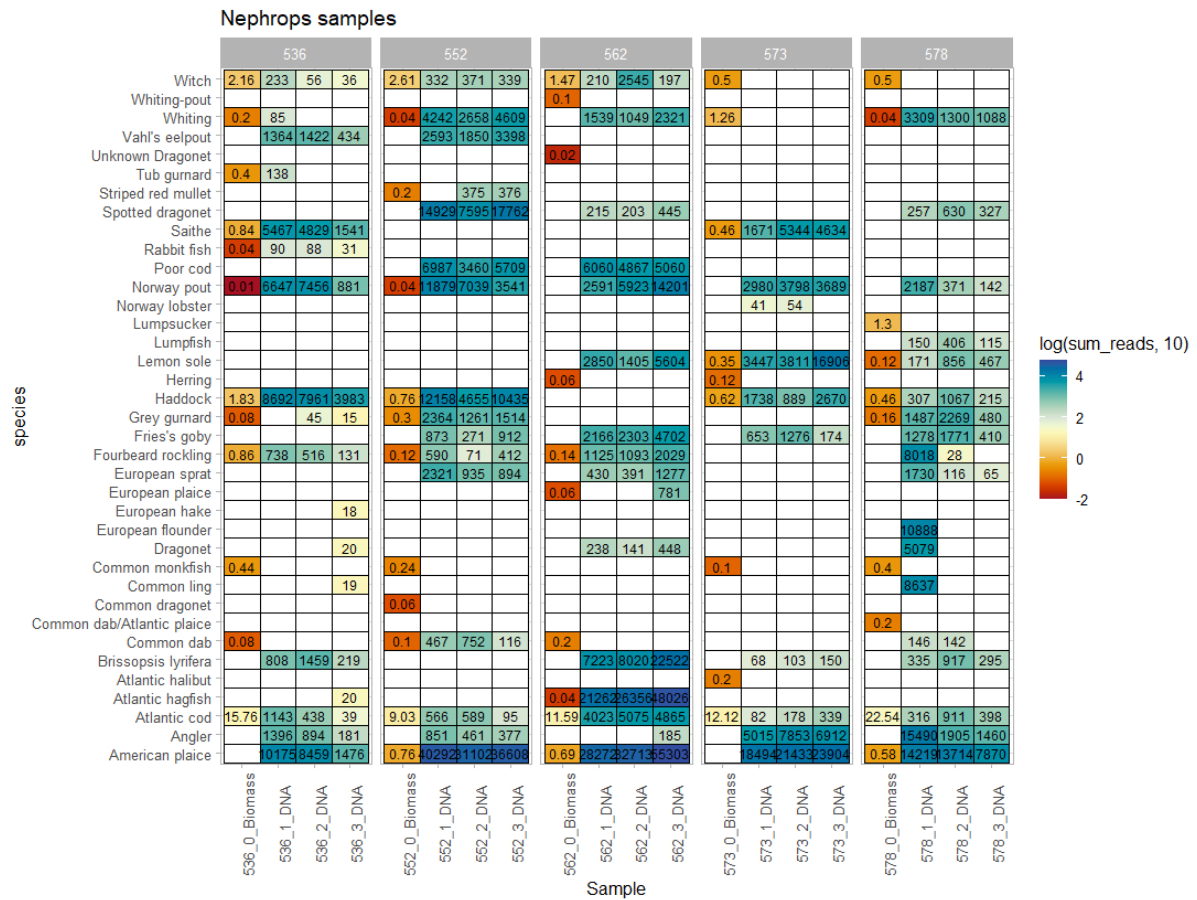
Figure 6.S1. Full overview of the species in the mock samples and the species detected in the eDNA samples. The numbers inside each cell represent cumulative weight (kg) of the species for the mock samples (samples names 'biomass') and number of reads in the eDNA samples (samples named 'DNA').
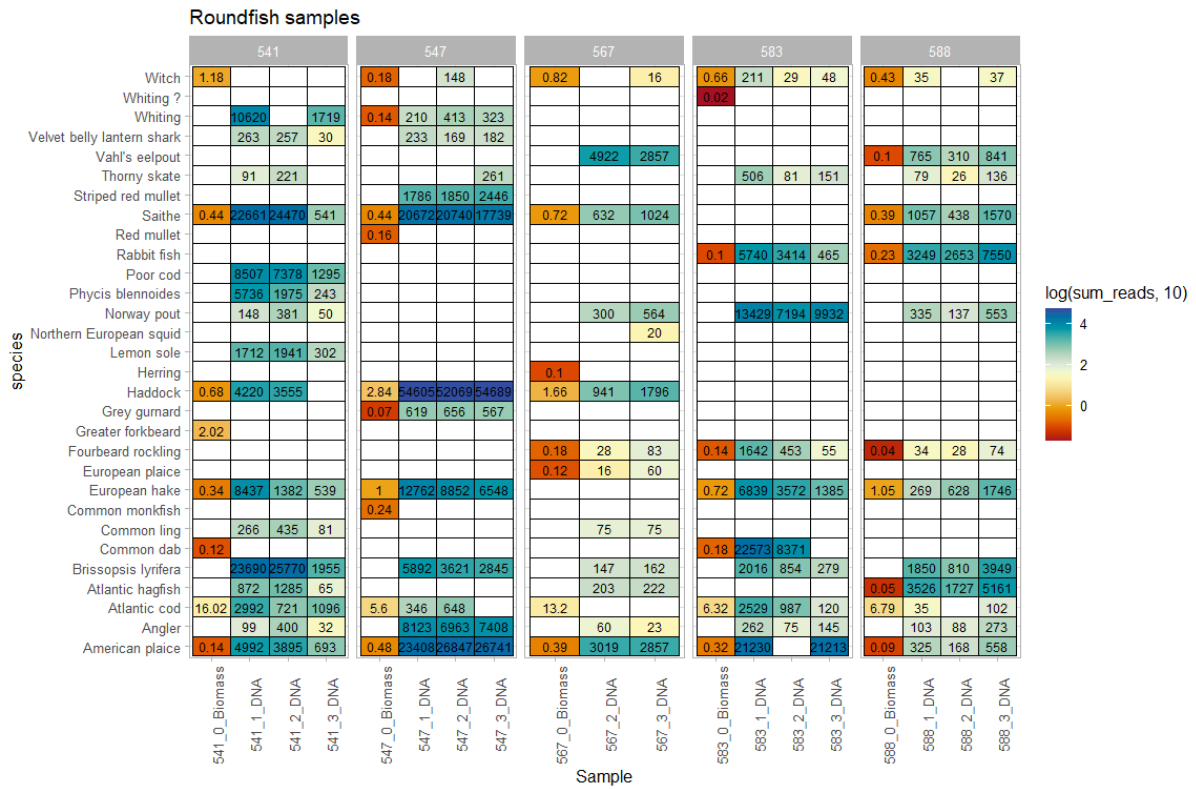
Figure 6.S2. Full overview of the species in the mock samples and the species detected in the eDNA samples. The numbers inside each cell represent cumulative weight (kg) of the species for the mock samples (samples names 'biomass') and number of reads in the eDNA samples (samples named 'DNA').

Figure 6.S3. Full overview of the species in the mock samples and the species detected in the eDNA samples. The numbers inside each cell represent cumulative weight (kg) of the species for the mock samples (samples names 'biomass') and number of reads in the eDNA samples (samples named 'DNA').