Project StockDNA;
Combining marine eDNA and hydroacoustics to improve the accuracy of pelagic fish monitoring surveys.

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Submitted in partial fulfilment of the requirements of Liverpool John Moores University for the degree of Doctor of Philosophy.

This research programme was carried out in collaboration with the Centre for Environment, Fisheries and Aquaculture Science

January 2023

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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Acknowledgements

“Firmitas, Utilitas, and Venustas”

Vitruvius, Rome, 70-60BC

Stefano Mariani, WhatsApp, 06/06/19

Strength, Utility and Beauty is the quote I received from my supervisor when I asked, “How will I get through this PhD?”. I have found that this quote does not only apply to my research and thesis structure but also to life as an academic and in a poetical ‘*Ottava rima*’ style I will continue the use of this quote to structure my acknowledgements.

Strength

Like the architectural origins Vitruvius wrote about, strong foundations were needed for me to weather the stress that is inertly attached to the PhD academic and personal life. At the base of my foundations is my family and soon to be wife Hilary. Without the love, care and support given to me by every member of my family I would likely not be in this position today. The foundations of my strength have been continually supported from friends, both within and outside of academia. I would personally like to thank my childhood group of friends (SFSA) and university friendships that have always been there when I have needed you. I would also like to personally thank Prof Gary Carvalho who initially motivated me to apply for Bangor University when I was still in high school. Without Gary’s guidance (and occasional motivational speeches) I would not have been able to open as many doors, meet as many wonderful people and advance my scientific career.

The strength to complete this PhD was also provided by some key people during the past 4 years. Although they have occasionally disagreed with each other, I do believe that I had the perfect supervisory triforce in Prof Stefano Mariani, Dr Veronique Creach and Dr Jeroen Van Der Kooij. Both individually and as a team they have helped solve many pieces to the PhD puzzle, emotionally supported me through hard times, and forged me

into the academic I am today. I will be forever grateful that you chose me for this PhD and forever thankful for the opportunities and support you have all provided. I would also like to thank the past and present Salford, cefas, and LJMU lab teams and support staff. Without the many academics from these institutes my PhD would have been a lonely, boring, and calamitous experience so hopefully I will continue to also provide strength to each of you as we move forward with our careers.

Utility

To borrow the words of Cher, I would like to take this opportunity to “Turn back time”, back to when I first sang this song in front of my new lab partner Charles Baillie in the eDNA lab in Salford University. Like many other recent PhD students my academic experiences have been somewhat marred by the changes brought about by covid that also coincided with my transition from Salford University (where I spent the first year of my PhD) to Liverpool John Moores University. Little did I know that the ‘Utility’ of my PhD would refer both to my thesis topics but also to the adaptability of myself as an academic to work from home and rapidly “grow up” as an academic researcher. Without the guidance and support of Charles Baillie and Peter Shum in the lab and remote bioinformatics troubleshooting help from Rupert Collins I would not have achieved the many great results in this thesis and would have infinitely struggled without them.

Beauty

Sadly, I do not believe that this part of the quote refers to my Hawaiian shirt wearing receding hairline charms but more of the beauty of the project. Thus, I would like to acknowledge the funding bodies (NERC, cefas, defra, LJMU, Salford University, Thunnus UK, and the fsbi) that enabled me to create this “beautiful” thesis and more importantly facilitated my progress as an academic so that I can continue to pursue more beautiful ideas and projects in the future.

And of course, I can’t not mention my beautiful fiancée Hilary. We may have struggled at times through the stress and hardships that the PhD lifestyle brings but we got through it together and we are all the stronger for it.

Contributions to Data Chapters

Chapter II

Christopher Brodie designed the sampling protocols and collected the samples with Marine Cusa onboard the RV Endeavour. The lab protocols were co-designed and conducted by Christopher Brodie and Charles Baillie. Bioinformatics pipelines were designed by Rupert Collins and troubleshooted by Christopher Brodie and Rupert Collins. The Chapter was written by Christopher Brodie and reviewed by Veronique Creach, Jeroen Van Der Kooij and Stefano Mariani. All aspects of the chapter were also developed with advice from Veronique Créach, Jeroen Van Der Kooij and Stefano Mariani.

Chapter III

Christopher Brodie, Tom Horton, and Matthew Witt designed the sampling protocols and Tom Horton collected and filtered the sample water. The lab protocols were conducted by Christopher Brodie and Peter Shum. Bioinformatics was troubleshooted by Christopher Brodie. The Chapter was written by Christopher Brodie and reviewed to by Veronique Créach, Jeroen Van Der Kooij and Stefano Mariani. All aspects of the chapter were also developed with advice from Veronique Creach, Jeroen Van Der Kooij and Stefano Mariani. David Righton, Jeroen Van Der Kooij, Matthew Witt was responsible for obtaining the funding.

Chapter IV

Christopher Brodie designed the sampling protocols and collected the samples in 2018 and 2019 and with Marine Cusa in 2018. The lab protocols were co-designed and conducted by Christopher Brodie, Charles Baillie and Peter Shum. Bioinformatics pipelines were designed by Rupert Collins and troubleshooted by Christopher Brodie and Rupert Collins. The Chapter was written by Christopher Brodie and reviewed by Veronique Créach, Jeroen Van Der Kooij and Stefano Mariani. All aspects of the chapter were also developed with advice from Veronique Créach, Jeroen Van Der Kooij and Stefano Mariani.

Abstract

The vast and dynamic swathes of pelagic ecosystems in the North-East Atlantic house multiple ecologically and economically important fish species. To ensure that humans do not over exploit these fish, and their habitats, multiple routine monitoring surveys take place. These surveys typically involve the use of hydroacoustic echosounders with trawling methods and collect information on the age, sex, size and weight of species that is used in dynamic models to predict changes in the mortality rate and stock size of cohorts of species. Although this method can be used to target pelagic fish, it has difficulty separating fish schools by species, typically requiring trawling validation methods to confirm the identity of the fish. However, trawling does not guarantee a representative catch of the fish schools which can skew the partitioning of hydroacoustic data, resulting in non-valid estimations of pelagic fish populations. Environmental DNA (eDNA) is a tool that has been utilised to detect pelagic fish species and is not impacted by the same bias as trawling. To investigate the use of eDNA to become a “net with no holes”, validating fish schools detected by hydroacoustics, this thesis first aimed to refine the eDNA sampling strategy for marine pelagic fish. By collecting eDNA during routine pelagic hydroacoustic surveys this thesis first demonstrates that the sampling depth and hydrological conditions of the water do not significantly impact the number of pelagic fish species, communities, or the number of eDNA reads. Using these inferences on the eDNA sampling strategy for pelagic fish facilitated further investigations into the temporal utilisation of eDNA sampling for the highly migratory Atlantic bluefin tuna (ABT). Whereby surface water eDNA samples were collected from smaller, more cost effective, research vessels from summer to winter. I found that using this method ABT was detected across seasons while also revealing the presence of important potential prey species and marine predators providing useful information for future monitoring surveys. Additionally, the results of this thesis revealed significant correlations between the current trawl-validated and eDNA-validated hydroacoustics of pelagic fish. Fish species that were more abundant and widespread had stronger correlations, yet there were still large variances between species and sample years. Overall, this thesis demonstrates that eDNA can be used to verify the abundance of pelagic fish and suggests that the eDNA methods for

validation are more appropriate for specific, high abundant, fish species where the DNA in the water column is more easily detected.

Chapter I

General Introduction

1.1 Monitoring Marine Pelagic Fish using Molecular Methods

Marine pelagic ecosystem functioning, and health are under increasing pressures from climate driven effects such as microalgal blooms, invasive species, and regime shifts in pelagic populations, which are currently being exacerbated by human activities (Verity et al., 2002; Halpern et al., 2008; Ruckelshaus et al., 2011; Bruno et al., 2018). These activities include overfishing, plastic pollution, introductions of invasive species, oil spillages and water contaminants as well as, human induced climate change that influences; salinity, oxygen, nutrient concentrations, and pH changes across pelagic ecosystems (Sarmiento et al., 2004; Beaugrand and Kirby, 2018). The ecological ripples created by these impacts, if left unchecked, could form waves of knock-on effects throughout entire trophic webs (Scheffer et al., 2005), such as the loss or significant reduction of important socio-economic species and ecosystem services (Möllmann et al., 2009; Last et al., 2011; Cebellos et al., 2015). Monitoring surveys act as our first line of defence against these changes to our marine pelagic systems by providing a suite of information that can be used to predict changes to biological and ecological parameters over time and inform future actions (Patrício et al., 2016). Notable parameters include abiotic factors such as water temperature, salinity, and pH, as well as biological features such as species composition, biomass, age, sex, and distribution ranges (Legrand et al., 2003; Danovaro et al., 2006). Using the inferences from these surveys we can produce data series to identify trajectories that can then predict the future changes in the marine environment; this can help inform policies that develop strategies to reduce the risks posed to our oceans (Sukhotin & Berger., 2013; Nygård et al., 2016; Danovaro et al., 2016).

Pelagic fish are one of the most abundant macro-organisms that inhabit marine pelagic waters, because of their high abundance they underpin major food webs in the marine environment which maintains the ecological functioning of their ecosystems (Jennings et al., 2009). A common monitoring practice for pelagic fish is hydroacoustics, a semi-quantitative method that continually scans a water body using energy pulses that reflect off fish. The reflections, also known as echoes, create backscatter energy that can be detected by a receiver and translated into a visual representation of the fish in the water (MacLennan et al., 2002; Popper et al., 2006). Hydroacoustic methods are more efficient for the detection of swim-bladdered species because swim bladders best reflect backscatter energy ensuring a stronger and clearer representation of individuals in the water column (Foote, 1980). The hydroacoustic frequency of these energy beams can be accurately adjusted, or run in parallel, for different size species. A wider beam at a frequency of 38 kHz is used for small pelagic fish or juveniles and a narrow beam at 120kHz for larger pelagic fish (Baran et al., 2017 Coetzee et al., 2008).

Hydroacoustic technology has been a significant development in marine pelagic ecology monitoring, facilitating more accurate research into the ecology of multiple different species, particularly for those in inaccessible areas of the ocean (Benoit-Bird, 2015). The use of hydroacoustics has allowed monitoring to shift away from weather reliant, more destructive and species-specific capture-based methods such as gill nets and bottom trawling to a fast, multispecies, and cost-effective way of routinely monitoring marine ecology (Mayer et al., 2002; Polunin et al., 2009; Benoit-Bird, 2015; Doray et al., 2018; Egerton et al., 2018; Kok et al., 2021). It is also a dynamic method that can be used for fish biomass calculations by analysing the density and area of fish schools in-situ or can be adjusted to observe other aspects such as the hydrology of the water or other species e.g., phytoplankton, meso-zooplankton, and other megafauna such as cetaceans and seabirds (Benoit-Bird, 2015; Doray et al., 2018).

However, the main disadvantage of this method is that sometimes it is difficult to verify whether a fish school is solely composed of one species. To partition the fish schools into different species components monitoring surveys use pelagic mid-water trawling to catch these schools, which are also used to collect information on the age, sex, and size of the

pelagic fish (Boldt et al., 2018; Bean et al., 2017; Rowell et al., 2019; Fig.1.1). Monitoring methods that use trawling and hydroacoustic based equipment are highly costly to set up, time consuming, requires vessels that are produce low mechanical noise that does not interfere with the hydroacoustics (Urick, 1978) and can have the equipment needed to deploy and haul a pelagic net. Even with the correct set up and vessel, the trawling component of this monitoring method used to ground truth the hydroacoustic data can be unreliable. This is because trawling is not guaranteed to catch a sample of pelagic fish that has the same proportions of species that is found from the fish schools in the monitored region (Kaartvedt et al., 2012; Bassett et al., 2018). Although there are algorithms that aid in the identification of certain fish species, such as the Atlantic mackerel (*Scomber scombrus*), it is difficult to quantify pelagic fish that lack a swim-bladder or that have peculiar backscatter properties (Korneliussen, 2010; Popper et al., 2006). In addition, fish species with sleeker body types such as eels, scabbard fishes, sandlances, garfishes etc, have significantly lower catchability rates from a pelagic trawl in comparison to other orders of pelagic fish (Heino et al., 2011). When fish schools have mixed age classes within midwater-trawls, depending on the trawl net design, are more likely to catch larger adults than smaller juveniles (Williams, 2013; De Robertis et al., 2021).

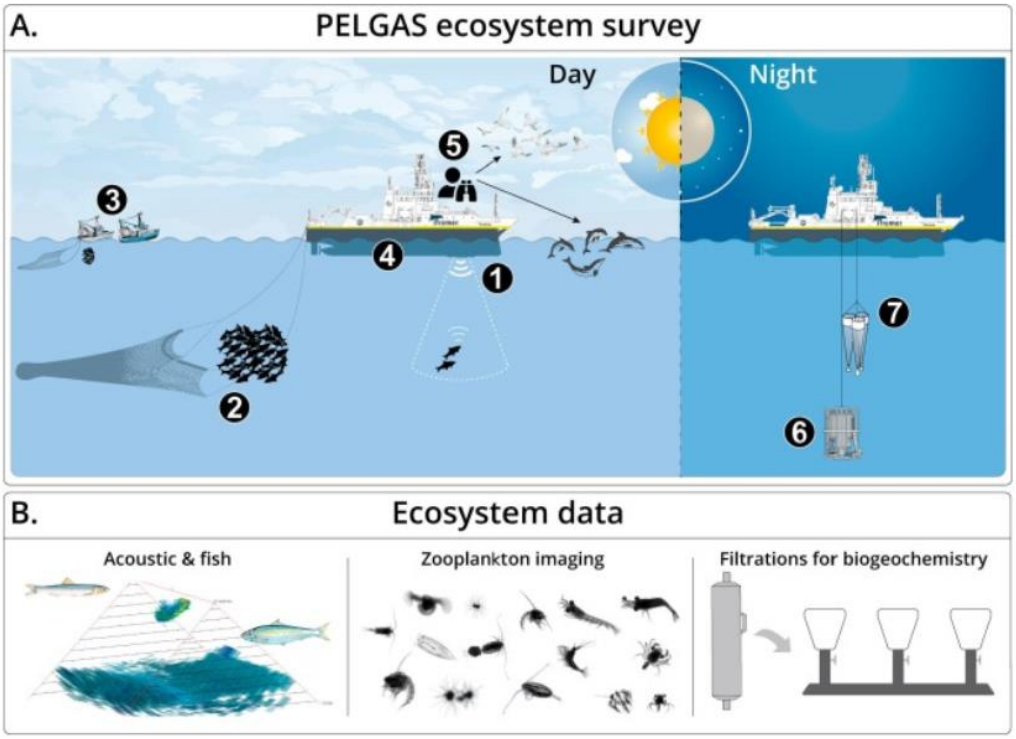


Figure 1.1 Model of the Bay of Biscay pelagic ecosystem survey (PELGAS).
A.) Ecosystem data collection; During daytime along a transect: 1. Hydroacoustics is conducted detecting the presence of fish schools, 2. When a school of the target fish species is detected midwater trawling is carried out, 3. Consort commercial pair trawlers fishing, 4. Hull-mounted thermo salinometer, seawater is pumped in from here and ran through a FerryBox system. The FerryBox system is a unit that consists of multiple sensors that is hooked onto the seawater pump from under the vessel. This is used to assess the temperature, salinity and pH of the area , 5. Onboard observers are actively looking for sightings of megafauna and recording the number of species and number of individuals sighted on each daytime transect. During night-time, at fixed stations: 6. CTD rosette deployment to collect water samples from depth and record abiotic variables such as temperature, salinity and stratification, 7. Meso-zooplankton nets are deployed.
B.) Onboard ecosystem data pre-processing: Backscatter energy of the fish schools are assessed to determine what species the schools could be and the density of the schools is observed, this information is then used to decide whether to conduct a midwater trawl. Zooplankton images using a camera mounted microscope are taken from the catch of meso-zooplankton nets after the zooplankton have been washed with distilled water and biogeochemistry is recorded using the water samples from the CTD rosette deployment. (Doray et al., 2018 [Edited]).

There have been growing calls for the implementation of environmental DNA (eDNA) into marine biodiversity monitoring practices. This method utilises the trace DNA of macro-organisms found in the environment to become a new tool in the ‘utility belt’ of monitoring

surveys (Darling et al., 2017; Gilbey et al., 2021; Norros et al., 2022). Organic matter and DNA is released from an organism when it moves, respires, reproduces, defecates, feeds, and decomposes (Taberlet et al., 2012; Kelly et al., 2014; Hansen et al., 2018). After which, any DNA that is in the surrounding environment outside of the origin organism is termed eDNA. In principal microbiologists have used similar techniques since the 1980's by extracting the DNA found in soil to indicate the presence of certain bacteria (Torsvik, 1980; Ogram et al., 1987) but its potential to detect marine macro-organisms only started to gain traction in the last decade (Thomsen et al., 2012; Foote et al., 2012; Olds et al., 2016).

The rate and amounts of DNA marine macro-organisms release into the surrounding environment varies. There are multiple factors that contribute to this variation such as the size, metabolic rate, surface area, behaviour, and morphology of the organism, which can fluctuate across the organism's life span and will be different between species (Maruyama et al., 2014; Kelly et al., 2014; Andruszkiewicz et al., 2021). In seawater eDNA exists in several forms; intra-cellular (within prokaryotic or eukaryotic cell membranes), extra-cellular (free/dissolved) and attached or absorbed to detrital and/or mineral particles (Maruyama et al., 1993; Sassoubre et al., 2016; Jo and Minamoto, 2020). The most use genes used for eDNA analysis of intra- and extra-cellular DNA is mitochondrial DNA (mt-DNA) over nuclear DNA (nu-DNA) because there is an order of magnitude more gene copies per cell of mtDNA in eukaryotic cells and also more target regions of the gene that can be used to target different taxa (Deiner et al., 2017; Jo et al., 2019). Furthermore, nu-DNA has been shown to degrade faster in seawater under increased temperatures in comparison to mt-DNA which could make detecting multiple species more difficult (Jo et al., 2019).

To collect and isolate the DNA from a sample of seawater requires filtration through a material that the DNA can be extracted from through chemical and mechanical cell lysis (Taberlet et al., 2018; Fig.1.2). In offshore marine environments 77-99% of DNA is sized between 0.2 and 1.0μm and can be over 1μm in coastal and estuarine environments (Paul et al., 1985). Based on this, filter pore sizes should be at least >0.2μm to retain higher quantities of DNA from a water sample (Spens et al., 2017; Fig.1.2).

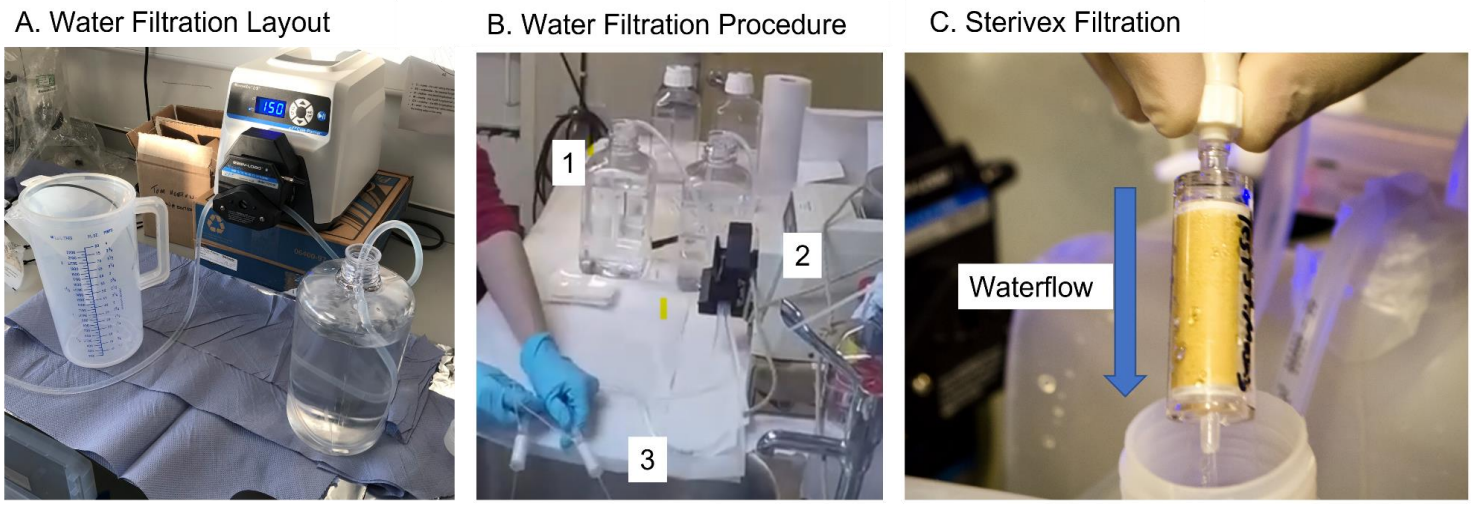


Figure 1.2 Workflow of seawater filtration to trap DNA onto a sterivex filter.
A.) Typical set-up of a eDNA water filtration bench. Sea water is pumped through tubing by a peristaltic pump at 100-200 ml/min through and onto a sterivex filter. (Photo by Matthew Witt)
B.) Filtration procedure; 1) Sea water sample 2) Peristaltic pump that creates a vacuum in the tubing to pump the seawater from the sample to the filter 3) seawater passes through the sterivex filter and DNA collects onto the filter material. Tubing is secured into place with a zip tie or tube luer-lock. (Photo by Marine Cusa, onboard the RV endeavour)
C.) Sterivex filter cartridge. The outside plastic cartridge houses a polyether sulfone membrane which sieves out particles smaller, and captures particles larger than 0.22µm. Up to 2 litres of water can pass through. (Photo by Caitlin Bailey, The Hidden Ocean 2016: Chukchi Borderlands; https://oceanexplorer.noaa.gov/explorations/16arctic/logs/july31/july31.html)

After DNA isolation and extraction, the DNA is then amplified through polymerase chain reaction (PCR), using oligonucleotides, known as primers, that target marker genes (generally mitochondrial) for the taxon of interest (Dieffenbach et al., 1993; Creer et al., 2010). These primers can be species-specific, using a method called quantitative real time PCR (qPCR), that amplifies a gene region specific to an individual species allowing exact quantification of the DNA concentration of that species in a sample (Doi et al., 2015; Salter, 2018; Langlois et al., 2020). On the other hand, to simultaneously detect multiple taxa from environmental samples the primers must have some degree of “universality”, which means that they will anneal on a broad spectrum of DNA templates (Collins et al., 2019). This will amplify the homologous “DNA barcode” region of multiple taxa, which will in turn be sequenced in parallel, using high-throughput platforms (Liu et al., 2020; Fig.1.3). This process is referred to as ‘DNA metabarcoding’ as species are deciphered

by clustering sequences from the same taxa together, and matching each lot of amplicons against DNA reference libraries (Fig.1.3). Traditional visual and capture-based marine monitoring provides information on the biological diversity and species abundances in the marine environment, which is a key aspect of ecosystem services, such fisheries (Manley et al., 2004; Hilborn and Walters., 2013). It is also needed to check for invasive species that might threaten local ecological processes, the potential loss of keystone species that are important for ecosystem functioning, and in general rare or vulnerable species of conservation priority (European Commission, 2010; Borja et al., 2010). Information gathered from monitoring provides evidence that the marine environment, its functions, and its biodiversity are being maintained, in accordance with the environmental targets and laws set out by governments for example the Marine Strategy Framework Directive set by EU governments (MSFD; Cochrane et al., 2010) and the Marine Strategy UK set by the UK post-Brexit (MSUK; Merchant et al., 2022).

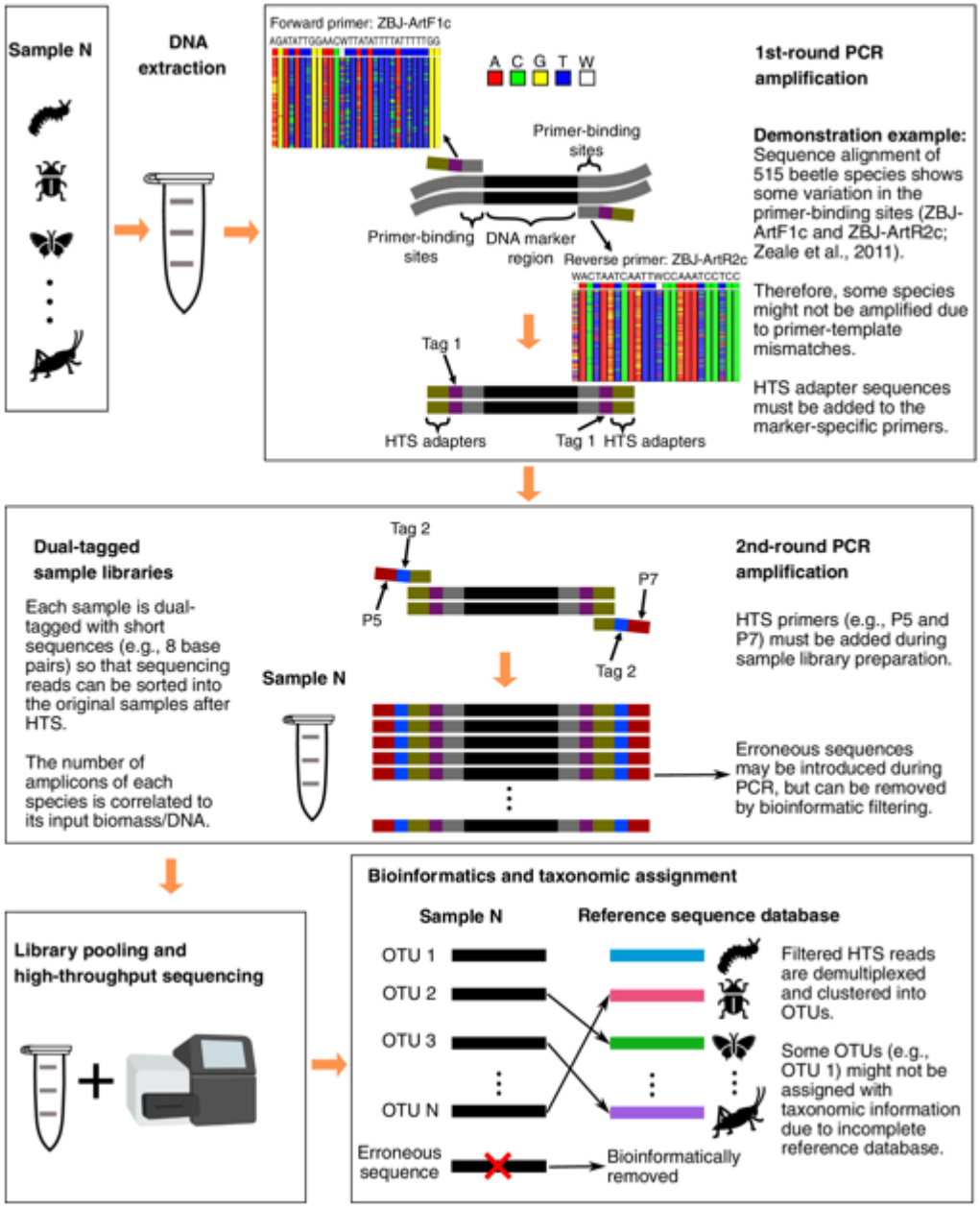


Figure 1.3 Example workflow of the metabarcoding pipeline, from DNA extraction to species identification. (Liu et al., 2020)

1.2 Powers and Pitfalls of Using eDNA in Marine Pelagic Water Bodies

Environmental DNA applications are poised to be useful for monitoring fish in marine ecosystems. Across marine habitats, fish are some of the most important species for ecosystem health and functioning (Kaiser et al., 2011). In the pelagic realm, fish are not only modulators of energy flow through marine trophic webs but are also an important food and valued commodity for humans, with over 66.7 million tonnes of marine finfish caught in 2020 alone (The State of World Fisheries and Aquaculture, SOFIA, 2022). The effectiveness of eDNA analysis for the detection of fish species at sea was first demonstrated by Thomsen et al (2012) who found that eDNA metabarcoding can detect several coastal fishes. When the eDNA detections were compared with a range of other sampling methods (including seining, angling, underwater visual census, etc.) eDNA samples always yielded more species than any of the other methods (Thomsen et al., 2012; Knudsen et al 2019). In recent years, comparisons between monitoring using capture-based methods and eDNA methods have been repeatedly studied in the pelagic realm (Closek et al., 2019; Stoeckle et al., 2021; Sanchez et al., 2022). The consensus from these studies also suggests marine eDNA can detect more species that traditional capture-based methods because struggle to efficiently identify taxonomically similar species, especially at juvenile stages, and are only designed to capture certain species. Whereas eDNA is not limited by these factors because all species from all life stages release DNA (Handley, 2015).

Despite eDNA having great potential, just like any other approach, it is not without its caveats and technical challenges, which can complicate the integration of eDNA methods with pre-existing monitoring strategies. For example, eDNA does not have the capability of determining the size, sex, or age of species. These are vital metrics used for interpreting fish population health by predicting natural mortality and growth of fish stocks (Roberts and Polunin, 1993). Furthermore, eDNA cannot generally determine the various

ways the detected DNA has been shed and released (Barnes & Turner 2016), so we cannot distinguish if the DNA is derived from a living fish and thus eDNA could not be used to infer natural or catch mortality rates in stock assessments. Marine eDNA fish studies have false negative detections and/or missing species (Thomsen et al., 2012; Thomsen et al., 2016; Fraija-Fernández et al., 2020; Maiello et al., 2022), which are caused by both molecular or biochemical processes and computational constraints. After the DNA has been extracted from the seawater sample, the DNA of certain fish species have better molecular affinity to the specific primers being used, a mechanism known as amplification bias (Krehenwinkel et al., 2017). This can result in the amount of DNA from some species being more prevalent, out-competing less abundant DNA, increasing the likelihood of some species not being detected (O'Donnell et al., 2016). This bias is more likely to be exaggerated in offshore pelagic ecosystems because of the increased dilution factor of DNA in seawater. For example, coastal areas have between 2-15mg of DNA per m^{-3} released from all species in comparison to offshore areas that had an overall DNA concentration between 1-5 mg per m^{-3} (DeFlaun et al 1987; Danovaro et al., 2006). The lower the concentration of fish DNA in seawater, the lower the likelihood of detecting fish species that have a low DNA output in the pelagic water column. Furthermore, when high-throughput parallel DNA sequencing (HTS) outputs are taxonomically assigned, there may be better coverage in the reference database for certain species than others. A lack of mitochondrial references for eDNA may result in the DNA not being assigned efficiently to its associated species meta-data, also causing a false negative detection (Marques et al., 2021).

Environmental variables can affect the persistence and transport of eDNA (also known as the ecology of eDNA) in the marine pelagic environment. The rate of this persistence can be dependent on environmental factors such as water temperature, pH, and salinity (Barnes et al., 2014; Thomsen and Willerslev, 2015). These factors can promote cell lysis to release DNA into the water column, where the rate of degradation and DNA fragmentation increases the breakdown of amino acids (Pietramellara et al., 2009; Torti et al., 2015). After the DNA has been shed by the organism the mt-DNA gene fragments can persist for 48 hours in UK marine water before it is degraded past the point of detection, the rate of this degradation will change over time and seasonality depending

on the temperature of the water (Collins et al., 2018; Jo et al., 2019). After which there is a rapid drop off in the likelihood of eDNA being able to be traced back to the organism that shed it (Collins et al., 2018; Wood et al., 2020; Holman et al., 2021).

The eDNA particles can also be transported in multiple different directions and in three dimensions (across longitude, latitude, and depth) by water flow regimes (Harrison et al., 2019; Goldberg et al., 2016). The interplay between the distance over which eDNA can be transported (either passively, through oceanographic features, or actively, through animal movements), and the time it takes to degrade, will determine the physical space and context within which eDNA detections can be interpreted (Hinz et al., 2022). The further eDNA is transported the more difficult it will be to carry out fine-scale species mapping studies (Darling et al., 2021). For example, coastal and shelf habitats are shallower waters where particulate movement is significantly affected by waves and wind (Jones and Davies, 2008), potentially displacing the eDNA signals up to 12 km away from their release location/organism (Bonfil et al., 2021). In open oceans Andruszkiewicz et al (2019) showed that using a particle tracking model the DNA of the Northern Anchovy (*Engraulis mordax*) could have travelled up to 40km in 4 days post release. This creates the potential for significant mixing and unpredictability across sample sites in coastal areas.

However, small scale retention of the eDNA signal has also been observed; O'Donnell et al (2017) found that the communities detected from eDNA sampling in coastal areas can significantly differ between sample sites that are even just 50 metres away from each other. In offshore pelagic water eDNA distribution is highly variable from area to area and between seasons (Andruszkiewicz et al., 2019) which means that the environmental influences should also be monitored to understand the distribution, and potentially detection, of eDNA from marine samples. In offshore pelagic water columns, understanding the vertical distribution of eDNA is important to ensure eDNA sampling detects as many pelagic species as possible. Although pelagic fish can move and release DNA throughout different depths of the water column, the concentration of their DNA at different depths, and therefore the likelihood of detection, can also fluctuate. This is best demonstrated by the diel migrations of pelagic fish that migrate between upper and lower

pelagic waters, following the movement of prey species. Canals et al. (2021) found that these fluctuations in fish populations within a sample area can be detected from eDNA sampling at different times of the day. Other environmental influences like water column stratification, that is the creation of two or more water bodies within the water column, has been shown to have varying impacts on the detectability of species between differing sample depths (Closek et al., 2019, Jeunen et al., 2019).

These variables add background noise and increase susceptibility of outliers skewing the number of DNA amplicons (reads) in a sample. This means that the number of reads is not a direct quantitative proxy for the number or biomass of the fish species they are assigned to. For example, 100 reads of fish X compared to 1000 reads of fish Y in a single sample does not necessarily mean that there is a higher population of fish Y than X, or in any case that the 1:10 ratio reflects the actual biomass proportion of those two species. The background noise created by these variables can be overcome through multiple sampling sites and replications within an ecologically similar and connected pelagic water body. DNA metabarcoding, as well as real time single-species quantitative PCR (qPCR), studies that have conducted adequately replicated efforts have still found significant correlations between trawl captured pelagic fish biomass and the number of amplicons reads or DNA concentration of pelagic fish (Thomsen et al., 2016; Knudsen et al., 2019; Salter et al., 2019; Stoeckle et al., 2021; Pont et al., 2022). For marine pelagic fish species, in terms of quantification, qPCR has shown stronger relationships to pelagic trawling biomass in comparison to metabarcoding- which is likely caused by the higher sensitivity of qPCR and impact of non-target species DNA decreasing the detection rates and accuracy of quantification from metabarcoding (Yu et al., 2022).

On the basis that the species read proportions inferred from eDNA is comparable to the proportions of different species found in the trawl, which takes place over a relatively small spatial scale (1-2 km) in comparison to the vastness of the pelagic environment, it begs the question whether eDNA can be applied to methods of continuous monitoring such as hydroacoustics that are over larger spatial scales. Especially given that trawling is used for hydroacoustic ground truthing fish schools of multiple species and is known to have catchability variances between species and size classes of fish (Heino et al., 2011;

Bassett et al., 2018). In the past three years, studies in both freshwater and marine habitats have started to investigate the potential of eDNA being used as a method of ground truthing and quantifying fish schools detected through hydroacoustics. These studies verify fish species and estimated biomass from eDNA concentration by means of using species-specific qPCR methods rather than a capture-based method (Coulter et al., 2019; Berger et al., 2020; Rasmuson et al., 2021; Li et al., 2022; Shelton et al., 2022). If eDNA read abundance is equivalent to the catch proportions from trawling in the pelagic realm, eDNA methods of ground truthing hydroacoustics have instant advantages over midwater trawling methods. For example, eDNA can be used to help develop quantification methods for species that are normally not detected by trawling, such as eels, scabbardfish and garfish that may not be considered species of economic interest but are an important prey species needed for energy transfer through pelagic trophic webs (Haugland et al., 2006; Santos et al., 2013; Berger et al., 2020; Alter and Peck., 2021). Furthermore, eDNA can detect the traces of abundant fish species that inhabit an ecologically connected region within 48 hours of release, meaning that the species detected from eDNA samples are likely resident marine pelagic species of that region rather than accidental detections (Collins et al., 2019). Shelton et al. (2022) found that Pacific hake (*Merluccius productus*) biomass estimated from hydroacoustics, and trawling has a significant correlation with eDNA qPCR concentration estimates across the Californian coastal ecosystem. Other eDNA methods such as qPCR can be used to further support hydroacoustic monitoring, species-specific qPCR assays could be multiplexed for multi-species approach but would increase the costs per additional target fish species. Although this is a viable method (Ushio et al., 2017; Yu et al., 2022), we must also consider the cost effectiveness of this approach if we wanted to upscale marine monitoring with eDNA applications for multiple species monitoring. Preliminary investigations into the effectiveness of metabarcoding amplicon reads as a proxy for DNA concentration in the marine environment have shown positive correlations between qPCR eDNA concentrations of Japanese anchovy (*Engraulis japonicus*) and Japanese jack mackerel (*Trachurus japonicus*) and the number of reads (Ushio et al., 2017). Findings from Yu et al (2022) also found similar positive correlations between qPCR concentrations and amplicon reads for six marine pelagic species. However, in both studies the strength

of these correlations varied from species to species. The underlying cause of this variation in unclear but suggests that background noise affects the quantification of eDNA from qPCR and/or metabarcoding methods across different species. Li et al (2022) has compared the hydroacoustic data and eDNA metabarcoding for multiple fish species that inhabit a freshwater reservoir. But found that various biotic and abiotic background noise resulted in a lack of correlation between capture-based verified hydroacoustics and eDNA read abundance. Although it is unclear whether eDNA can become an accurate standalone method of monitoring, if we can optimise eDNA sampling and bioinformatic pipelines to overcome the background noise, it could provide a useful tool to support current capture based monitoring.

1.3 Thesis Rationale

This thesis aims to evaluate whether eDNA can be integrated into hydroacoustic based marine monitoring practices of swim-bladdered pelagic fish by acting as a proxy for trawling-based verification methods of fish schools. To investigate this, eDNA samples were collected throughout two annual pelagic monitoring surveys that use trawling-verified hydroacoustics (Fig.1.1). The thesis focussed on finding whether abiotic variables and different sampling methods significantly impact species detections and number of reads between and within sample sites [Chapter 2]. As well as how eDNA metrics compare to the mid-water trawl caught biomass and abundance of fish species [Chapter 2]. This thesis also assesses the sensitivity of eDNA to detect seasonal fluctuations of the highly migratory Atlantic Bluefin Tuna (ABT) and whether we can use eDNA to monitor the ecological structure of a sample area over time [Chapter 3]. The eDNA data [from Chapters 2 &, in part, 3] were then used to partition hydroacoustic backscatter to estimate the species density of six economically and ecologically important pelagic fish: sprat (*Sprattus sprattus*) European pilchard (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), Atlantic herring (*Clupea harengus*), Atlantic horse mackerel (*Trachurus trachurus*), and boarfish (*Capros aper*) [Chapter 4]. This thesis then aims to determine if the eDNA approach could be used to add fish diversity information to the hydroacoustic

data collected during the survey. Ultimately, the two types of validation (eDNA and trawling) for hydroacoustic data are compared when possible [Chapter 4].

1.4 Data chapter summaries

These summaries represent a brief introduction to each data chapter that outline how each chapter address the thesis rationale.

1.4.1 Data Chapter 2; Refining the eDNA Toolkit for Pelagic Fish Detection

In Chapter 2, seawater was sampled in tandem with recordings of the abiotic variables of the sampling area during the 2018 pelagic monitoring survey around the South-West of the UK (PELTIC18). The abiotic variables, temperature, and salinity were measured by an onboard Ferrybox System, as they are known to impact the persistence of eDNA in a marine environment (Collins et al., 2018; Legrand et al., 2003; Danovaro et al., 2005). The influences of abiotic variables on the number of reads and diversity of species detected between sample sites was investigated. In this Chapter I also investigated whether sampling depth significantly affected the eDNA results by looking at the differences in species richness, beta diversity, and read numbers between surface, upper pelagic and lower pelagic water samples within and between sample sites. Surface water samples were collected from surface water sampling using the FerryBox system when the vessel was underway, and the upper and lower pelagic water column samples were collected using a CTD rosette that also recorded the abiotic variables at the different depths. I also conducted this experiment when the water column of the sample area was stratified into two water bodies to measure the differences between eDNA samples detected from each water body. If these variables impacted the eDNA results, then this would impact the comparisons between trawling metrics and the eDNA read number. Moreover, we compared eDNA reads with the trawling metrics of biomass, number of individuals and surface area of the individuals to find which metric fitted more closely.

1.4.2 Data Chapter 3; Complementing Bluefin Tuna Monitoring in the UK with eDNA analysis

Weekly eDNA seawater samples were collected from July to December 2019 in two locations across the South-West coast of the UK. The sample sites were chosen from pre-existing knowledge on the presence of Atlantic Bluefin Tuna (ABT) in those areas. From the eDNA metabarcoding data, the presence of ABT and their small pelagic fish prey species were used to predict seasonal migration in comparison to known sighting data of ABT and fish catch data from that area. To investigate whether eDNA metabarcoding could be used to find ecological predator-prey relationships between marine species, the relative read abundance of ABT and other species detected from eDNA were used in a species co-occurrence matrix.

1.4.3 Data Chapter 4; Validating pelagic marine fish identity and abundance from hydroacoustic backscatter using environmental DNA

In Chapter 4 we use the eDNA data from two annual trawl-verified hydroacoustic pelagic surveys from 2018 and 2019. Because temperature, salinity, sample depth and stratification of the water column had no impact on the eDNA species richness or read number, no adjustments were needed for eDNA data. During these surveys the hydroacoustics backscatter densities were averaged into a distance-based sampling unit (EDSU) that represented a 1 x 1 nautical mile water section. The hydroacoustic densities recorded at each EDSU was then partitioned by fish species using the nearest eDNA sampling point. To see if eDNA could be used as a proxy for trawling, the eDNA validated hydroacoustic densities of fish species were then statistically compared to the trawling validated densities using a linear model for both sampling years to investigate the interannual variability.

Chapter II

Refining the eDNA Toolkit for Pelagic Fish Detection

2.1 Abstract

Harnessing environmental DNA to simultaneously detect multiple fish species could be used in tandem with current monitoring practices to increase the accuracy of the detections of pelagic fish species. However, our understanding of the variables that affect eDNA sampling methods need refining for pelagic fish detection on the continental shelf. Multiple other marine eDNA studies have shown that varying methodological procedures and hydrological variables affect the qualitative and quantitative outputs of eDNA. This chapter investigated the impacts of eDNA sampling depth, stratification of the water column and abiotic variables on the detected pelagic fish species richness, evenness, community assemblages, and quantity of eDNA reads. Water was sampled across a four-week small pelagic fish stock survey (PELTIC) around the South-West continental shelf of the UK. Samples were taken from the surface, upper and lower depths at sampling sites in coastal and offshore areas and during times when the sample site had either stratified or mixed water bodies. This chapter finds that stratification, sampling depth and abiotic influences do not affect the species richness or evenness (spread of species). We do observe a significant uplift in pelagic species detected in the surface water and in the upper water column in comparison to demersal species but no significant difference in community structure from stratification or sampling depth. This chapter suggests that future eDNA studies looking to encompass the pelagic fish communities from within a single season or single time point from relatively hydrologically consistent continental shelf seas can sample from surface water without the necessity to check the stratification of the water column or process large amounts of abiotic variables to ensure eDNA is comparable between sampling areas. Although, it is still recommended to collect these variables if the study is looking to investigate changes over season, annual surveys or

across multiple marine habitats where abiotic influences can significantly vary. Furthermore, this chapter also found significant correlations between the pelagic fish caught in a mid-water pelagic trawl (that was conducted simultaneously to the eDNA sampling) and the number of eDNA reads.

2.2 Introduction

Effective mitigation of the impacts of overfishing and climate induced changes requires robust monitoring of biotic and abiotic components of the ecosystem (Berg et al., 2015; Addison et al., 2018; Magliozzi et al., 2021). A new biodiversity assessment approach, environmental DNA (eDNA), can be used to significantly increase the taxonomic resolution of monitoring practices for pelagic fish species (Stephenson, 2020; Miya, 2022; Thomsen & Willerslev., 2015). This method harnesses the DNA naturally shed by organisms in the water, which can be retrieved from water samples, amplified and sequenced to identify which species the DNA belonged to (Taberlet et al., 2018). In simulations of marine water a majority of DNA shed by organisms significantly reduces past the point of detection after 48 hours and is completely undetectable (Collins et al., 2018; Holman et al., 2021).Although, the detection rates of species can be intermittent if the species are rarer or in low abundance compared to the bacdrop of multiple high abundance species. Ely et al (2021) showed that *in-situ* dection rates for DNA in costal reef sites disappeared after 7.5 hours for DNA that was spiked into the environment. This increases the likelihood that faunal detections of low abundance or invasive species through marine eDNA are relevant to the time of sampling in ecologically connected areas. Because eDNA metabarcoding is not restricted to species-specific designs of traditional catch-based methods, like trawling or gill netting, multiple marine eDNA studies were able to detect a considerable proportion of fish species that would not normally be detected (Thomsen et al., 2012; Stat et al., 2019; Fraija-Fernández et al., 2019; Bernatchez, 2020; Gold et al., 2021; Gilbey et al., 2021). Not only does this method detect a higher number of species but does so in a relatively cost-efficient manner, which for marine monitoring is often the constraint that limits the spatial and temporal scope of surveys (Edwards et al., 2010; Nygård et al., 2016; Aylagas et al., 2018; Goodwin et al., 2019).

Although there has been much progress towards the use of eDNA for marine organisms, it has yet to be refined to ensure eDNA sampling provides an accurate reflection of pelagic fish assemblages (Palialexis et al., 2019; Pawlowski et al., 2021). One obvious factor that would guide much of the future sampling methodologies, is the depth at which water should be collected, to encompass the highest percentage of fish species that are present in the ecosystem at the time of sampling. There have been contradicting findings amongst the studies that have investigated the impact of eDNA sampling at different depths; for example, Andruszkiewicz et al (2017) and Jeunen et al (2021) found deeper sampling will pick up more species from deeper habitats, whereas Closek et al (2019) and Mariani et al (2021) found no significant difference between sample depth and the species detected. Govindarajan et al (2021) showed that communities of species detected using eDNA, significantly differed between shallow water (0-200 metres) and deeper mesopelagic water (200-800 metres), yet there was no significant difference between deeper water categories; 100-200m, 200-500m and 500- 800m.This was likely due substantial drop off of zooplankton abundance after 200m depth and thus a change in the ecological trophic structure of the species within the first 200m and 200-800m depth (Govindarajan et al., 2021). There is also evidence of eDNA being able to detect the diel migrations of fish species, resulting in the fluctuations of detection for certain species at different times of the day from the same water column depth (Easson et al., 2020; Canals et al., 2021). Hydrological factors and water regimes can also play a part in what species assemblage eDNA can detect, for example, water column stratification. Stratification is where two or more water bodies within a water column are horizontally separated by a chemical barrier; caused by salinity (halocline) and temperature (thermocline) gradients changing at a certain point of the water column (Rippeth et al., 2005; Bourgain et al., 2011). Other studies from around the world that have tested marine eDNA sample depths with stratification have either observed clear partitioning of fish communities and differences in the number of species between stratified water bodies (Jeunen et al., 2020) or no impacts at all (Closek et al., 2019) thus the effects of stratification on eDNA findings are likely to be specific to depth, hydrological and ecological structure of a sample area. To ensure that eDNA methods of sampling encompasses the highest number of pelagic fish species from sample areas across spatial scales marine eDNA sampling efficiencies must

be tested at different depths in water columns and during both stratified and unstratified water bodies.

As well as refining where eDNA should be collected in the water column, recent eDNA studies have started to investigate the quantitative links between the outputs of eDNA and fish catch methods. Fish catch methods such as trawling is a prominent method for pelagic fish identification and used for the calculation of pelagic fish biomass and abundance. In marine waters quantitative links between trawling metrics and eDNA have used single species targeted methods (quantitative PCR) but also study general diversity analysis using high throughput sequencing (HTS) (Knudsen et al., 2019; Salter et al., 2019; Stoeckle et al., 2021). These studies have found varying relationships between eDNA (qPCR and HTS) and trawling methods, yet only Knudson et al (2019) investigated the impacts of abiotic variables (temperature, salinity, water depth) on the eDNA concentrations and whether it affected the comparison between eDNA qPCR and trawling.

In this study we conducted eDNA sampling over a four-week period small pelagic fishery-independent survey on the Northeast Atlantic continental shelf in the Celtic Sea. To refine how and where in the water column eDNA should be collected, we investigated the impact of sampling depth and water column stratification on the number of species eDNA can detect and the species communities. To understand the influences of abiotic variables on our sampling we tested whether the relative proportions of HTS reads and communities between different depths are impacted by temperature and salinity. Accounting for the inferences made from the impacts of depth, stratification, temperature, and salinity we also compared detections from pelagic trawling data with eDNA. To further refine the uses of eDNA as a monitoring tool for European pelagic shelf habitats, based on Stoeckle et al (2021), we hypothesised that there is a quantifiable link between eDNA HTS reads and fish catch metrics.

2.3 Materials and Methods

Environmental DNA water samples were collected during the annual pelagic ecosystem survey in the Western Channel and Celtic Sea (PELTIC), run by the Centre for

Environment, Fisheries & Aquatic Sciences, aboard the RV “Endeavour”, which took place between the 7th of October and the 8th of November 2018 (Fig.2.1).

2.3.1 Sample Collection

During this survey, information on species and size composition of fish schools was obtained by means of pelagic trawl, and continuous data was collected on the biochemical and hydrological states of the marine environment. Methodological details are provided in Doray and Boyra (2021) but a summary is included here. During the trawling process a Marport netsonde rigged at the centre of the headline at the mouth of the trawl was used to monitor the vertical opening of the trawl, the depth of trawl (relative to the seabed) and any fish schools entering the trawl net. The vertical opening of the trawl net varied depending on the deployment depth and towing direction relative to the tide but was generally approximately 12 m and the trawl duration was generally fixed to 30 mins. Once aboard, all species in the catch were identified, measured, individually weighed, and their biological life history parameters (including maturity and age) collected for up to five specimens per 0.5 cm length class. Large catches were subsampled, ensuring that a sufficient length range was collected for all species.

At night, oceanographic profiles were collected of temperature and salinity: at ~100 fixed stations, regularly spaced in the survey grid, mini CTDs were deployed while the vessel was stationary. Deployment of a full CTD and Rosette with Niskin bottles occurred at a subset of these stations, to collect further information including dissolved oxygen, chlorophyll, salinity, water nutrient concentrations and phytoplankton abundance and diversity. Before each deployment, the Niskin bottles were flushed through for at least 30 seconds using a freshwater hose. At all times during the survey, sub-surface oceanographic variables (temperature, salinity, oxygen, fluorescence, turbidity) were recorded by a FerryBox system at 1-minute intervals, using an inflow on the hull located at 4m depth.

A total of 224 seawater samples were taken from 51 different sites for eDNA extraction, using two different methods. The first method was used at 26 of the 51 stations and involved sampling of water using a rosette (Fig.2.1) at 20% (“Upper”) and 80% (“Lower”)

of the total depth of the sample site (minimum total depth of 54 metres, maximum total depth of 164 metres, average total depth of 100 metres). This was conducted to explore the variability in detectability of species' eDNA in the water column, including the boundary effects of stratification which has affected marine eDNA studies differently (Closek et al, 2019; Jeunen et al 2020; Canals et al., 2021).

Stratification is the formation of a chemical barrier within a water column that separates a body horizontally into two or more zones (Kaiser et al., 2011). This chemical barrier is created when the salinity or temperature drastically changes, and depth creates "layers" of marine organisms as they separate into more favourable habitats which can form the aforementioned boundary effects.

The second method was used at the remaining 25 stations which were sampled 'underway' (hereafter known as underway sampling) through the existing FerryBox system, which continuously pumps seawater from 4 metres depth. This method was deployed during trawling operations when deployment of the rosette was not possible. In both cases, the seawater samples were filtered through a 200-micron mesh before being collected in 2.5 L previously decontaminated Nalgene bottles, then stored at 4 °C less than 24 hours before processing. The samples were taken in triplicates at each sample depth.

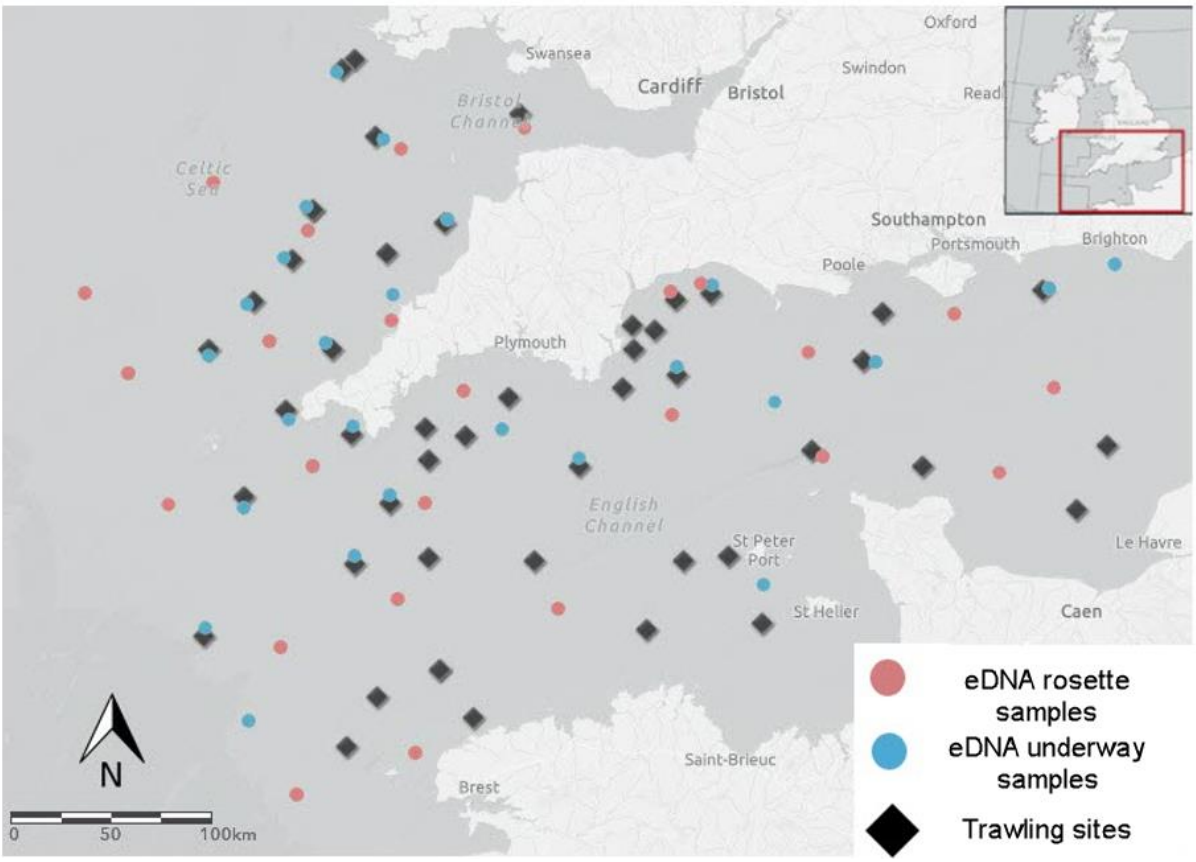


Figure 2.1 Environmental DNA and PELTIC 18 sampling area across the Southwest coastline of the UK and Northern coastline of France. eDNA surface sampling (blue dots) was taken during trawling events representing 3 replicates and at sample sites where the water was too rough for a CTD deployment. Rosette sampling (red dots) representing 3 replicates at 2 depths.

2.3.2 eDNA Sample Processing

On board of the research vessel, the seawater samples were pumped (100ml/min) through a sterile tube (MasterflexTM L/S, Cole-Palmer, UK), using a peristaltic pump (MaterflexTM L/S modular Cole-Palmer, UK), into a sterile 0.2 µm SterivexTM filter capsule (Merck, UK). After removing the seawater by pumping air through the Sterivex unit, each cartridge was placed inside a sterile bag and then wrapped in aluminium foil to be stored at -80 ºC. After sampling the Niskin tubing, collection funnels, Niskin collection bottles, peristaltic pump tubing were then submerged in a 4% hydrogen peroxide solution for least 12 hours and rinsed with deionised water three times to remove excess peroxide.

There was a total of 16 field blanks taken during the survey, once every 48 hours, by collecting and filtering deionised water that had been flushed through the cleaned sampling funnels and into niskin collection bottles. A total of 205 eDNA samples were collected from 50 eDNA sampling sites across the survey.

2.3.3 eDNA lab work

DNA was isolated following the water MU-DNA protocol (Sellers et al., 2018) and each extraction was stored into a 200µL elution buffer. The nucleic acid yield per sample was checked on a Nanodrop 1000 (ThermoFisher Scientific). A ~167bp fragment of the mitochondrial 12S rRNA gene was amplified through PCR using the 'Tele02' primer set (Miya et al., 2015; Taberlet et al., 2018), which were customised with 8bp unique indexing oligo-tags on both the forward and reverse ends of the primers, and additional 4-6 randomly degenerate nucleotide positions (Ns) at the beginning of each tag to increase sequencing diversity. Each sample was PCR-amplified in triplicate, in a total volume of 20 µl, which consisted of 10 µl Amplitaq Gold Master Mix (ThermoFisher Scientific), 0.16 µl BSA, 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer. This was added to a scaled ddH20-to-template input depending on the nucleic acid yield of each sample, which ranged from 0.1ng/ul to 72 ng/ul, from the Nanodrop. The samples were split into 3x82 sample plates with three PCR replicates per plate, PCRs were performed following the protocol in Taberlet et al. (2018) with an initial denaturation step of 94°C for 10 min, 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final extension step of 72°C for 5 min. The PCR products were checked on a 1.5% electrophoresis gel and pooled per plate. The pooled PCR products were cleaned using a 0.7x bead clean-up with AMPure magnetic beads (Beckman Coulter) and quantified on a Qubit using the dsDNA high sensitivity assay. Each pooled plate became a dual-indexed Illumina library made following the KAPA library preparation guide (Roche) and quantified using a NEB quant kit (Illumina) on the MIC RT-PCR machine (Bio Molecular Systems). The libraries were then pooled at an equimolar concentration (4nm) with 10% PhiX control, with a final

sequencing molarity of 8pM on an Illumina MiSeq platform using (v2) 2×150 paired end chemistry.

2.3.4 Bioinformatics and downstream data analysis

From the PCR free library preparation, the forward and reverse fastq files were reorientated to 5'-3' for each sequenced library. The sense and antisense orientated sequences were independently filtered for PCR primers tags and demultiplexed (cutadapt v2.10; Martin, 2011), followed by PCR primers and adapter trimming. The sequencing run yielded 12.1 million raw reads of which 7.8M were retained that contained tags on both ends of the amplicon without mismatches. Reads were then denoised, dereplicated and chimaeras removed using the DADA2 workflow (DADA2 v1.17; Callahan et al., 2016) leaving 3.98 million reads. Non homologous reads (10,300~reads) were discarded as noise (HMMER v3.3) with a homology filter that aligns the amplicon sequence variants (ASVs). This uses a profile hidden markov model that uses all possible alignments of the sequences to the expected fragment to define which sequences are non-homologous from insertions and deletions.

ASV taxonomic assignment was carried out by performing a local blast against the entire ‘nt’ blast database (ftp.ncbi.nlm.nih.gov/blast/db/v5; 21 March 2019) using blastn (v2.90) on each ASV, filtered to retain the best blast percentage identity score. Then a secondary blast was performed against a curated 12S UK fish database (Collins, 2021). To maximise identification of the query sequences against the reference sequences we used and Evolutionary Placement Algorithm (EPA-ng v0.3.5, gappa v0.6-0; Barbera et al., 2018; Czech, Barbera & Stamatakis., 2019). Species were then assigned based on the following parameters: 1) Aligned matched length of EPA to blast score of 97% or 2) the best scoring blast hit with an EPA probability of over 90% and a blast identity over 97%. Non-UK fish species and ASVs identified to lower than species level (<90% identity) were later re-assessed with individual nucleotide blasts to investigate whether they were UK fish species that are underrepresented in the nucleotide database or if they could be vagrant or alien species. The species heterogeneity and read numbers per species were observed between the PCR-, extraction- and field-blanks.

Contaminant ASVs were identified by using the decontam R package (https://github.com/benjjneb/decontam) and all of their reads that were present in the PCR-, extraction- and field-blanks were removed from their corresponding samples. To counteract false positives induced from sequencing any ASVs that amounted to less than 0.1% of the average total read number per sample were removed from the results.

Downstream analysis was performed in R v4.0-0 (R core team, 2020). To reduce the impact of disproportionate reads between samples, read counts over the upper quartile read range were reduced to the 75th percentile (4500 reads) and were normalised using the relative abundance of reads per sample replicate (Handley et al., 2019; Majaneva et al., 2018). Sample replicates were compared for similarity in species richness, detected within each sample site and were combined through averaging the relative abundance of the species detected, rounding up.

The effects of sampling depth and the stratification of the water column (stratified water bodies n=28 and mixed water bodies n=50) on species richness and Pielou's evenness from eDNA sampling were compared using boxplots. To ensure that the species richness and evenness values were as accurate as possible for the regions samples the sampling depth data set was separated into five ecoregions based on a sea surface temperature change of 0.6˚C from a 10 nautical mile zone to the next (Supplementary Fig.1). To make the heatmap and separate the zones uniform kernel density mapping of the sea surface temperature was conducted in QGIS, the radius of the points were adjusted to 10 nautical miles, and polygons generated based on the temperature change. Sea surface temperature was used as it is one of the most important factors for determining the distribution of zooplankton species, a significant prey in small pelagic fishes' diets (Rutherford et al., 1999). To make this comparison only data where there were at least three sets of three, or a total of nine replicates per ecoregions from surface water, upper water bodies and lower water body sampling were retained. From the ecoregions only zones 2 and 3 had enough replicated to make this comparison. The data from both ecoregions were normalised using a log normalisation and the relationships were statistically analysed using a one-way ANOVA. The data for the stratification data sets were not normally distributed; thus, a Kruskal-Wallis H test was used to test the overall

relationships. This was followed up using a pairwise Wilcoxon test to investigate the differences between each sample depth using the pairwise Wilcoxon function from the stats package (R Core Team, 2020). For species richness the number of unique species in each sampling replicate was combined per sample site depth. Species richness is used as an indicator of biodiversity of an area and doesn't consider species abundance of distribution. Pielou's evenness was calculated from the Shannon-Weiner diversity index using the mean combined relative abundance values of each replicate per sample site depth using the stats package (R Core Team, 2020) and diversity function from vegan (v2.4-2) in R. Pielou's evenness is another measurement of biodiversity but is a count of those individuals in that area, in this case amplicon read numbers were used, and the distribution of the counts of individuals. The more even Pielou's evenness the more equally split the amplicon reads were per species at that sample site.

To investigate whether water temperature (°C) or water salinity levels (ppt) were significantly impacting the species richness or Pielou's evenness at each sample site depth a multiple linear regression was calculated using the lm function from the stats package in R. A multivariate linear regression was also used with sample depth and stratification as a nested variable to find whether temperature and salinity impacted species richness or Pielou's evenness between the different sampling conditions. An analysis of variance (ANOVA) on the residuals was also calculated for each regression.

Differences in species assemblages between sample sites based on eDNA sample depth and water column stratification was investigated using Bray Curtis dissimilarity (read number was used as a proxy for abundance) and Jaccard indices from the vegdist function in the vegan package (Oksanen et al., 2013). To visualise the Bray-Curtis and Jaccard distances, a Principal Coordinate Analysis (PCoA) using cmdscale from the package stats and plotted using ggplot2 (Wickham et al., 2016). To identify the species that were the source of variation between sample sites, we conducted a homogeneity of dispersal test using permutest then used a permanova with 1000 permutations using the adonis function both from the vegan package in R (Oksanen et al., 2013). The species that statistically affected the species assemblages in the data was extrapolated using fviz_contrib function from the R package factoextra and visualised using a principal

component analysis (PCA) plot using the pca function from the ade4 package (Dray and Dufour., 2007).

2.3.5 Pelagic trawl catch composition and eDNA

During daytime midwater pelagic trawls, eDNA was collected from the FerryBox flowthrough system and night-time CTD rosette casts at fixed stations. The comparisons between the pelagic catch composition and eDNA were only used using eDNA samples that had been taken at the time of trawling to increase spatial relevance.

The frequency of the species detected using both methods was summarised using a bar plot and all species found from both methods displayed using a Venn diagram. Boxplots of species richness and Pielou's evenness were used to compare between eDNA, and pelagic trawling methods and a kruskal-wallis H test was applied to assess differences using the Kruskal test function from stats package (R Core Team, 2020). To maintain temporal and spatial relevance, this comparison only included underway water eDNA sampling sites where trawling took place at the same time.

For each fish species in a trawl, number of individuals caught, catch weight (kg) and the body surface area (BSA, in m^2) were compared with the relative proportions in metabarcoding read counts of those species in corresponding eDNA samples (see below). BSA was included, as lots of smaller fish are often expected to release more genetic material than a few larger fish with the same weight (Kelly et al., 2014; Wang et al., 2021). The most accurate way to determine fish BSA is to use body weight in combination with measurements from the full caudal length and all fin measurements (O'Shea et al., 2006); however, in our data only fork length and weight of each specimen were measured. Thus, for simplification and rough standardisation across all pelagic species, an edited version of the Monsteller calculations (Mosteller, 1987) was used:

(Full length(m)*(average weight of fish species * number of individual fish at that length size)/3600)1/2

To compare with the underway samples, the pelagic trawling metrics were then changed to relative catch abundance data by making the number of individuals, biomass and BSA of each species caught per trawl a percentage of the trawl catch. There were 47 sample sites where eDNA underway sampling and pelagic trawling took place either at the same time, or within a 2 nautical mile radius and within a 72-hour time frame. Species relative abundance data was averaged over each trawling transect across both data sets. Only pelagic fish species that were detected by eDNA and caught through pelagic trawling in the same trawling transect were used to compare reads and catch metrics. Read counts were not normally distributed so a Spearman's rank correlation coefficient was used to calculate the association between species catch metrics and read count data using the cor.test function from the base R stats package.

Species assemblage differences between eDNA data and the trawl catch were analysed using bray-curtis dissimilarity and Jaccard similarity using the vegdist function from the vegan package in R. This data was visualised with Principal Coordinate Analysis (PCoA) using cmdscale from the base R stats package.

2.4 Results

A total of 164 of the original 224 samples from 49 of 51 sampling sites (23 underway sampling out of 25 and the full 26 rosette stations) were retained after. These remaining samples contained 1.28 million UK fish reads with a total of 66 unique fish species.

The one-way ANOVA for both ecological regions 2 and 3 found no significant difference in species richness ($F(2,82.5)= 0.38$, $p=0.7$; Fig.2.2a, $F(2, 34.25)=0.78$, $p=0.5$; Fig.2.2b) and Pielou's evenness ($F(2,2.86)= 0.06$, $p=0.5$; Fig.2.2c, $F(2,57)= 1.19$, $p=0.6$; Fig.2.2d) from eDNA sample depth.

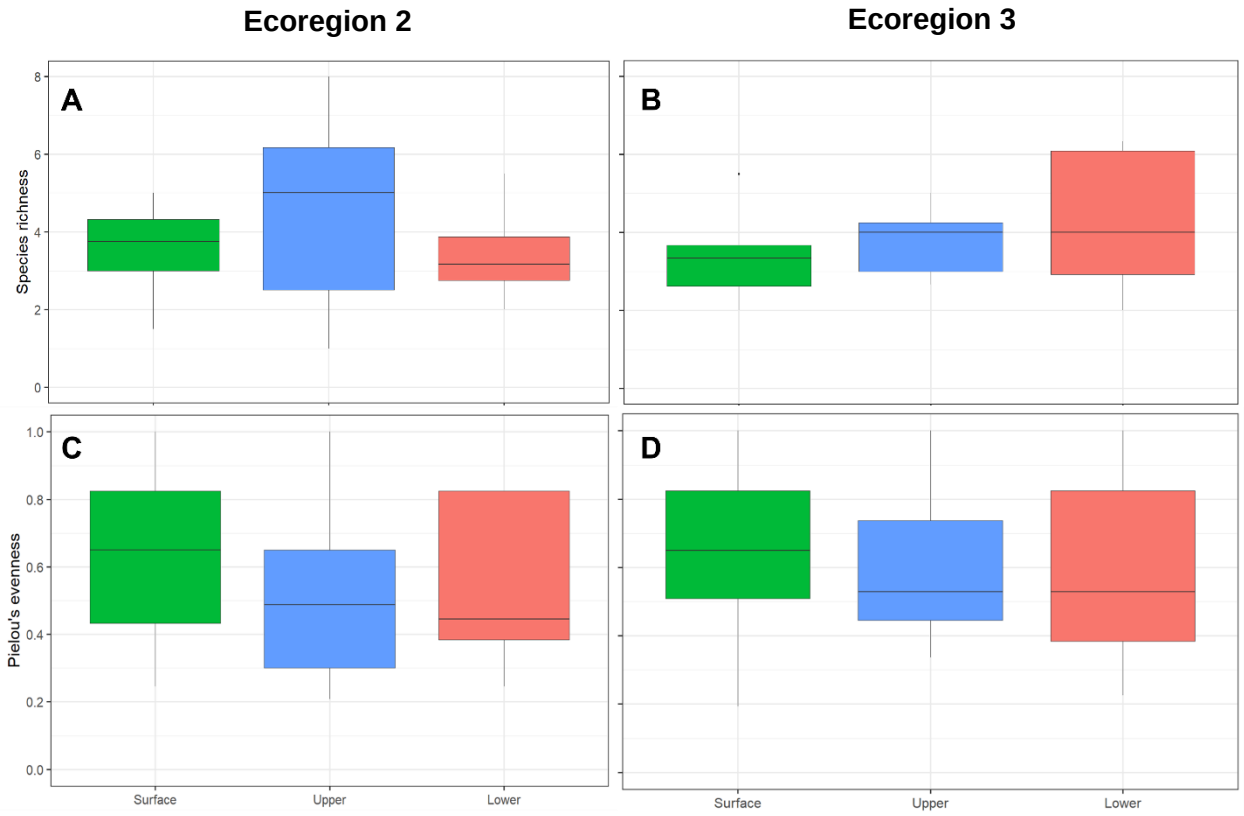


Figure 2.2 Boxplot of species richness from ecoregion 2 (A) and ecoregion 3 (B) Pielou's evenness between eDNA sampling depths from ecoregion 2 (C) and ecoregion 3 (D); Underway surface water (within 4m of the sea surface), Upper (20% depth of water column) and Lower (80% depth of the water column)**.**

Across all data, when separated into pelagic species or strictly demersal species (Fig.2.3), there is a significant increase in species richness of pelagic fish species found from eDNA underway water sampling (H=6.8, df= 1, p=0.008) and upper water column sampling (H=5.1, df= 1, p=0.02). There was no significant difference in species richness (H=1.5, df= 1, p=0.22) or Pielou's evenness (H= 2.6, df= 1, p= 0.1) between eDNA samples taken in stratified or in mixed water bodies (Fig.2.4). Stratified water bodies did

not impact the number of pelagic or demersal species detected when sampling from the surface, upper, or lower water column (Fig.2.5).

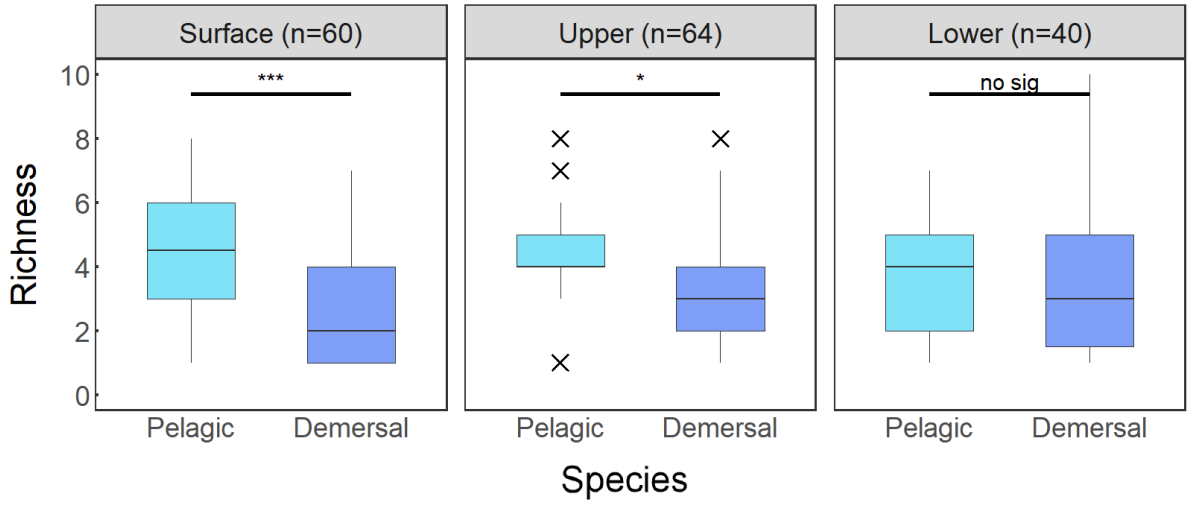


Figure 2.3 Boxplot of species richness of pelagic and demersal fish species detected from surface water, upper water column and lower water column eDNA sampling. The n represents the number of sample sites and outliers marked with X. Underway surface water (n=60) medians between pelagic and demersal fish species were significantly different (Kruskal-Wallis H test, H= , p<0.001). Upper water column samples (n=64) medians between pelagic and demersal fish species were significantly different (Kruskal-Wallis H test, H= , p<0.05). Lower water column samples (n=40) medians between pelagic and demersal fish species were not significantly different (Kruskal-Wallis H test, H= , p=0.7) Fish that were classed as coastal, or where known have daily migrations between demersal and pelagic zones were excluded for this analysis.

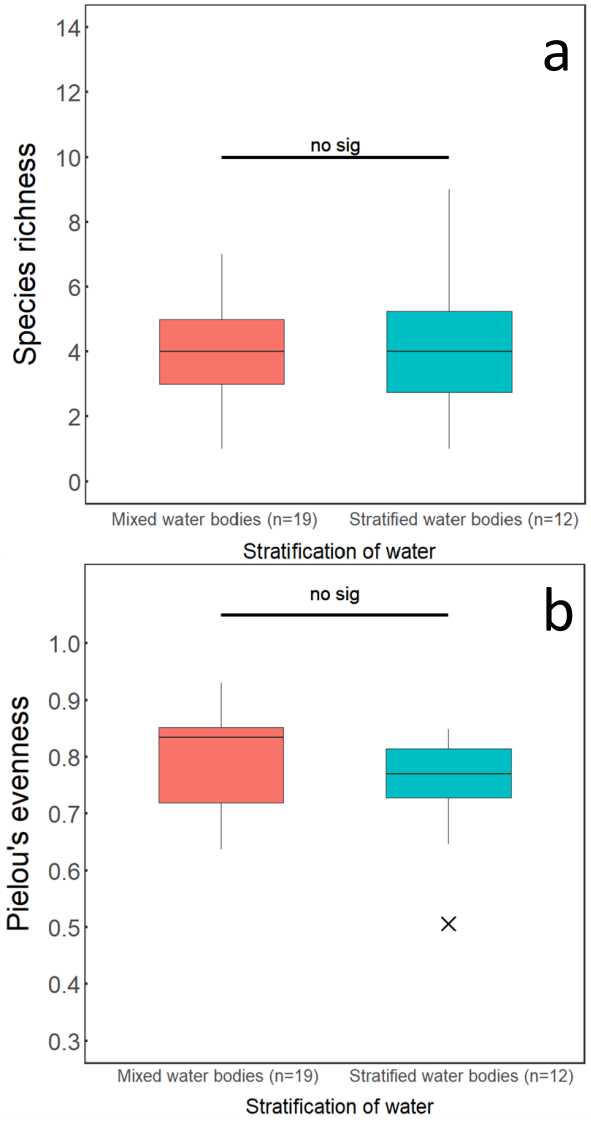


Figure 2.4 Boxplot of species richness (a) and Pielou's evenness (b) from eDNA in mixed or stratified water bodies within the water column, n represents the number of sample sites and outliers marked with X. (a) Medians between water body stratification not significantly different (Kruskal-Wallis H test, H=1.5, df= 1, p=0.22). **(**b) Medians between water body stratification are not significantly different (Kruskal-Wallis H test H= 2.6, df= 1, p= 0.1).

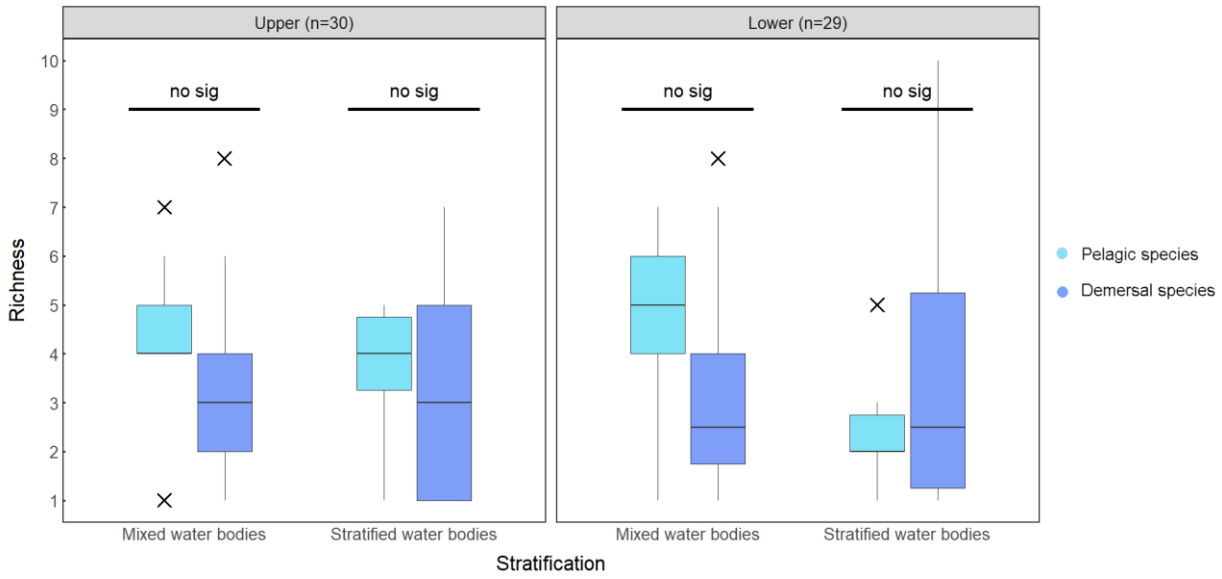


Figure 2.5 Boxplot of species richness of pelagic and demersal species between mixed and stratified water bodies from upper water column and lower water column eDNA sampling. The n represents the number of sample sites and outliers marked with X. Fish that were classed as coastal, or where known have daily migrations between demersal and pelagic zones were excluded for this analysis.

Overall, linear regression found no significant effect on eDNA inferred species richness or Pielou's evenness by the water temperature (richness; F= 0.74, p=0.329, evenness; F=3.2, p=0.07) and salinity (richness; F= 0.39, p= 0.53, evenness; F=0.33, p=0.56). Multivariate linear regression models showed that temperature and salinity had no significant influence on the species richness or Pielou's evenness from different sampling depths (stratified water upper sample depth; richness p=0.5; evenness p=0.4, stratified water lower sample depth; richness p=0.47; evenness p= 0.83, mixed water bodies upper sample depth; richness p=0.7; evenness p=0.41, mixed water bodies lower sample depth; richness p=0.94; evenness p= 0.27).

We found that the assemblages between the stratified and mixed water bodies were compositionally dissimilar based on the bray-curtis index ($p< 0.01$, SS= 1.01, $R^2= 0.09$; Fig.2.6a) but not significantly different based on the Jaccard indices (p= 0.07, SS= 0.42,

R2 = 0.04 Fig.2.6a). Out of the 66 species eDNA detected we found that four species were significantly influencing the mixed water species assemblages; European plaice (*Pleuronectes platessa*), Daubed shanny (*Leptoclinus maculatus*), sand sole (*Pegusa lascaris*), lesser sand eel *(Ammodytes tobianus*). And another four species were directly influencing the stratified water species assemblages; Whiting pout (*Micromesistius poutassou*), Atlantic bluefin tuna *(Thunnus thynnus),* Norway pout (*Trisopterus esmarkii*), *John Dory (Zeus faber*), (Fig.2.6b).

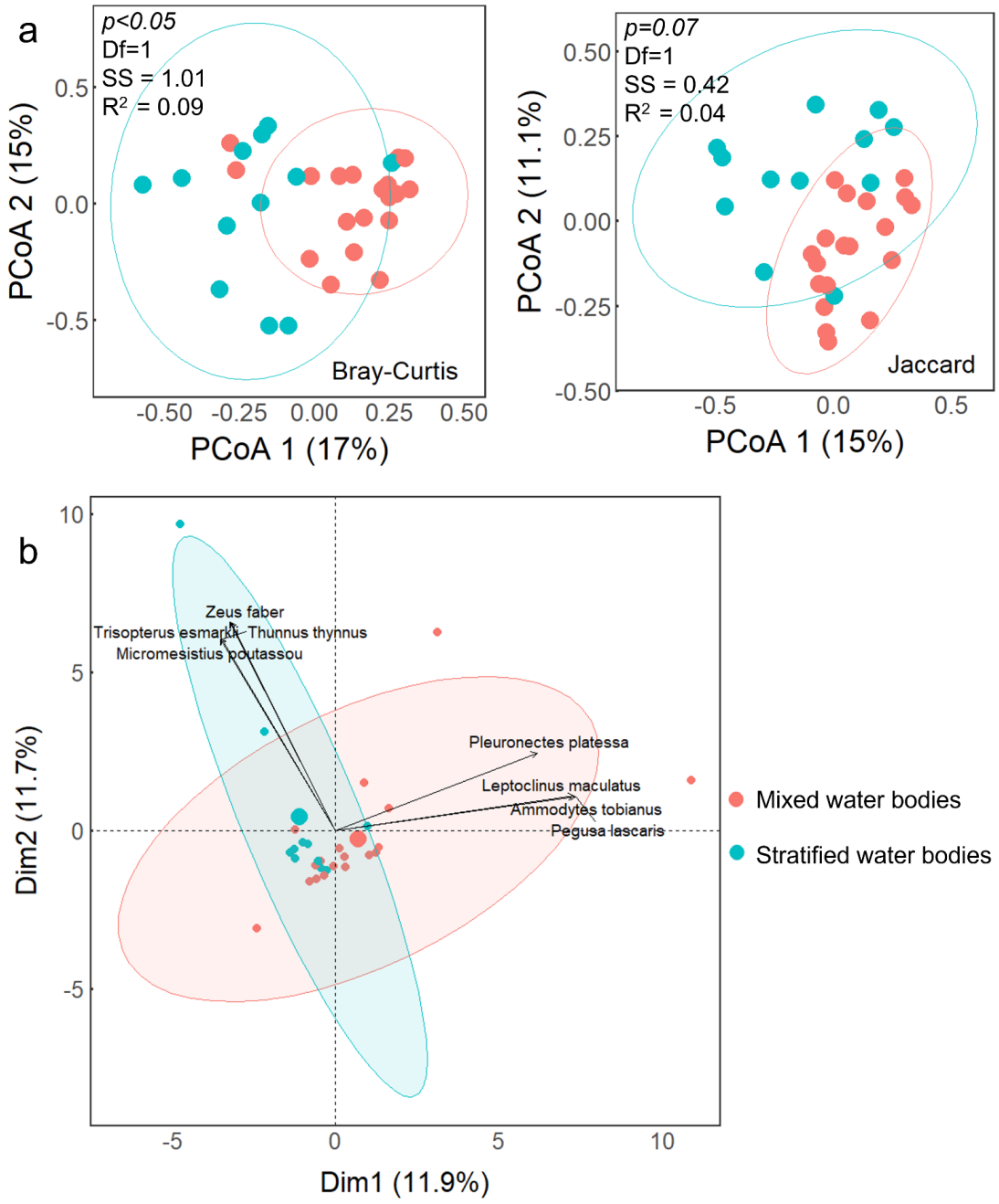


Figure 2.6 Biplot of distance matrices between mixed and stratified water columns. a) Principal coordinate analysis (PCoA) of the species assemblages between stratified and mixed water column using the Bray-Curtis dissimilarity and Jaccard similarity distances. b) Principal component analysis (PCA) of the species that were significantly (p=0.05) impacting the species assemblages of stratified and mixed water columns.

2.4.1 Comparisons between eDNA and pelagic trawling

Overall, eDNA detected 40 more fish species compared to pelagic trawling (n=66 to n=26, Fig.2.7) and had a larger number of unique benthic fish species (n=9 to n=2). Six of the species collected in the trawl could not be unambiguously detected by eDNA: the Atlantic bonito (*Sarda sarda*), black sea bream (*Spondyliosoma cantharus*), great pipefish (*Syngnathus acus*), tub gurnard (*Chelidonichthys lucerna*), grey gurnard (*Eutrigla gurnardus*), and the lesser weever (*Echiichthys vipera*) (Fig.2.7).

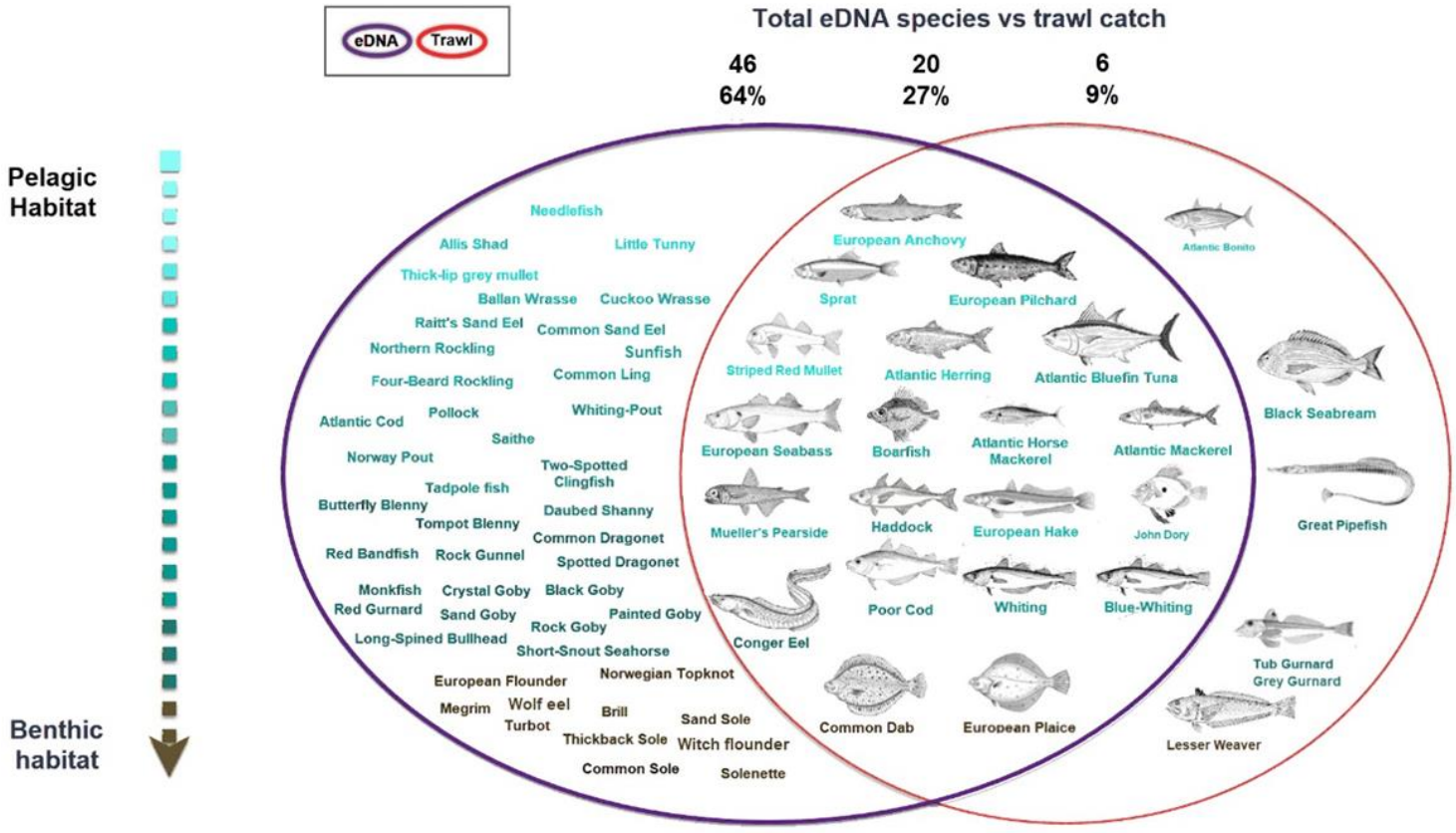


Figure 2.7 An overall Venn of all UK fish species detected by eDNA metabarcoding of the water samples (purple ellipse) and in the pelagic trawl net catches (red ellipse) collected during the 2018

PELTIC survey. Habitat depth categories were assigned to the most common depth utilised by each species during adult life stage and drawings from FishBase (2020)

Of the 20 species detected in the eDNA data and mid-water trawls, only 8 species were contributing to more than 1% of the total catch from trawling whereas eDNA had at least a 1% coverage across all 20 species (Fig.2.8).

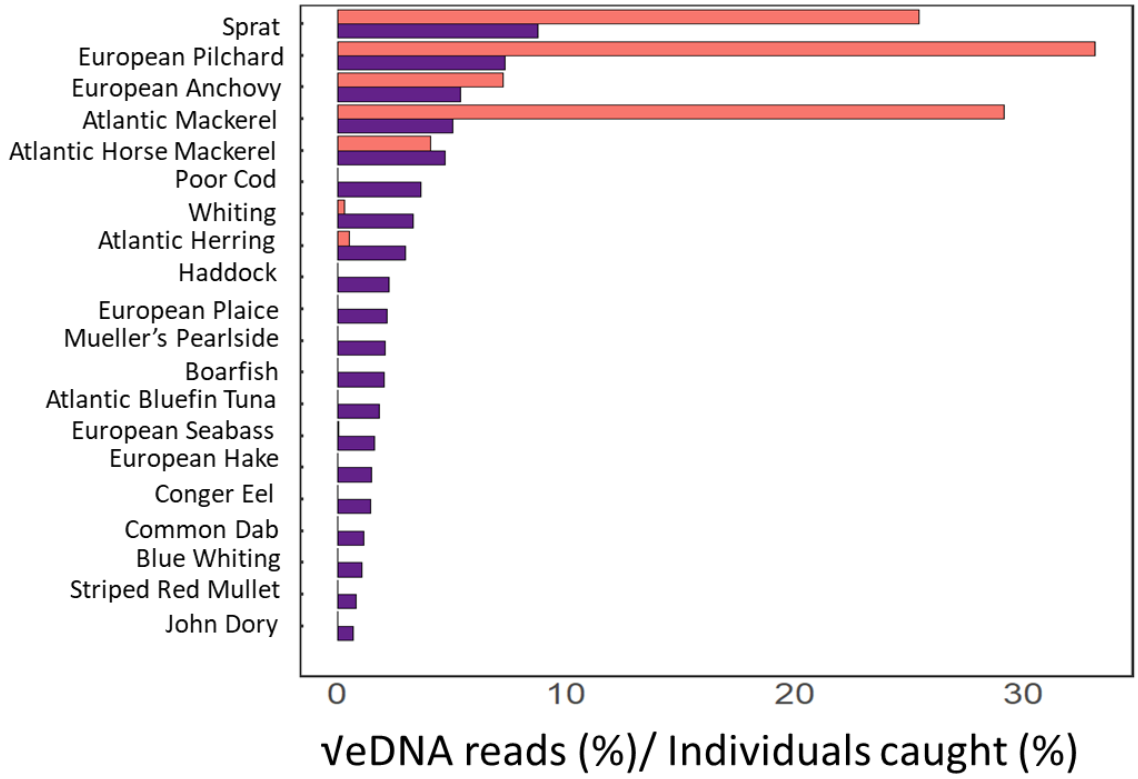


Figure 2.8 Bar plot of the frequency of detection across all samples from the twenty species detected in both the eDNA (purple) and pelagic midwater trawls from the PELTIC18 survey (red). The eDNA data was normalised using square root and is displayed in the descending order of percentage of reads. All the pelagic midwater trawls from the PELTIC18 survey were combined (n=52).

Underway eDNA sampling had significantly higher species richness per site ($H= 18.1$, $p< 0.001$, Fig.2.9a) but significantly lower Pielou's evenness index ($H= 14.8$, $p<0.001$,

Fig.2.9b) than trawling catches in the same sample area. At 3 sample sites six species; Atlantic herring (*Clupea harengus),* European anchovy *(Engraulis encrasicolus),* sprat *(Sprattus sprattus),* Atlantic mackerel *(Scomber scombrus),* European pilchard *(Sardinia pilchardus),* and Atlantic horse mackerel *(Trachurus trachurus*) were detected by both sampling methods.

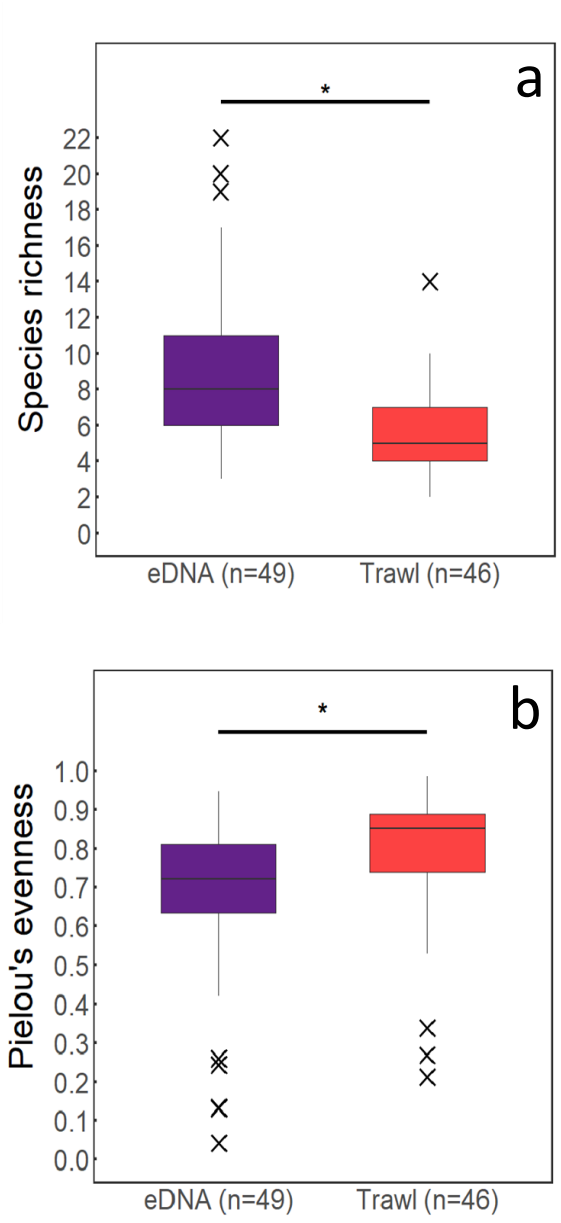


Figure 2.9 Boxplot of species richness (a) and Pielou's evenness (b) between eDNA and pelagic trawl, n represents the number of sample sites and outliers marked with X. (a) Medians between species richness of eDNA and the trawl significantly different (Kruskal-Wallis H test,H= 18.1 ,df=1, p<0.001). **(**b) Medians between Pielou's evenness of eDNA and trawl significantly different (Kruskal-Wallis H test, H=14.8 ,df=1, p<0.001)

The eDNA read percentages per species were compared against each different type of trawl catch metric (number of individuals, biomass (kg) and BSA (m^2). The Spearman's rank correlations for the overall trends found significantly positive but with weak explanations of the variance between eDNA and: individuals (R^2=0.18,p=0.01), biomass (R^2=0.2, p=0.01) and BSA (R^2=0.19, p=0.01) (Fig.2.10).

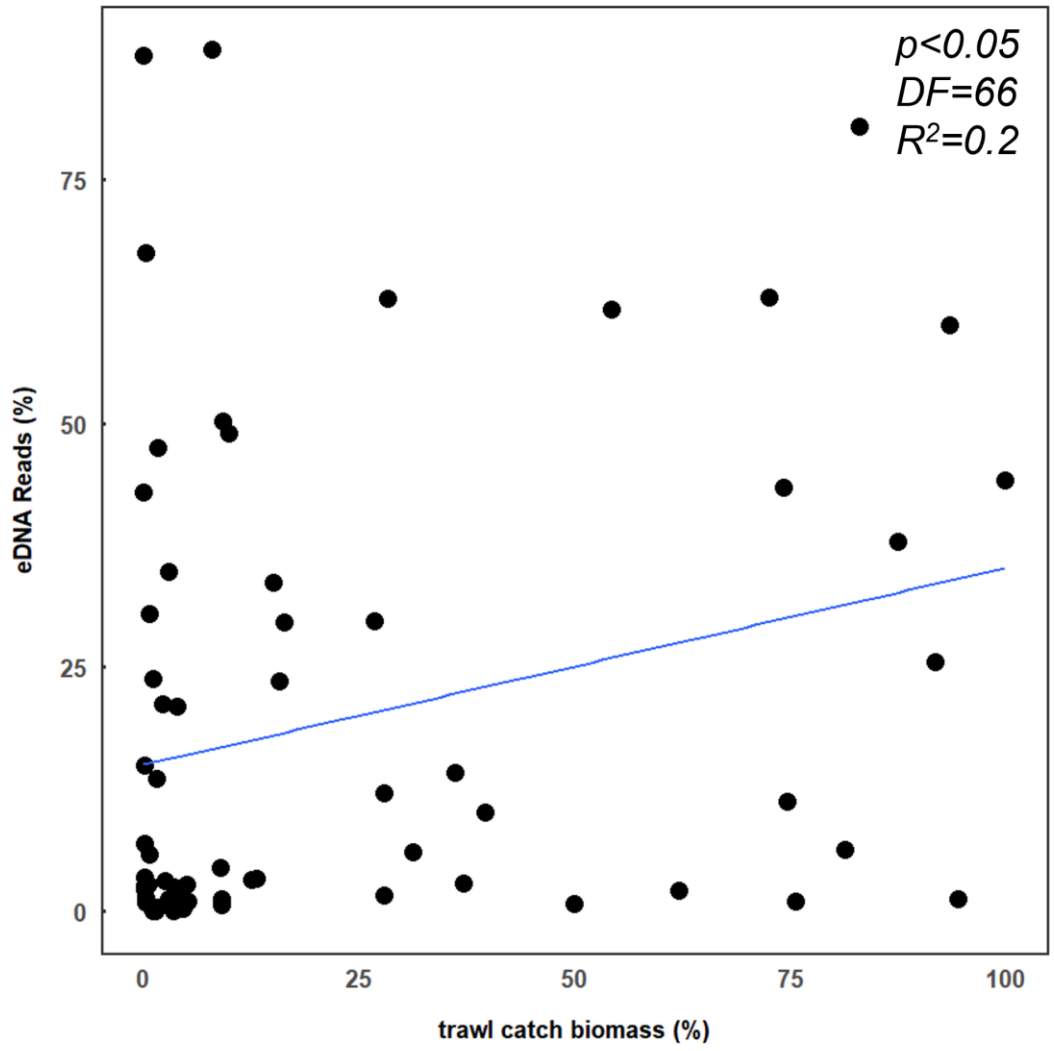


Figure 2.10 Regression plot with a fitted R value between percentage of eDNA reads and fish catch biomass (kg) of species caught in the pelagic trawl at 15 sampling stations. Biomass was used to represent the correlation between eDNA and fish catch metrics because it had the strongest fit to the regression model (R^2=0.2) and because the trendlines of BSA (m^2) and individuals caught had the same statistical significance and incline ($p<0.05$, R^2=0.18). The points represent a single species that was found at least 3 times in both the eDNA data and trawl catch metrics simultaneously. Includes data of species with more than 0.1% trawl catch biomass and 0.1% eDNA read proportions. Species include the Atlantic herring, European pilchard, European anchovy, Atlantic mackerel, sprat and Atlantic horse mackerel.

2.5 Discussion

Recent studies have already demonstrated the potential environmental DNA (eDNA) methods could provide by endowing monitoring surveys with greater power to detect a higher taxonomic resolution from a single survey. However, there is a lack of consensus on the optimal eDNA sampling strategies for the detection of pelagic fish in water bodies on a continental shelf. In this study, we found that neither sample depth, water temperature, or salinity levels of the water column significantly affects estimates of biodiversity metrics, which is needed for UN sustainability and environmental impact assessment monitoring goals, and beta diversity indices detected by eDNA sampling. There was also a weak positive correlation between relative abundances of trawl-caught fish and the relative proportion of sequence reads retrieved from marine eDNA across all sample sites combined, although this relationship broke down at the sampling station level. In terms of taxonomical variety our results clearly demonstrate that eDNA can detect most of the pelagic fish species known to inhabit the North-East Atlantic continental shelf.

Our results suggested that sampling depth had negligible impact on the number of species detected which was congruent with similar marine eDNA studies (Closek et al., 2017; Fraija-Fernández et al., 2020; Stoeckle et al., 2021). However, underway surface sampling (4 metres) and in the upper 20% of the water column resulted in detection of significantly more pelagic fish than demersal species. In contrast, sampling in the lower 80% of the water column did not have significantly more demersal to pelagic species. This is likely to be a result of pelagic fish diel vertical migrations (Easson et al., 2020) which has been observed in many pelagic fish families found in the area (e.g. *Carangidae, Clupeidae, Scombridae*). Pelagic fish move up and down in the water column following the movement of zooplankton or other prey (Bohl., 1979; Gauthier and Rose., 2005; Arndt and Evans., 2022; Cardinale et al., 2003). Demersal species on the other hand tend to be more restricted in their movements, as most of their prey can be found

on or near the seabed, for example Atlantic cod (*Gadus morhua*) remains within 10 metres from the seabed when feeding (Skaret et al., 2020). Furthermore, during our sampling times it was a spawning season for pilchards (*Sardina pilchardus*) and Atlantic herring (*Clupea harengus*), that may have increased mt-DNA being released across the water column, from the surface to the seabed (Ganias and Nunes., 2011; Maravelias et al., 2000). This would increase the vertical spread of pelagic fish DNA in the water column in areas such as the Bristol Channel, Lyme Bay and coastal Plymouth areas where these spawning activities took place (Hansen et al., 2020; Canals et al., 2021).

We also found that stratification had no significant impact on the number or evenness of species detected within a water column and between sampling areas. Like the findings of Closek et al (2017), who sampled in the Pacific Ocean targeting similar pelagic fish families, but within a sample area which had significantly larger total depths (1000-2000m). The stratification of the water column in the North-East Atlantic is rather more tied to seasonal hydrological events (Colebrook., 1984; Lozier et al., 2011), which is relevant, as our sampling took place in the Autumn, when wind and storms increase the likelihood of water bodies mixing across certain locations before “re-stratifying” which may have resulted in the DNA being mixed more evenly in and between stratified water bodies (Simpson et al., 1977; Holt & Umlauf., 2008). Through the analysis of the communities within stratified and mixed water body eDNA sampling we see that the Bray-Curtis indices suggest that there is a significant difference in community composition between the stratified and mixed water bodies. However, only 9% of the variance is explained by this model suggesting that the difference seen may not be fully explained by stratification alone. The data for the bray-curtis indices are inferred from read numbers acting as a proxy for abundance. This might not be an ideal metric to use because we are assuming read abundance is an accurate reflection of actual fish abundance in and between sampling areas, which is not affected by unknown bias, and that the sampling sites have equal distributions of fish communities. Jaccard indices, based on the presence and absence of species, found no significant difference between the community composition across eDNA sampling sites, which further suggests that stratification of the water column is not a significant descriptor of community differences within our sampling areas. To further investigate the effect of stratification on species compositions within the eDNA

samples we used a nested approach (stratification as a variable within sample depth) PCA and showed no significant difference between the species assemblages within mixed or stratified waters. Although there was no significance overall, stratified water bodies were more likely to have the presence of predominantly pelagic species; blue whiting (*Micromesistius poutassou),* Atlantic bluefin tuna *(Thunnus thynnus),* Norway pout (*Trisopterus esmarkii)* and John dory (*Zeus faber*). This further indicates that the exact sample depth on the continental shelf is not a priority for the future of marine eDNA sampling in areas where there is high seasonal mixing of the water column. However, we speculate that in sampling areas where the water column stratification is more pronounced and less variable certain species, such as the four pelagic species we see more often in the mixed water bodies, may have a stronger impact on the community assemblages.

2.5.1 Biodiversity comparisons to the pelagic trawl

Due to the selectivity of pelagic trawling (Kotwicki et al., 2018), and in the context of other aquatic eDNA studies (Rourke et al., 2021), it is unsurprising that eDNA detected significantly more fish species than pelagic trawling and that the species assemblages between trawling and eDNA are not similar between sample sites. By using Pielou's evenness scores we find that the frequencies of species from the trawl are significantly more even (equally distributed) than the detection of fish species from eDNA. Which is indicative of the midwater trawling being a more specific targeted method of sampling where it aims to catch specific schools (Doray et al., 2018). Whereas eDNA (as is the main point of this chapter) is a more opportunistic "catch all" method that is not specifically targeting a single school but rather the diversity of an area. As expected, the eDNA method did not fully overlap with the trawling methods (Thomsen et al 2012), where 6 marine fish species which were present in the pelagic trawl catches were not found in the eDNA: Atlantic bonito (*Sarda sarda*), black sea bream (*Spondyliosoma cantharus*), great pipefish (*Syngnathus acus*), the lesser weever (*Echiichthys vipera*), grey gurnard (*Eutrigla gurnardus*) and the tub gurnard (*Chelidonichthys lucerne*). These species have well-covered mitogenomic reference sequences in both the NCBI and the curated UK fish database and are optimised for teleo2 PCR amplification, which suggests that failed

detection in these species likely stems from low amounts of DNA from these species in the water and were opportunistically caught by the trawl. This could be because of low abundance at the sampling sites, certain specific behaviours, or physiology. While only 1 individual each of the tub gurnard, grey gurnard, lesser weever, and the great pipefish were caught in the pelagic trawls, local beam trawl surveys confirmed that most of these species, as well as black seabream, are generally widespread and abundant in the area (van der Kooij et al., 2011). Apart from the bonito and black seabream, the species that eDNA failed to detect are non-gregarious, low activity organisms that are often near or on the seabed (Gökçe, 1998; Vasconcelos et al., 2004; Howard and Koehn, 1985), with lower aerobic capabilities (Killen et al., 2016) and thus lower metabolic rates (Chabot, Steffensen, and Farrell, 2016); thus, their detection chances in relation to abundant schooling fish, could be lower. In such a scenario, rare templates are easily 'outcompeted' by the more common sequences dissolved in the water column (Hatzenbuhler et al., 2017).

Therefore, to ensure that we can detect the full spectrum of fish species present by sampling the water column, PCR biases for certain fish species should be checked under mock simulations and during sampling a greater number of PCR replicates, deeper sequencing of eDNA samples and/or filtering larger volumes of sample water could increase the probability of detecting low abundant pelagic and demersal species (Jo et al., 2019: Yates et al., 2021; Kirtane et al., 2021).

2.5.2 Not just a tool for biodiversity

In our study, we show that eDNA can provide information on understudied and migratory species. For example the surface-dwelling garfish (*Belone belone*) and the lesser sand eel (*Ammodytes marinus*) that are both key prey species for marine birds (Cox et al., 2016; Paiva et al., 2010), piscivorous pelagic fish (Logan et al., 2011) and cetaceans (Gosch et al., 2020), which modulate energy flow from the lower to upper trophic levels in the Celtic Sea and North-East Atlantic (Camphuysen, 2005; Furness, 2002). No existing monitoring tool captures garfish effectively and information on their distribution

within European waters is scarce (Veneranta & Urho, 2021). Like the garfish, little is known about the distribution of the lesser sand eel outside of their fished areas (Langton, Boulcott, & Wright 2021), which limits the available information we have on their migration in relation to climatic changes. If eDNA can reliably detect species like garfish and sand eels that are understudied, any data found on the distribution of these species would significantly improve our current information on these important species.

Furthermore, at eight different sampling sites eDNA detected Atlantic bluefin tuna (*Thunnus thynnus*), a species which has recently reappeared around UK waters after decades of absence, but their exact migratory routes and locations they are visiting in UK waters are unknown (Horton et al., 2021). This further suggests that eDNA may be a useful tool to track phenology and movement of migratory organisms in the pelagic environment.

2.5.3 Quantifying eDNA from the trawl

As we did not find an impact on the relative eDNA read proportions or the diversity of fish species, from our sampling methods we were able to compare the eDNA data directly with the trawling data without the need for many adjustments. Like previous studies (Kelly et al., 2014; Stoeckle et al., 2021) we found a significant correlation between the overall trawling catch metrics of individual abundance (N), biomass (kg) and the body surface area (bas; m^2) of the species caught and eDNA relative read abundance from surface water sampling (Fig.2.10). This reiterates the findings from Stoeckle et al (2021), but covering deeper, less coastal, pelagic water columns from a single “on the spot” collection rather than a multiple collection, time-series study. However, the relationship has a high spread of variance (with all three metrics) thus these correlations are not a precise prediction of the observed relationship. Other variables such as hydrological influences the transport of DNA (Carranza et al., 2018; Easson et al., 2020), dilution factor impacting the detection of species (Eble et al., 2020), PCR primer choice (Shelton et al., 2016), and catchability issues from the trawl (Fraser et al., 2007), as well as other unknown variables, or a combination of, could be influencing the variation within this model. Current literature suggests that BSA should be a more accurate measure of eDNA release as it scales with

the metabolic rate of fish and thus eDNA read abundance (Kelly et al., 2014). Seeing that each of the trawl metrics had similar variability, with very little impact on the overall correlations observed, we assume that there is a combination of other unknown variables impacting the comparison between eDNA and trawling. It could also suggest that there are methodological problems, for example the use of the modified Mosteller equation for body surface area for multiple species, even though it is likely a useful proxy to use than raw biomass, could be an inaccurate assumption, especially because the measurements used for the size ratios of the fish species caught from the trawl may not be an accurate reflection of the school size classes. Which would also be a safe assumption seeing that the catchability differences from mid water pelagic trawling tends to skew towards larger size classes which may have lower metabolic rates and lower surface area (Fraser et al., 2007; Doray et al., 2018; Yates et al., 2021). Future predictions of body surface area through trawls should use adjusted size class data that accounts for missing smaller size classes and smaller species.

Other possible causes of discrepancies between trawl and eDNA metrics in our study could be the result of inflated eDNA representation from certain species because of spawning activity, including the presence of fish larvae (Takeuchi et al., 2019; Garcia-Vazquez et al., 2021). For example, in our eDNA data set, we consistently found a higher number of reads from sardines (*Sardina pilchardus*) and their presence in more sample sites than the trawling data. At the time of sampling, sardines are known to be spawning in these areas (Stratoudakis et al., 2007; Coombs et al., 2010) and the ichthyo- and zooplankton samples obtained during the survey confirmed sardine eggs and larvae were prevalent throughout the survey area. To further refine marine pelagic eDNA methodology, future studies should look to investigate the effects of other co-variables such as spawning season, and fish larvae composition and concentrations that may be affecting the relative abundance of the mitochondrial eDNA signals in the pelagic water column. For pelagic water column sampling, especially in more coastal areas it is necessary to establish a better understanding of the spatial parameters for sampling marine eDNA under different hydrodynamic conditions. Using the icthyo-plankton eggs and larvae from routine plankton netting could be a potential way to incorporate these measurers into future research by baselining changes in mt-DNA reads across spatial

areas - adjusting the HTS reads of the species by the relative percentage of eggs and larvae of that species were found in the water. Although models of particle movement in the marine environment have been developed (Harrison et al., 2019), the complexity of hydrological features in the pelagic water column stresses the need for further *in-situ* application of these models to further estimate the distribution and fate of eDNA. This could involve *in-situ* use of predictive particle tracking models to help better understand where the DNA or cellular DNA has drifted from to create a more well-defined sampling zone and better define the spatial parameters of the results. For example, the modelling framework could be built based on the surface water movement (measured in meters per second; m/s^{-1}) from satellite data based on the previous 48 hours. This will give us an area or transect that a sample taken from one point could be representative of. These sample areas, depending on water movement could be used to examine the impacts on the number of reads and biodiversity detected to see to test the quantitative and qualitative impacts of transport and dilution of marine eDNA samples.

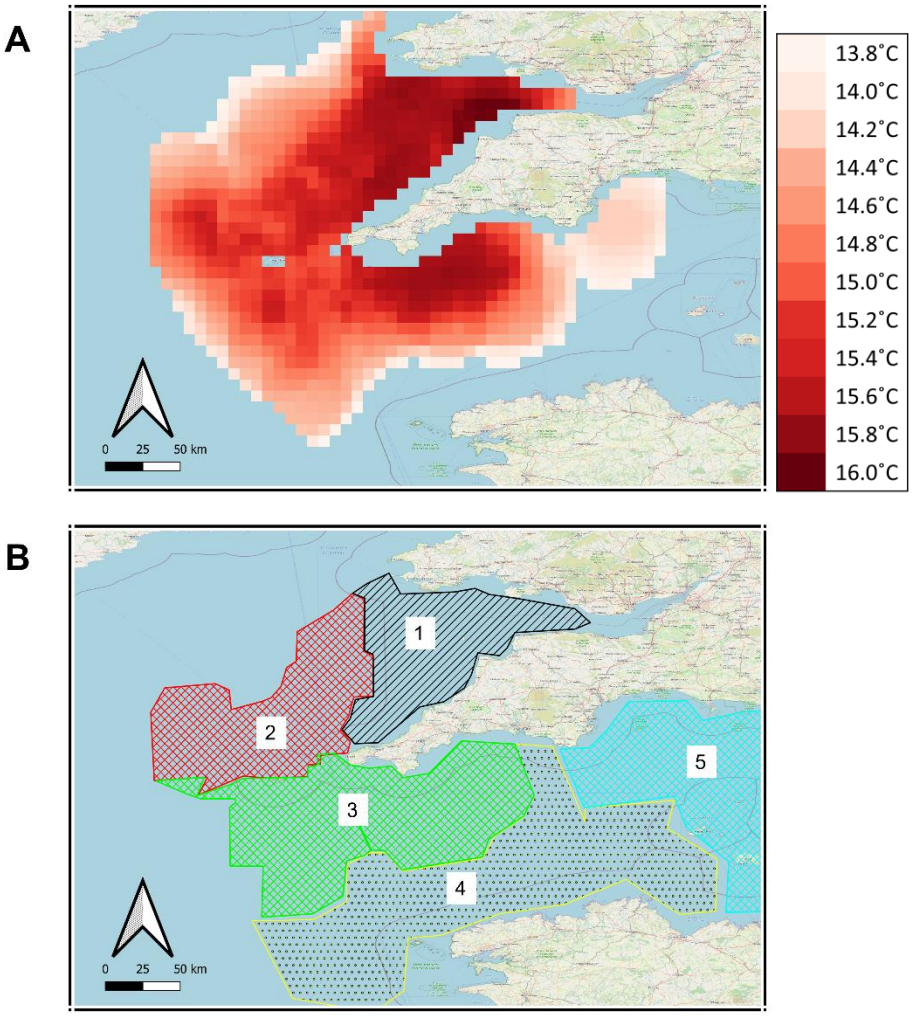
2.6 Conclusions

Through this study we have highlighted that eDNA methods can be used in the pelagic environment to detect a multitude of fish species and crucially that sampling at different depths and within stratified water bodies (that can have different temperature and salinity profiles) does not significantly impact the number of species and pelagic fish communities when samples are collected within one season in ecologically similar areas across the North East Atlantic Continental shelf. There were significantly more pelagic fish species in the surface and upper part of the water column suggesting that future sampling can take place from the surface and still provide sufficient coverage on the pelagic fish communities of that sample site. However, this study also highlights the several unknowns of eDNA sampling that could still be impacting comparisons between current methods and species-specific variances such as the where the eDNA has moved from since release from the organism.

2.7 Chapter II Supplementary Figures

Supplementary table 1.0 Frequencies of the main commercial pelagic fish species detection between eDNA and trawl in regions of interest. Frequencies in bold highlight areas where there is a mismatch in presence of a species between trawl and eDNA.

	Bristol Channel		Inner Celtic sea		Outer Celtic Sea		Western English Channel		Eastern Celtic Sea		Eastern English Channel	
Small Pelagic Fish Species	Trawl	eDNA	Trawl	eDNA	Trawl	eDNA	Trawl	eDNA	Trawl	eDNA	Trawl	eDNA
Sprattus sprattus	98.7%	69.5%	81.5%	39.2%	**0.0%**	46.9%	83.9%	7.9%	0.4%	0.7%	47.3%	19.6%
Clupea Harengus	0.7%	0.5%	2.8%	1.1%	**0.0%**	1.2%	**0.0%**	2.8%	**0.0%**	0.7%	**0.0%**	2.8%
Trachurus trachurus	0.4%	2.2%	5.3%	0.9%	11.6%	0.7%	3.9%	2.2%	25.8%	1.0%	27.3%	7.4%
Scomber scombrus	0.1%	1.0%	10.0%	1.4%	14.5%	4.2%	1.2%	3.0%	15.3%	0.7%	0.3%	8.7%
Engraulis encrasicolus	**0.0%**	0.2%	0.2%	6.5%	4.2%	0.9%	7.0%	2.0%	17.7%	12.8%	17.5%	16.1%
Sardina pilchardus	**0.0%**	19.7%	0.1%	43.8%	**0.0%**	35.2%	3.7%	40.9%	40.8%	73.4%	7.5%	26.5%
Micromesistius poutassou	0.0%	0.0%	**0.0%**	0.1%	0.0%	0.0%	0.0%	0.0%	**0.0%**	0.6%	0.0%	0.0%
Merlangius merlangus	**0.0%**	1.3%	**0.0%**	0.6%	**0.0%**	1.5%	0.3%	1.0%	0.0%	1.6%	0.1%	0.1%



Supplementary Figure 1.0 (A) Sea surface temperature map of the sample area measured from a continuous monitor on the vessel. Each square represents a 10 nautical mile area where temperature has been averaged across (B) Ecoregion map split into zones where there is more than a 0.6˚C change in seas surface temperature.

Chapter III

Complementing Bluefin Tuna Monitoring in the UK with eDNA analysis

3.1 Abstract

Atlantic bluefin tuna (*Thunnus thynnus,* ABT) are one of the most valuable fish species in the world, but because of their highly migratory nature the management of their stocks is complex. The Eastern ABT stock spawns in the Mediterranean and migrates across multiple European and North African nations' EEZs. In 1963 the Eastern ABT stock collapsed, leading to its disappearance from Northern European coastlines in the decades following. From 2014, observational and tagging evidence indicates that the Eastern ABT stock are returning to UK waters, but it is unclear whether this is as a result of population increase or because of change in prey distribution and climatic favourability. Assessing this change in ABT distribution requires adequate monitoring of their distribution ranges and time spent in UK waters, which typically involves tagging ABT to track their behaviours as well as observational data from aerial surveys, fishery surveys and the public. Although these methods are well established, they require significant time and financial investment, especially over large spatial areas. Environmental DNA can provide reliable, monetary and time efficient data on ABT is eDNA, by potentially detecting the presence of ABT DNA as well as a wide range of other marine organisms in the water column. Here we show that 12s eDNA metabarcoding can be used to verify the observational data on ABT in UK waters, but also detect their presence in areas where observers had previously not seen them. Furthermore, eDNA sampling can be used as a weekly sampling technique to detect ABT presence within an area over time. We discuss

discrepancies between eDNA and visual observations and place them in the context of ABT migratory behaviour. This study indicates that eDNA should be considered as an integrative tool to further investigate and support observational and tagging monitoring of ABT stocks.

3.2 Introduction

Atlantic bluefin tuna (*Thunnus thynnus*, henceforth ABT) are the largest species of the family Scombridae and has been a desirable seafood product in both Western and Eastern cultures, (Bestor, 2004; Campling, 2012; Jacquet, & Pauly, 2007). Although there is variation between different ABT populations (Rodriguez-Marin et al., 2015), they can grow up to 3 metres in fork length, weigh over 600kg and live for over 30 years (Safina, 1993; Kitagawa and Kimura, 2015; Secor et al., 2009). They are a powerful, fast, continuous swimming species, that display seasonal migratory behaviour, slow population growth (Fromentin & Fonteneau., 2001; Neilson et al., 2008), wide thermal tolerance (Arrizabalaga et al., 2015) and a broad habitat range (Fig.3.1a, Kell et al., 2003).

In the Atlantic Ocean there are two main ABT stocks, the Eastern and Western, that are delimited by the breeding and spawning locations on the opposite ends of their Atlantic distribution range (Fig.3.1). The Western Atlantic stock spawns in the Gulf of Mexico and the Eastern stock spawns in the Mediterranean Sea (Fig.3.1, Fromentin, 2009; Muhling et al., 2017). These stocks can migrate over thousands of kilometres (Fig.3.1b) within a season and are considered ‘straddling stocks’ as they cross multiple nations’ exclusive economic zones (EEZs) (ICCAT, 2002). Both stocks have been known to migrate into Northern European waters (Mather et al., 1995, Fromentin and Powers, 2005; Muhling et al., 2017; Rooker et al., 2019).

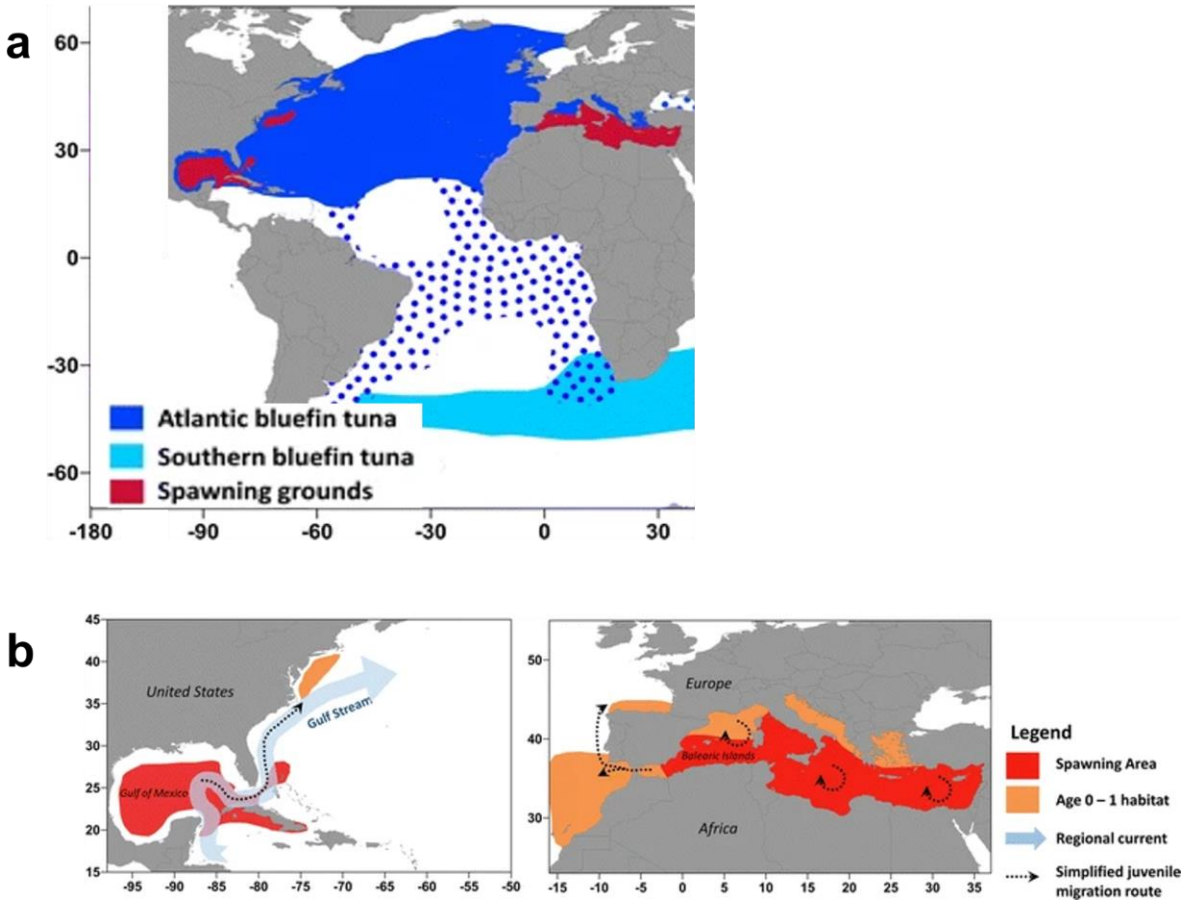


Figure 3.1 Maps of Spatial distribution, spawning grounds and juvenile migration pathways of East and West Atlantic bluefin tuna stocks a) Adult distributions and spawning ground of Atlantic bluefin tuna (ABT) and Southern bluefin tuna in the Atlantic Ocean and Mediterranean sea. Places where ABT are rarely spotted or have low abundance is represented by the blue dots. b) Mean juvenile migration pathways, circular arrows indicate where there is an overlap between juvenile and adults and larvae (Muhling et al., 2017) [Edited]

3.2.1 Fall and Rise of the Eastern ABT stock

Although data on ABT pre-1970 are not well documented, historical records and archaeological finds show that ABT was targeted by fisheries in the Mediterranean since the 8th century CE (Cort and Abaunza, 2019; Andrews et al., 2022). Multiple sources also outlined the common presence of ABT in Northern European waters in the early 1900's (Le Gall, 1927; Wolfe Murray, 1932). Due to their migratory behaviour and wide distribution, multiple century-long fisheries have been set up in countries such as Spain, France, Italy, Norway, and the UK that all caught the same Eastern ABT stock, which for the early parts of the 1900's was largely unregulated (Fromentin, 2009; MacKenzie &

Myers, 2007; Longo, 2011). Such unregulated ABT landings led to the overfishing of ABT in 1963 (Fromentin and Powers, 2005), which prompted the 1966 set up of the International Commission for the Conservation of Atlantic Tunas (ICCAT) to monitor and regulate tuna fishery resources.

However, the continued decrease in ABT spawning biomass since 1963, eventually led to the collapse of Northern European ABT fisheries in the 1980s by which time ABT also had disappeared from Northern European waters (Nøttestad and Graham, 2004; Cort & Abanuza 2015). The overall consensus on the cause of this points towards the overfishing of ABT from the industrialization of fishing fleets, driven by increasing demand from the japanese markets and an increase in their landing price in the mid-20th century (Safina and Klinger, 2008; Cort & Abanuza, 2015, Andrews et al., 2022).A decrease in prey abundances (Dickey-Collas et al., 2010) compounded by overfishing led to poor recruitment, and a loss of 60% in spawning stock biomass of Eastern ABT in the 1970's (Fromentin, 2009; ICCAT, 2007).

3.2.2 Return of ABT to UK waters

Since the late 1990's ICCAT have shown that Eastern ABT catches have started to make a recovery (Cort and Abaunza, 2019), with a year-by-year increase in stock biomass throughout the 2000's (Boge, 2019; Tensek et al., 2018). More intriguingly, ABT has also been detected in 2014 from observer data in the English Channel for the first time since the 1980's fishery collapses (Pinnegar et al, 2020; Horton et al., 2021).

Little is known about the reasons for the reappearance of ABT in the English Channel. It could be that the increase in Eastern ABT stock has induced a density-dependent distribution change in ABT, like the 1960-1990 shifts in *Thunnus* species geographic range observed by Worm & Tittensor (2011). Or there could be other factors such as a change in climate suitability (Failletaz et al., 2019) that has resulted in a shift in preferable prey populations like herring (*Clupea harengus*, Pinnegar et al., 2020), mackerel (*Scomber scombrus,* Fromentin, 2009) or squid (van der Kooij et al., 2016) making the English Channel a new suitable feeding ground for mature ABFT (Druon et al., 2016).

3.2.3 Monitoring the return of ABFT in UK waters

Current fisheries independent tools employed to monitor ABT include sightings from the general public and observer's on-board fishery surveys. These surveys count individuals and schools of ABT, but it is a situational approach, requiring the ABT to appear at the surface during daylight hours and during favourable weather conditions (Polacheck et al. 1999; Bauer et al., 2015). Tagging data is used for tracking migrations and residence time within UK waters. However, tagging methods can take many months before the data can be processed and are generally costly (Bauer et al., 2015; Di Natale et al., 2017; Horton et al., 2021).

One prominent method that is highly effective in the capture of marine organisms, and importantly is cost and time efficient, is environmental DNA (eDNA) metabarcoding. As mentioned in previous chapters, this method identifies species through the genetic material left behind by organisms in the water column (Taberlet et al., 2018; Thomsen & Willerslev, 2015). Using eDNA can detect a greater range of marine fish species than other traditional capture methods and is an easily deployed method, requiring only the simple collection of seawater (Bessey et al., 2020; Zinger et al., 2019), therefore potentially 'tracking' ABT over time and space, while simultaneously informing on a wider range of fish species occupying the studied habitats.

In this study we explored whether weekly eDNA water sampling can capture the temporal changes in ABT presence in two fixed sites in the Western English Channel. We also integrated these findings with observational data and additional survey data from across the South-West coast of the UK to explore the potential role of eDNA in ABT monitoring.

In addition, we explored whether eDNA detections can provide information about the ecological drivers that may be influencing the presence of ABT in UK waters, under the hypothesis that the presence of ABT will be associated with the presence of their known prey species.

3.3 Materials and Methods

eDNA samples were collected during two fieldwork programmes: the Pelagic ecosystem survey in the Western Channel and Celtic Sea (PELTIC) and the Thunnus UK project (University of Exeter and cefas). The PELTIC integrated ecosystem survey collects information on key pelagic species such as small pelagic fish, marine birds, cetaceans, and zooplankton using hydroacoustics, midwater trawling, onboard observers and plankton nets.

Through the use of satellite tagging and tracking devices the Thunnus UK project aims to provide information on when and where ABT are, feeding, breeding, and migrating through in UK waters.

3.3.1 Weekly sampling eDNA collection

Water samples were taken in triplicate 2L samples once a week from one of two locations off the South-West coast of the UK from July to the end of December 2019 for a total of 68 samples (Fig.3.2). Sample sites were selected from known historical sightings from previous surveys (Horton et al., 2021) and because the same sampling vessels were being used for current ABT tagging activities around those areas.

The water samples were collected using a single Niskin deployment to 4 m depth from a vessel, as it left port to conduct ABT tagging fieldwork. Samples were collected before tagging and a deionised water field blanks were taken at each site. Each replicate was collected into sterile Nalgene 2L bottles and stored in a temporary ice box, until later filtration of the water within 24 hours onto a 0.22um Sterivex filter (Merck) using a peristaltic pump and stored in a -20°C freezer.

3.3.2 Fixed stations eDNA collection

To capture the spatial features of ABT distribution, eDNA water samples were collected during two annual pelagic ecosystem surveys in the Western Channel and Celtic Sea (PELTIC) that took place between October and November. Samples were either collected from surface waters while the vessel was steaming (using a continuous flow-through

system) or from different depths, while stationary from a Rosette (see Chapter 2 for more details). A total of 472 eDNA water samples were collected, of which, 210 from the underway sampling collection method (4 metre depth) and 262 by rosette (from depths between 20-112 metres). From the 2018 survey (PELTIC18) a total of 205 samples were collected and a total of 270 samples were collected from the 2019 survey (PELTIC19). As mentioned in Chapter 2, the eDNA water samples were filtered through a 200-micron mesh before being collected in 2.5 L Nalgene bottles, then stored at 4 °C for less than 24 hours before filtration of the water through a 0.22um Sterivex filter (Merck) using a peristaltic pump and stored in a dedicated -80°C freezer.

3.3.3 DNA extraction to sequencing

For the weekly sampling, the frozen Sterivex filters (Merck) were removed from the cartridge and one half of the filter was extracted using the Mu-DNA protocol (Sellers et al., 2018). The other half of each filter was stored at -20°C. For the weekly sampling and PELTIC19 samples, the Mu-DNA protocol was adapted to exclude the use of garnet beads, through optimisation tests, as previously mentioned(chapter 2), PELTIC18 extractions followed the Mu-DNA water protocol. The samples were amplified through PCR targeting the 12 region by using the ‘Tele02’ primer set (Miya et al., 2015; Taberlet et al., 2018). All samples from the weekly and fixed station sampling were adjusted and pooled for a target library concentration of 1µg total DNA making 1 sequencing library for the weekly samples, 3 libraries from PELTIC18 and 4 libraries from PELTIC19. Following DNA clean up and purification libraries were quantified and diluted in a 20µl library. The weekly samples were sequenced on an Illumina iSeq 100 platform using the i1 (v2) 300 cycle kit with a 10% PhiX (Illumina) control at a final sequencing molarity of 50pM. The fixed station samples were loaded onto a Illumina MiSeq platform using (v2) 2×150 cycle with a 10% PhiX control and a final sequencing molarity of 8pM. A more detailed recount of the extraction to sequencing methods is detailed in the supplementary methods.

3.3.4 Bioinformatics

Both the weekly and fixed stations (PELTIC18 & 19) eDNA data followed the bioinformatic pipeline described in Collins et al., (2021). Fastq files were demultiplexed using paired combinatorial demultiplexing from 'cutadapt' v3.40 (Martin, 2011) then denoised using the DADA2 v1.17 pipeline (Callahan et al., 2016). Taxonomy was assigned through a three-step process: first, by using the syntax output from a nucleotide blast to filter the best bitScore (highest quality matches), then through a secondary blast against a curated 12S UK fish database (SeaDNA database); lastly, sequences were assigned again using likelihood based phylogenetic inference using RAxML-NG, gappa (Czech et al., 2019) and epa-ng (Barbera et al, 2019). ASV sequences that were unassigned were removed from the data.

3.3.5 Blank filtering and downstream analysis

Contaminant ASVs In both the spatial datasets contaminant ASVs and all of their reads that were present in the PCR-, extraction- and field-blanks were identified by using the decontam R package (https://github.com/benjjneb/decontam) and were removed from their corresponding samples. There were no contaminant ASVs in the PCR-, extraction- and field-blanks of the weekly sampling. To counteract false positives induced from sequencing any ASVs that amounted to less than 0.1% of the average total read number per sample were removed from the weekly sampling and spatial data. Sample site replicates were then combined, and the read numbers averaged. The spatial data was then transformed into binary data for the presence or absence of ABT. For the weekly sampling data to reduce the impact of disproportionate reads between samples, read counts over the upper quartile read range were reduced to the 75th percentile (>371 reads). The number of reads in the weekly sampling data was then square root transformed to account for large variances in the data set.

The spatial distribution of detected ABT was then mapped using arcGIS (ESRI) and compared with ABT surface water sighting data from observers on the PELTIC 18 & 19 surveys. For the weekly sampling a heat map of the square root number of reads of ABT compared against other vertebrate species detected by eDNA across the sample months

was plotted in R (v4.1.1, R core team 2021) using ggplot2 (v3.3.3; Wickham, 2016). Prey species of the ABT were assigned from diet analysis papers from ABT caught in the Mediterranean Sea (Gunther et al., 2021), northeast and the northwest Atlantic (Logan et al., 2011; Estrada et al., 2005), but because of ABT's opportunistic feeding habits, other similar small pelagic fish were also included. ABT and their associated prey species detected from both the fixed location samples and from the spatial data (PELTIC19) were then compared across the sampling months against the ABT sighting data from the South-West UK coast from Horton et al., (2021) and prey species catch data from commercial catch data, specifically the landing weight (tonnes) of pelagic fish from over and under 10 metre vessels, from ICES statistical rectangle 29e5 (MMO, 2021). Species co-occurrence analysis was conducted in R using the package co-occur (v1.3, Griffith et al., 2016).

3.4 Results

Atlantic bluefin tuna (ABT) DNA was found 21 times across 10 sampling stations and 6 times across 4 stations, during PELTIC18 and PELTIC19 fixed sample stations respectively (Fig.3.2). This means that ABT was found at 20% of the stations from the PELTIC18 survey and 6.5% of the stations from PELTIC19. At the fixed stations weekly sampling data detected ABT DNA in 11% of all samples, from 6 sampling replicates across 5 sampling weeks (Fig.3.3).

The fixed station samples (PELTIC18 and PELTIC19) MiSeq run yielded a total of 7.8 million reads for PELTIC18 and 18.3 million reads for PELTIC19. After trimming and filtering a total of 3.98 million UK teleost fish reads across 143 samples from PELTIC18 and 6.72 million UK teleost fish reads across 159 samples from PELTIC19. From the fixed sampling stations there were a total of 68 identified fish teleost fish species, 3 elasmobranch species and 2 marine birds.

The weekly sampling iSeq 100 run yielded a total of 1.49 million reads, of which a total of 428,000 reads remained after trimming and filtering. Taxonomic assignment to 97% percentage identity found 77,297 reads were from UK teleost species. The ASV for ABT also had the same 100% percentage identity score from the nucleotide BLAST as yellowfin tuna (*Thunnus albacares*, YFT). This ASV was assigned to ABT because the

YFT is an extremely rare occurrence in UK waters. This left a total of 92,600 reads from 27 teleost fish species, 3 bird species, 3 marine mammals and 2 elasmobranchs, with an average of 2,760 reads per sampling event. Of the 68 samples, 40 samples had no fish species reads and a total of 5 of the 23 weeks sampled contained no data, but there was sample representation for every month. Most samples that had no reads were from late August and September.

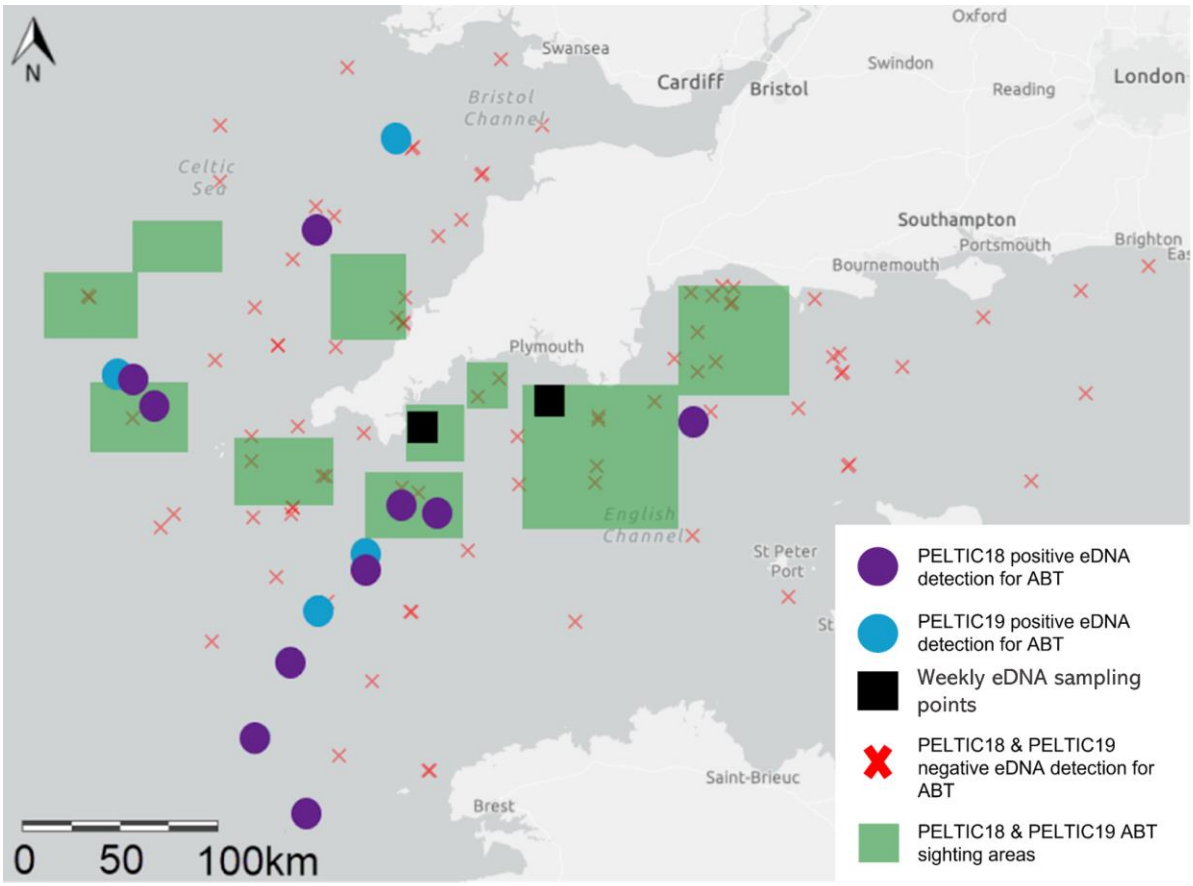


Figure 3.2 Map of eDNA samples showing the spatial distribution of ABT from October- November 2018 and 2019 around the South-West coast of the UK and the sample locations of the weekly eDNA sample locations. The black squares indicate where eDNA has been sampled across July-December 2019. Purple points show where eDNA samples found ABT during the 2018 survey and blue points show where eDNA samples found ABT during the 2019 survey. The red crosses are where there was no presence of ABT in the eDNA samples from the 2018 and 2019 surveys. Green transparent boxes indicate the areas during daylight hours where observers during the survey spotted ABT at the surface, sightings were carried out along transects across the entire map. The transects were used to draw the rough length and width of each sighting area.

Atlantic bluefin tuna (ABT) was detected in 5 of the 23 weeks sampled, once in July, twice in August and twice in November (Fig.3.3). Sighting data and catch data shows that ABT and their prey species are present within the sample site and the surrounding area across all sampling months., Prey species detected from eDNA show generally higher read numbers across August and November (Fig.3.4).

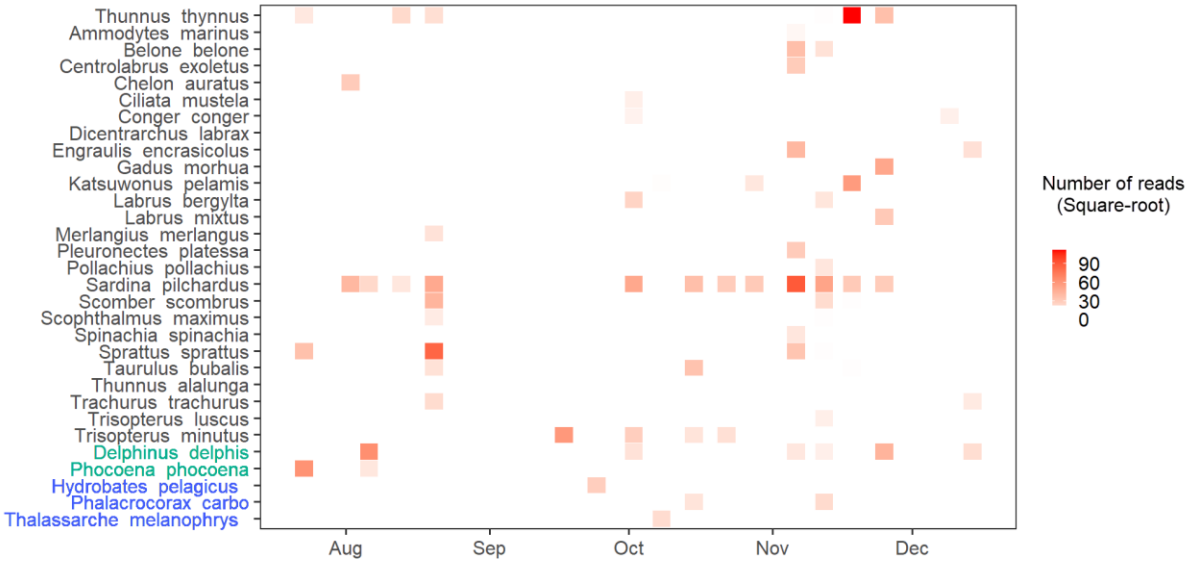


Figure 3.3 Heat Map of the number of weeks each eDNA detected species were present at the fixed sample areas from the months of July-December 2019. On the Y-axis we start with ABT then other fish species in black text colour, in green text font is marine mammals and blue text font is marine birds. Only weeks with data from at least 2 of the three sample replicates were used in this figure.

Diversity of marine pelagic fish species was highest when eDNA bycatch species such as marine birds and cetaceans such as the great cormorant (*Phalacrocorax carbo*), European storm petrel (*Hydrobates pelagicus*), black browed albatross (*Thalassarche melanophrys),* and the common dolphin (*Delphinus delphis*) were also present. Although, no other more common bird species were detected. Co-occurrence analysis found no significant relationship between ABT and other species, although we found a significant positive correlation between the common dolphin (*Delphinus delphis*) and European pilchard (*Sardina pilchardus*), as well as European pilchard and Atlantic mackerel

(*Scomber scombrus*) and European pilchard and sprat when there was high sequencing depth of the sample (supplementary Fig.3.1).

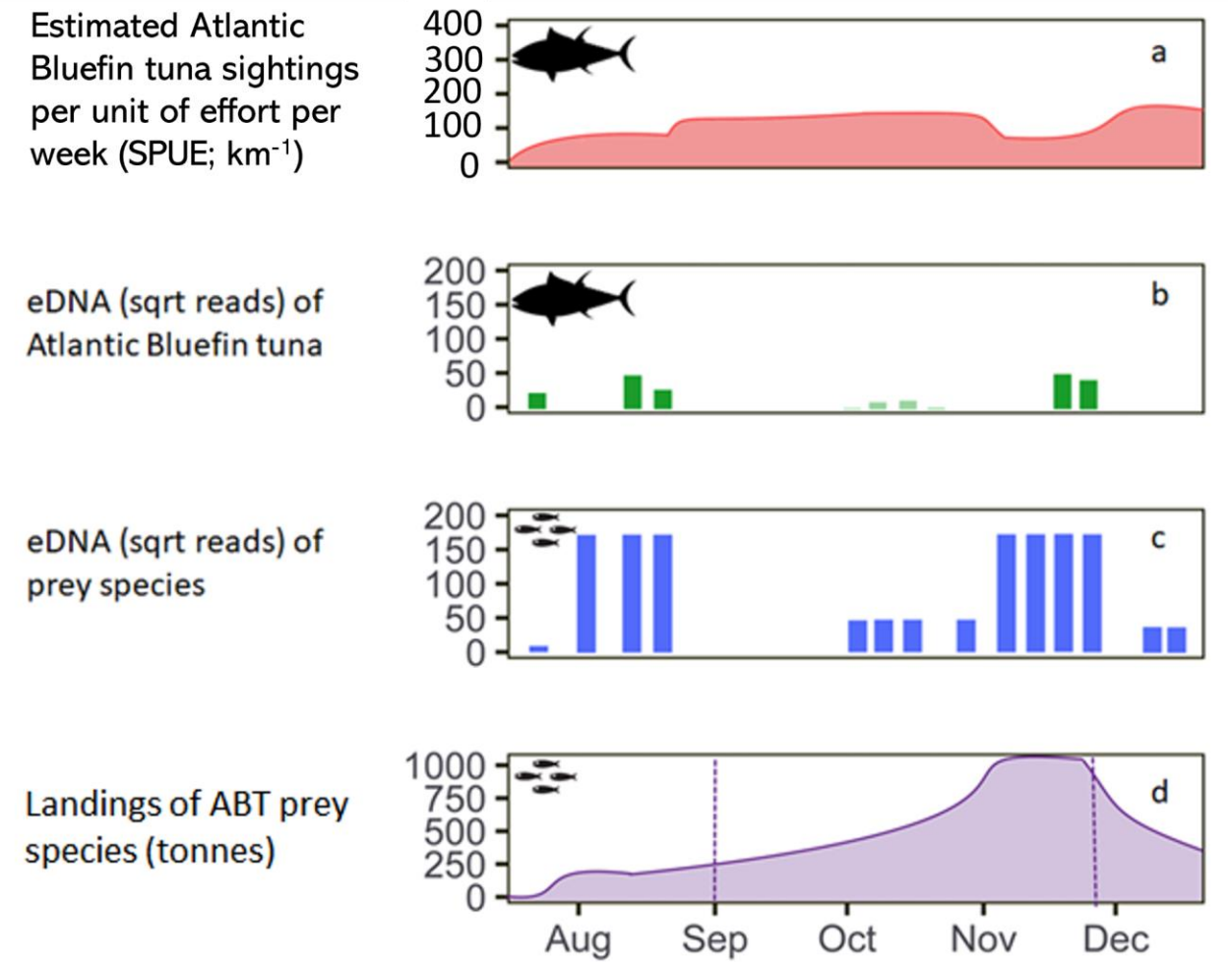


Figure 3.4 Bar chart, Line and area graph of the eDNA data compared against the estimated ABT sightings percentage from different sources per month and prey species catch data from ICES statistical rectangle 29e5 through July to December 2019. **a**) The estimated number of ABT sightings per unit of effort across each week from: visual surveys (n=319 sightings), and general public sightings from 'Ecotours' and sightings around Cornwall and 29e5 (n=255 sightings), estimated using data from Horton et al (2021). **b**) The square root of normalised reads of ABT eDNA from the temporal sampling in 29e5 (dark green) and from the PELTIC19 survey during October to November 2019 (light green). **c**) The square root of normalised reads of ABT pelagic prey species from the eDNA data, likely prey species were identified through Gunther et al (2021), Logan et al (2011) and Estrada et al (2005). Small pelagic species from similar families were also grouped in to represent ABT's opportunistic nature. **d**) Landings (tonnes) of the identified ABT pelagic prey species caught from both under and over 10m classed vessels (MMO, 2021). The dotted lines in September and the end of November represent the start and the end of the most active parts of the fishing season in terms of CPUE (MMO, 2021).

3.5 Discussion

There has been increasing visual and tagging evidence to suggest that the Eastern Atlantic bluefin tuna (ABT) are migrating through UK coastal waters for longer time periods. Although tagging data has proven to be informative, it is able to track a tiny portion of individuals within the population. Furthermore, sighting data from observers' onboard research vessels, or the general public, may not provide accurate information on ABT distribution and abundance as they rely on ABT breaching the sea surface, are affected by weather conditions and may be susceptible to misidentification. In this study we explored whether weekly eDNA water sampling would be able to track ABT presence over the period during which these fish are known to be around the South-West of England. We found that eDNA was able to detect some fluctuations in Atlantic bluefin tuna (ABT) presence in UK coastal waters against the backdrop of the broader fish community in the studied area but did not find ABT in all weeks where there were sightings.

3.5.1 ABT spatial distribution

The spatial sampling data from the PELTIC surveys showed some mismatches between eDNA and sighting derived ABT presence. These were typically in open ocean water of the Celtic Sea and were affected by adverse weather conditions for a total of 7 days across both PELTIC 2018 & 2019 surveys. Bad weather conditions are known to affect visual aerial surveys for ABT (Di Natale et al., 2017) and most likely had an impact on onboard observers' ability to spot ABT at the surface water, which highlights one of the benefits of eDNA sampling: its non-reliance on environmental conditions that impair visual observations from surface water.

Conversely, PELTIC eDNA data only detected ABT in two of the ABT sighting areas. For example, the eDNA data collected in the Eastern English Channel, where there were the

largest sighting areas of ABT, had no ABT eDNA detections. But it is worth noting that the eDNA sampling for ABT on the PELTIC18 & 19 surveys did not take place when ABT was sighted by onboard observers. Instead, samples were taken during trawling activities and at fixed sample stations during the night when observers are not actively looking for ABT. This suggests that spatial and temporal mismatches between eDNA water sampling and ABT visual observations are likely to occur, which are modulated by the interplay among the fast tuna movements, oceanographic dynamism, DNA degradation, and this should be noted for future sampling efforts. From this research it would suggest that visual surveys give more consistent data on ABT presence, which is also aligned with the prey catch data, but eDNA samples with higher sequencing depth also started to indicate that eDNA sampling could be good use as a support method. As shown in chapter 2, several small pelagic fish species dominate the waters off the South-West UK coastline including sprat (*Sprattus sprattus*), Atlantic mackerel (*Scomber scombrus*) and European pilchard (*Sardinia pilchardus*). Their very high biomass, and local spawning activity (Trenkel et al., 2014; Fernandes et al., 2020), and thus the amount of eDNA in the water, will far exceed that of ABT. Also ABT are more migratory compared to the local small pelagic fish community and this transient behaviour is likely to further reduce the amount of DNA in the water. Species-specific qPCR approaches may be a more accurate tool for mapping ABT distribution, and this could be verified by conducting simultaneous visual and eDNA collections.

3.5.2 Weekly eDNA sampling

The weekly sampling from July to December 2019 at the two fixed stations showed the highest number of ABT detections in August and November, which corresponded to the months where higher numbers of prey species were detected in the eDNA samples. However, we found no significant co-occurrence between these prey species and ABT in the eDNA data. This could in part reflect the opportunistic feeding behaviour of ABT, which is not strictly associated with one or few specific prey (Günther et al., 2021). But it is also likely due to only 5 sample week detections of ABT, even when additional data from the fixed sample stations were added we saw no significant relationships between ABT and other small pelagic species. ABT aside, weak significant species relationships

between the common dolphin and European pilchard as well as significant association between sprat and European pilchard was found. But this was only based on the read number of 4 sample sites, which may not be representative enough to make definite trophic links. Thus, due to the lack of consistent eDNA data across samples, it is currently unclear whether more data from eDNA metabarcoding can find indications of trophic relationships without additional information from diet analysis of the ABT.

The greater diversity detected in August and November should be interpreted cautiously, due to the significant gaps in the eDNA data that limits our inference. The most significant gap in the weekly sampling data is across September, where we know that ABT was spotted in those areas as well as the presence of other pelagic fish species as outlined in the commercial pelagic catch data from the MMO (2021) (Fig.3.4). In September, three of the four weekly samples failed to retrieve any data and the one week that did only detect poor cod (*Trisopterus minutus*) and storm petrel (*Hydrobates pelagicus*), with the unlikely absence of any of the usually abundant pelagic species. This suggests that some level of DNA degradation must have affected those samples, which could have resulted in false negatives in the data set because of low quality samples with potential technical issue in the collection, extraction, library preparation or sequencing of those samples. Another advantage of using 12S eDNA metabarcoding to detect ABT is that it can be used to find non-target 'bycatch' vertebrate species present at that sampling location (Mariani et al., 2021). From the weekly sampling data, a total of 27 other fish species, 2 marine mammals and 3 seabirds were detected. Interestingly, of these bycatch species we were able to detect the rare black-browed albatross, a southern hemisphere species (Wakefield et al., 2012), a few individuals of which have been recently documented to settle on North-East Atlantic coasts (Hager, 2021). The extra information provided from eDNA sampling data could thus also provide us with useful inferences on the biodiversity and ecosystem health of the sample areas (Yang et al., 2021, Ogden, 2022), but the samples did lack notable common marine bird species such as the common gull (*Larus canus*) that would have been detected using visual observational methods.

Like the spatial data, the weekly sampling data showed differences between the eDNA detection and ABT sighting data. This further supports the previously stated hypothesis

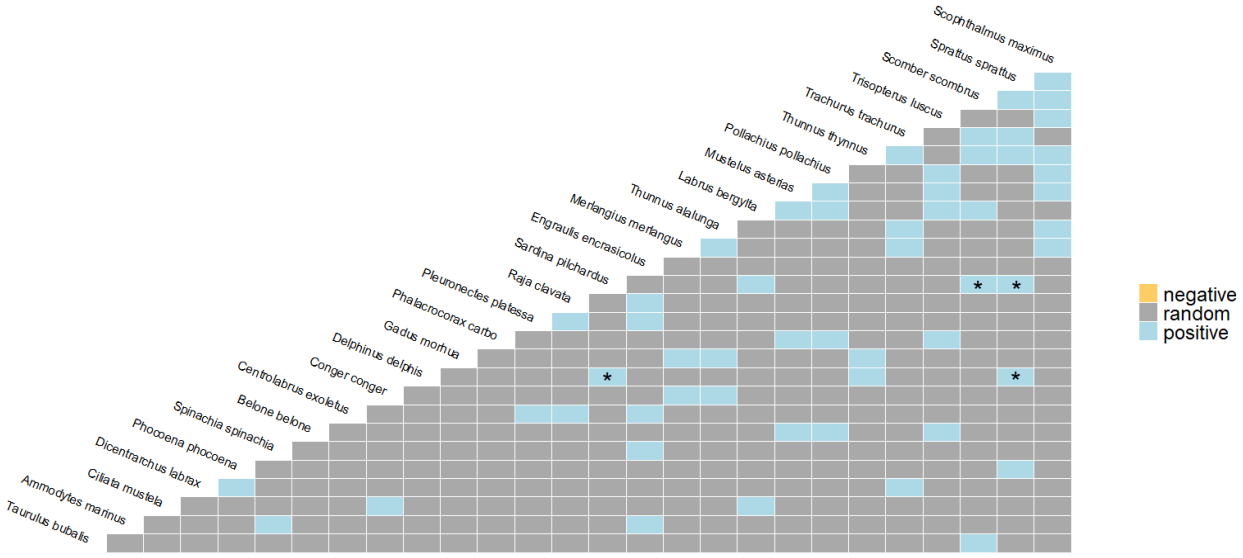
that ABT DNA in the water is patchier and thus eDNA sampling for ABT entails some level of stochasticity. The spatial and temporal patterns identified are difficult to ground truth via direct observational data, but eDNA could be used to detect ABT hotspots or ‘migratory corridors’ where ABT more consistently migrate or migrate in larger groups through. To increase the sensitivity of eDNA sampling and to increase the likelihood of detection of ABT - while retaining the valuable information contained in metabarcoding data sets - more than three ecological replicates, a larger volume of water, or a higher sequencing depth per sample, should be collected, as recommended by a plethora of other studies (Yates et al., 2020; Kirtane et al., 2021; DiBattista et al., 2017; Antich et al., 2021; Gold et al., 2021; Bani et al., 2020). It may also be advisable, given the pronounced mobility of this species, to increase the sample frequency, spatially or temporally, to increase detection likelihood and verify the presence or absence of ABT, or other highly mobile species, across large bodies of water. As well as use species specific qPCR methods that are more sensitive, and potentially more likely, to detect the presence of ABT and indicate their presence in UK waters by the quantification of the DNA per sample across temporal samples.

3.6 Conclusions

This study demonstrated that eDNA metabarcoding methods infrequently detected ABT, only occasionally finding their presence in areas where they are expected to be roaming, both when they were and were not sighted by visual observers. The metabarcoding approach also provided information on ABT prey species and broader information on the biodiversity of the area, including rare, serendipitous findings of non-target vertebrates but also failed to detect notable common fish species such as sprat and sardines and common bird species such as the common gull. Despite some failings, eDNA can provide supporting data that adds an additional tool to the repertoire of existing monitoring methods, which will improve our ability to understand movement, habitat use, and ecological traits of ABT without the restriction of daylight and human error from aerial and visual surveys. Nevertheless, much can be done to refine this technique as a tracking tool for ABT and other highly mobile pelagic species. This includes increasing the sampling effort, both in terms of replicates, amount of water volume filtered per sample, sequencing

depth and employing species-specific qPCR probes. Although the latter will not be able to procure additional information on the ecological community. In the future, an affordable, granular eDNA monitoring approach may be suited for covering large areas of the ocean, at different times of the year, and generate broad-scale distribution models, to understand the trajectories of this species' expansion, and provide managers and policy makers with further elements towards the stewarding of this resource. This could be achieved by deploying AUV eDNA samplers on platforms or buoys around the coastal areas of the UK and DNA collected over seasons. To target specific locations of ABT, qPCR methods should be used to increase the sensitivity of the assays and be combined with other data sets such as tagging or observer data and variables such as seas surface temperature and prey spawning seasons to build their seasonal distribution models.

3.7 Chapter III Supplementary Figures



Supplementary figure 3.1 Species Co-occurrence Matrix of the ABT eDNA data from 29e5 Asterix (*) represent significant relationships (p>0.05).

3.8 Chapter III Supplementary Methods

3.8.1 DNA extraction and amplification

For the weekly sampling, the frozen Sterivex filters (Merck) were removed from the cartridge and one half of the filter was extracted using the Mu-DNA protocol (Sellers et al., 2018). The other half of each filter was stored at -20°C. For the weekly sampling and PELTIC19 samples, the Mu-DNA protocol was adapted to exclude the use of garnet beads, through optimisation tests, the Proteinase K was adjusted to 40 µl per extraction

for optimum recovery of DNA and removal of potential contaminants and incubated on a thermomixer (Thermo-scientific) for at least 12 hours. As previously mentioned, (chapter 2), PELTIC18 extractions followed the Mu-DNA water protocol. Samples were extracted in batches of 12; for each batch a blank was taken, for a total of 6 extraction blanks for the weekly sampling, 20 for PELTIC18 and 24 for PELTIC19 samples. The sample extractions and blanks were then checked by loading the DNA on a 2% agarose gel stained with SYBR Safe and quantified for quality using a Nanodrop 2000 (Thermo Fisher Scientific).

A ~167bp fragment of mitochondrial 12S rRNA was amplified through PCR using the 'Tele02' primer set (Miya et al., 2015; Taberlet et al., 2018). At the 5' end of the forward and reverse primers, 8bp unique indexing oligo-tags and 2-4 degenerate positions (Ns) to increase sequencing diversity. Samples were PCR-amplified in triplicate in a 20 µl reaction, which consisted of 12 µl Myfi Master Mix (Bioline), 0.04µl BSA, 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer 5uL sample DNA and 0.96 µl ddH20. For the PCR positive, sample DNA from *Sebastes marinus* was used as this species is not found at our sampling area but will be amplified using Tele02 primers. For each batch of samples (72 for weekly samples, 82 for PELTIC18 and 86 PELTIC19 samples) a PCR negative, that had 5uL of ddH20 rather than the sample DNA, and a PCR blank, that just had the reagents, were also PCR-amplified.

The PCRs were performed following the protocol in Taberlet et al (2018) with an initial denaturation step of 94°C for 10 min, 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final extension step of 72°C for 5 min. The PCR replicates were pooled, and PCR products were checked on a 1.5% electrophoresis agarose gel stained with SYBR Safe. Following a double-sided AMPure (Beckman Coulter) magnetic bead clean up with 0.7:1 left sided to remove fragments larger than the target and 1:1 right sided to remove fragments smaller than the target, each sample was then quantified on a Qubit 4.0 Fluorometer using the dsDNA high sensitivity assay (Invitrogen™).

3.8.2 Library preparation and sequencing for weekly sampling

Each sample was adjusted and pooled at 14 ng total DNA for a target library concentration of 1 µg total DNA from the 76 total samples and blanks. After end repair and a-tailing and unique dual index adapter ligation, using the KAPA hyper prep kit (Roche), the library was checked on a tapestation 4200 (Aglient) and followed up with a 0.8:1 AMPure bead clean up to remove adapter dimers. A further 4 cycle amplification steps with 50 µl of the library was performed to stabilise the library for sequencing and increase the library concentration up to 1µg followed by a 1:1 AMPure bead purification. Lastly, the concentration was quantified with the NEBNext quant kit (New England Biolabs) with serial dilutions of 1000, 10,000 and 100,000 using a RotorGene Q RT-PCR machine (QIAGEN) and double checked using a Qubit 4.0. A diluted 20 µl library was loaded onto an Illumina iSeq 100 platform using the i1 (v2) 300 cycle kit with a 10% PhiX (Illumina) control at a final sequencing molarity of 50pM.

3.8.3 Library preparation and sequencing for spatial samples

These libraries were not prepared at the same time, PELTIC18 data was sequenced in 2019 and PELTIC19 was sequenced in 2021. Samples were separated and pooled for a target library concentration of 1µg total DNA which made 3 libraries of 86 from the PELTIC18 samples and 4 libraries of 82 for the PELTIC19 samples. The 3 PELTIC18 libraries and 4 PELTIC19 libraries were then pooled separately at an equimolar concentration (4nm). After end repair, a-tailing and unique dual index adapter ligation, using the KAPA hyper prep kit (Roche), the library was checked on a tapestation 4200 (Aglient). Each library was quantified using a NEBNext quantkit (Illumina) with serial dilutions of 1000, 10,000 and 100,000. The PELTIC18 pooled library quantifications were checked on the MIC RT-PCR machine (Bio Molecular Systems) and PELTIC19 pooled libraries were checked using a RotorGene Q RT-PCR machine (QIAGEN). For both PELTIC18 & 19 a diluted 20µl library was loaded onto a Illumina MiSeq platform using (v2) 2×150 cycle with a 10% PhiX control and a final sequencing molarity of 8pM.

Chapter IV

Validating pelagic marine fish identity and abundance from acoustic backscatter using environmental DNA

4.1 Abstract

High resolution hydroacoustic sampling paired with regular midwater trawling is a common method used to map and quantify pelagic fish. Although midwater trawling is a direct method of sampling pelagic fish, it is also expensive and does not always guarantee a representative reflection of the abundance of fish taxa present across a sampling transect. Using eDNA to help experts validate the identity and abundances of fish species from hydroacoustics is an enticing prospect for future fish monitoring strategies, unshackling the restrictions of current trawl-based validation methods.

Environmental DNA (eDNA) is a method of detecting marine pelagic fish species that is unburdened by the catchability bias induced by avoidance behaviours that affects pelagic fish detection from midwater trawling. In this study we combined amplicon reads from eDNA samples collected during two annual pelagic monitoring surveys around the South-West coast of the UK, with hydroacoustic data recorded across multiple transects. For each hydroacoustic sample point we partitioned the hydroacoustic backscatter values by the target pelagic fish species; European pilchard (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), Atlantic herring (*Clupea harengus*), Atlantic horse mackerel (*Trachurus trachurus*), boarfish (*Capros aper*) and sprat (*Sprattus sprattus*), by using the proportions of species found in the eDNA and the midwater trawl catch. When we compared the eDNA and midwater trawling backscatter values for each species we found that the strength of the correlations varied from species to species. A multiple generalised linear model revealed significant positive relationships between the trawl and eDNA partitioned hydroacoustics for Atlantic herring, sprat and European anchovy. Suggesting that eDNA could be used as a support tool to verify trawling data for some marine species and that some species are disproportionately effected by either primer bias, dilution or distribution of their DNA in the water column which may have resulted in the observed model variances for the Atlantic horse mackerel, boarfish and European pilchards.

4.2 Introduction

Evaluations of marine pelagic fish abundance are critically important for adjusting total allowable catch (TAC) limits to help ensure the sustainability of the fishing industry (Karagiannakos, 1996; Shephard et al., 2014; Johnson et al., 2021). Due to their patchy distribution and aggregative behaviour, small pelagic fish tend to be monitored using high resolution hydroacoustic methods: echosounders continually scan the water column using sonar beneath a research vessel (RV) as it sails along a series of transects (MacLennan et al., 2002; Georgakarakos et al., 2011; Doray and Boyra, 2021). Echosounders emits a pulse of energy in the form of a sound wave at different wavelengths (kHz) from a transducer at $1500m^{-s}$, giving an instantaneous profile of the water column. Information on the water column is received when the sound waves, like an echo, connect with an object that is denser than the medium around the object and is reflected back to a receiver, the returning echoes are termed backscatter (Simmonds & MacLennan, 2008). Each species has a specific wavelength frequency which can be used to target the density of fish from different taxa (Godø et al., 2009). The received backscatter energy is compiled onto an echogram that displays the density of the backscatter echoes against depth to reveal the location of the target taxa in the water column. Estimations of the target species density over a spatial area is calculated by using a cumulative average of the backscatter density over one nautical mile, also known as Nautical Area Scattering Coefficients (NASC) (Saunders et al., 2013).

The use of hydroacoustics was a significant turning point for fish monitoring methods, allowing previously inaccessible areas of the ocean to be monitored, moving away from environmentally damaging, weather reliant, time consuming, and costly methods such as bottom trawling and gill netting (Mayer et al., 2002; Polunin et al., 2009; Benoit-Bird, 2015; Egerton et al., 2018; Kok et al., 2021). While fisheries acoustics is a powerful non-invasive tool, the main disadvantage is the limited ability to identify multiple different species within schools of fish. Although the use of parallel frequencies and distinct, species-specific acoustic properties can help in partitioning the backscatter into species densities,

sometimes ground-truth activities, often by means of midwater trawl, is required (Bassett et al., 2018; Doray and Boyra 2021). Trawling also provides size, weight, sex and age information, which is important to convert backscatter to biomass and to provide biological information on the stock (Boldt et al., 2018; Bean et al., 2017; Rowell et al., 2019).

However, the fish composition caught from trawling can be affected by avoidance behaviour of fish and be biased towards size and age classes of certain fish species (Misund and Coetzee, 2000; Kaartvedt et al., 2012). Avoidance behaviour by fish, whether it be vertical or horizontal, can be triggered by the noise of the vessel, hydroacoustic equipment, or trawling equipment (Vabø et al., 2002; Gerlotto et al., 2004; De Robertis & Handegard., 2013; Brehmer et al., 2019). Although the relationship between noise and avoidance behaviour is not a linear relationship and is often complicated by other factors. For example, Atlantic herring (*Clupea harengus*), haddock (*Melanogrammus aeglefinus*), Chilean hake (*Merluccius gayi gayi)* and sprat (*Sprattus sprattus*) have each displayed varying avoidance responses depending on the trawling gear used, between seasons, and between mixed size classes of schools (Misund, 1990; Misund and Aglen, 1992; Fréon & Misund, 1999; Handegard et al., 2003; Jørgensen et al., 2004; Queirolo et al., 2010; Robert et al., 2020). Fish species with sleeker body morphologies and, depending on the trawl net size, juveniles within mixed age class schools have lower catchability rates from a pelagic trawl in comparison to other Orders and adult pelagic fish (Heino et al., 2011; Williams, 2013; De Robertis et al., 2021). Therefore, midwater trawling methods can, by random chance, inadvertently catch an over- and/or under-representative sample of the pelagic fish densities observed from hydroacoustics. Thus, the backscatter densities can be erroneously partitioned from midwater trawling, subsequently creating inaccuracies in fish biomass and stock calculations (Rosen et al., 2013; Bean et al., 2017; Rowell et al., 2019; Braun & Mehner, 2021). To ensure that sampling techniques collect accurate representations of pelagic fish populations, trawl-validated hydroacoustic monitoring applications must adapt to overcome this selectivity and catch biases.

Environmental DNA (eDNA) can provide a non-invasive *in-situ* method of detecting pelagic fish species, that is not influenced by environmental variables and, unlike trawling,

is unaffected by fish behaviour and can detect DNA shed by fish at all stages of their life cycle making it more likely to detect the ratios of whole taxa populations in comparison to trawling. As highlighted in chapter 2, eDNA data collected from our sample region and season is not impacted by the depth the sample is taken from or the environmental variables at, and between, sampling locations. Encouragingly this also means that eDNA samples can be simply taken from the surface water without the need for expensive equipment, such as the CTD rosette, to be deployed meaning that samples can be taken in parallel with hydroacoustics and making it a more cost-efficient method to midwater trawling. Furthermore, like other research (Stoeckle et al., 2021), in chapter 2 we also found a weak positive and significant correlation between eDNA amplicon reads and trawling biomass (R^2=0.2, p=0.01; Fig.2.10), suggesting that eDNA amplicon reads may be comparable to trawl verification methods of hydroacoustics if we are able to remove the noise impacting the comparison between the two methods. Here we consider whether additional sampling of water during hydroacoustic activities can yield comparable eDNA ratios of abundance in comparison to trawling and whether this could serve as a useful tool to support the identification of pelagic fish species.

Studies have only recently started to investigate the potential of using eDNA methods in combination with hydroacoustics for monitoring efforts, the majority using qPCR methods, with varying results (Fukaya et al., 2018; Coulter et al., 2019; Berger et al., 2019; Rasmuson et al., 2021; Li et al., 2022; Shelton et al., 2022). While freshwater studies have found either no linear relationships between the species eDNA and backscatter inferred fish biomass (Coulter et al., 2019; Li et al., 2022), studies on marine fish; Atlantic herring (*Clupea harengus*), capelin (*Mallotus villosus*), rainbow smelt (*Osmerus mordax*), Japanese Jack mackerel (*Trachurus japonicus*), and species of semi-pelagic *Sebastes* have been able to accurately match eDNA concentrations to backscatter densities in the water column (Fukaya et al., 2018; Berger et al., 2019; Rasmuson et al., 2021). Further still, Shelton et al (2022) found that the qPCR concentrations of eDNA also matched the backscatter density and spatial distribution of the Pacific hake (*Merluccius productus*), which shows that eDNA can be used over 1km transects with high accuracy. However, we do not know the horizontal spatial extent to which an eDNA sample is representative of, especially given that eDNA can be transported within the marine environment in

multiple directions and across tens of kilometres (Harrison et al., 2019; Andruszkiewicz et al., 2019; Bonfil et al., 2021). Using the data from two acoustic pelagic surveys we investigated whether eDNA can be used as an alternative source of species information to partition the backscatter densities into a more accurate proportions of fish species in comparison to the proportions of fish identified through midwater trawling.

We hypothesise that the ratios of fish species found from eDNA are less likely to be under- and/or over- represented by individual fish species in comparison to trawling, as eDNA is a more sensitive, less biased, method of detecting fish species. To establish the maximum applicable sampling range of eDNA to acoustics, we also compared differences between the proportion of species inferred by eDNA with those estimated by midwater trawling as distance from the eDNA sampling point increased.

4.3 Materials and Methods

To assess the ability of species identification from eDNA to partition the acoustic data, the eDNA partitioned backscatter by species was compared to the traditional trawl-derived backscatter by species.

4.3.1 Hydroacoustic backscatter and eDNA collection

The acoustic backscatter, trawl validated and eDNA water samples, as previously mentioned (chapter 2 and chapter 3), were collected during two annual PELTIC surveys that took place in waters to the South-West of the UK, from October to November, in both 2018 and 2019.

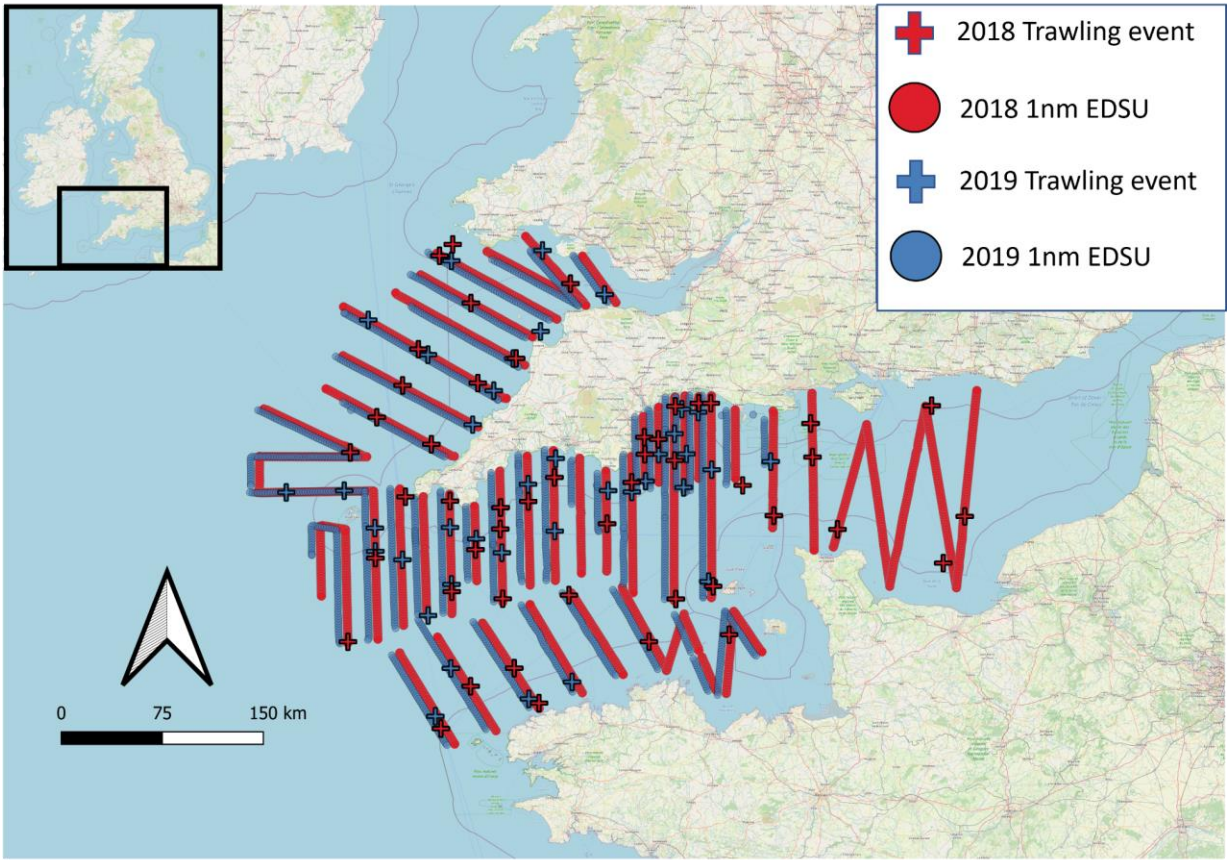


Figure 4.1 Map of the hydroacoustic transects and midwater trawling stations undertaken during the 2018 (PELTIC18) and 2019 (PELTIC19) surveys around the South-West coast of the UK. Each circle point represents where NASC values from the PELTIC survey transect have been averaged across a 1x1 nautical mile Elementary distance sampling unit (EDSU). The current EDSU markers are not to scale and are adjusted to show both the PELTIC18 and PELTIC19 transects side by side. PELTIC18 transects also covered the PELTIC19 transect routes, but only PELTIC18, extended to the Eastern English Channel.

Details of the methods applied during this survey are provided in Doray and Boyra (2021) but a short summary is given here. Acoustic backscatter, measured in Nautical Area Scattering Coefficient (NASC), was recorded during daytime only because several species exhibit diel vertical migration and disappear from the echogram at night and as schools disaggregate (Doray and Boyra, 2021; Plirú et al., 2012). The line transects that the vessel, the RV endeavour, sailed along in the PELTIC surveys were angled so they were perpendicular to the prevailing bathymetry to reduce the stochastic variability of fish schools across a transect (Fig.4.1). NASC values, that correspond to the density of fish derived backscatter, were aggregated over one nautical mile into an elementary distance sampling unit (EDSU). To increase the chances of catching a representative number of

fish for accurate biomass calculations, trawling events took place when there were a targetable number of fish schools detected over several EDSUs.

The acoustic backscatter was subsequently partitioned into different echo-types using an existing algorithm which considers the differences in frequency response and echo-strength (Doray and Boyra, 2021, van der Kooij et al., 2016) to distinguish four categories: fish with swim bladders (which generally have a strong signal at lower frequencies), mackerel, fluid-like zooplankton and jellyfish. The swimbladder fish category was then further partitioned by species using the trawl catch composition. This involved using the species and size information from the trawl to calculate an acoustic equivalent of the catch using published target strength-length conversions (Doray and Boyra, 2021). Schools were then allocated to a particular species based on the nearest trawl or, if no single species could be attributed with certainty to acoustic scatterers, the species ratio of trawl was used to partition the backscatter. The resulting backscatter for six pelagic fish species was then converted into biomass based on backscatter size and density: European pilchard (*Sardina pilchardus*), sprat (*Sprattus sprattus*), European anchovy (*Engraulis encrasicolus*), Atlantic herring (*Clupea harengus*), Atlantic horse mackerel (*Trachurus trachurus*) and, boarfish (*Capros aper*).

Across all transects, 2306 EDSUs and 49 trawls in 2018 and 1615 EDSUs and 40 trawls in 2019 were covered (Fig.4.1). The biomass indices and biological data for two species feed into the stock assessment: Celtic Sea pilchard (ICES area 7; Duhamel et al (2019) and English Channel sprat (ICES area 7de; Roel and General (2021).

As detailed in chapter 2, during both PELTIC surveys eDNA samples were collected either during daytime trawling activities, using an existing Ferrybox system that pumps water in from 4 metres below the water surface though a flow-through system and the night-time CTD rosette (Fig.4.2). Seawater samples were taken in triplicates, and within 24 hours, were filtered through 0.22 µM sterivex cartridges and stored in a -80˚C freezer. There was a total of 43 field blanks taken from both surveys, 16 from the PELTIC18 survey and 27 from the PELTIC19 survey (field blank and cleaning processes outlined in

chapter 2.3.2). The PELTIC 19 survey field blanks were taken once for every three sample stations for the flow-through and CTD rosette. Across both surveys there were a total of 111 eDNA sampling sites (50 in 2018 and 61 in 2019), with 475 individual eDNA samples collected: 205 in 2018 and 270 in 2019 (Fig.4.2).

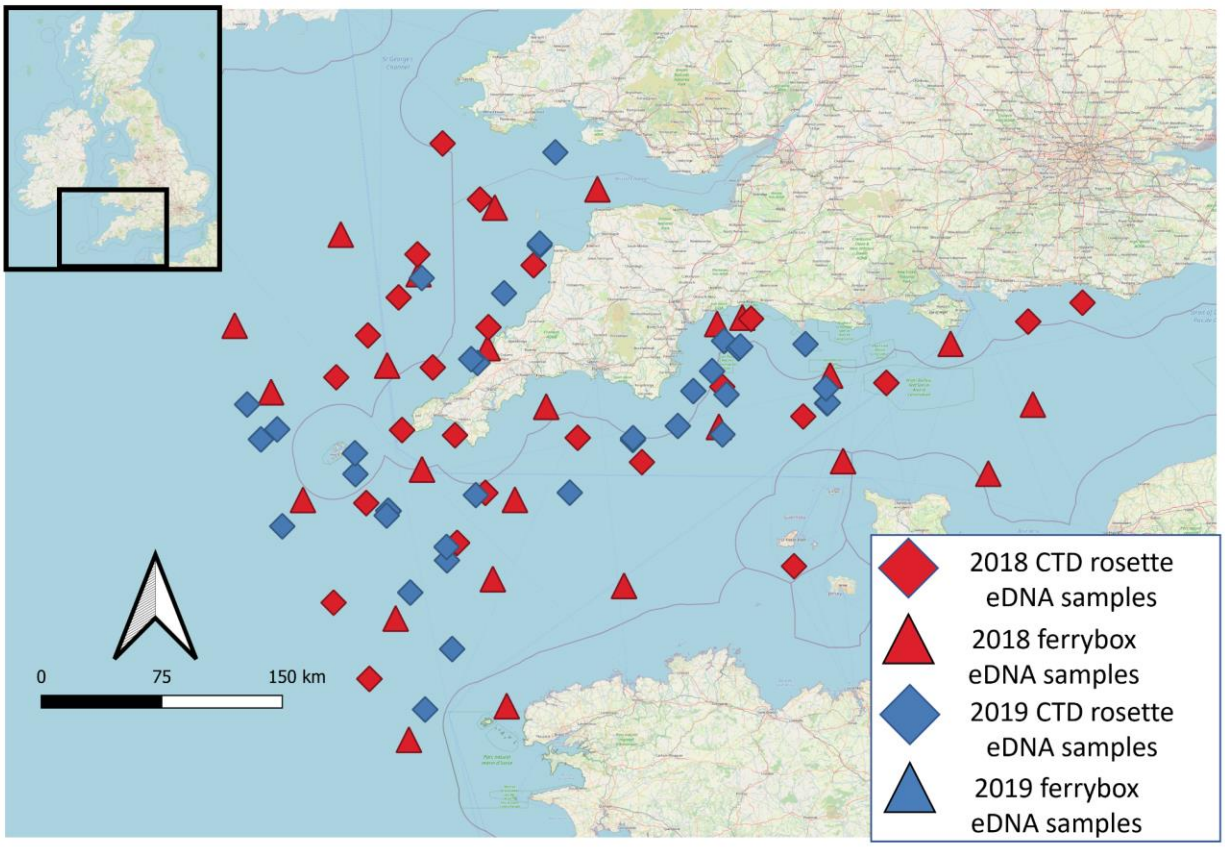


Figure 4.2 Marine eDNA sampling map around the South-West coast of the UK. The triplicate CTD rosette samples were taken at 20% and 80% total depth of the water column and were not always on the exact transect route as the RV endeavour but were annual fixed sampling points. FerryBox flow-through sampling always took place opportunistically at the same time as a midwater trawling event.

4.3.2 DNA extraction to sequencing

DNA was extracted from the filters in the same procedures outlined in chapters 2&3 using the MU-DNA protocols (supplementary Methods, Sellers et al., 2018). Following the protocol by Taberlet et al (2018), triplicate 20 µl PCR-amplification reactions with the 'Tele02' primer set (Miya et al., 2015; Taberlet et al., 2018) were carried out on each sample. As previously outlined (chapter3), a PCR positive, a PCR negative, and a PCR blank were also amplified for each batch of samples (per 86 PELTIC18 samples and per 82 PELTIC19 samples). PCR replicates were pooled and cleaned and a total of 7 libraries were prepared for sequencing (3 libraries for PELTIC18, 4 for PELTIC19), each library had a target concentration of 1µg. Libraries were quantified and pooled at an equimolar DNA concentration into two pools (one for PELTIC18 and one for PELTIC19, with 6 samples from the PELTIC18 pool also re-sequenced with the PELTIC 19, in order to ascertain consistency), each pooled library was diluted to 20 µl and was loaded onto an Illumina MiSeq platform using 2×150 cycle v2 chemistry, with a 10% PhiX control and a final sequencing molarity of 8pM. Further details on the methods used for DNA extraction to sequencing are provided in the supplementary methods.

4.3.3 Bioinformatics and downstream analysis

The Fastq files were demultiplexed, denoised and taxonomy assigned into amplicon sequence variants (ASVs), removing ASV's that could not be assigned to the species level using R and bash command line. These processes employed the DADA2 v1.17 (Callahan et al., 2016) bioinformatic pipeline, following the approach described in Collins et al. (2021), and using the SeaDNA UK 12S curated database for BLASTn as outlined in chapters 2&3 (Martin, 2011, Czech et al., 2019, and Barbera et al, 2019). Potential contamination ASVs that were present in the PCR-, extraction- and field-blanks were identified using the decontam R package (https://github.com/benjjneb/decontam) and removed from their corresponding samples. The amplicon reads were square root transformed to mitigate potential PCR amplification bias impacting the proportions of fish species reads.

Using data from previous annual monitoring surveys, the sample areas for 2018 and 2019 were split into different strata zones, each stratum representing a relatively homogeneous ecological area, where species and size composition is similar (Supplementary Fig.4.1-Fig.4.2). Because the ecology of these spatial areas changed between years the stratum zones between 2018 and 2019 are slightly different. There are also differences between the ecological strata zones for sprat and European pilchard that correspond with the adjustments that would be needed for stock assessments (ICES, 2010). The other small pelagic fish species: European anchovy, Atlantic herring, Atlantic horse mackerel and boarfish were assigned to the same stratum zones as the European pilchard. To assess the use of eDNA to validate acoustic backscatter, the backscatter values from each EDSU was partitioned by the closest eDNA sampling point within its designated strata zone (Fig.4.3). Partitioning was done by dividing the aggregated backscatter value of each EDSU by the proportions of species from the eDNA reads, this was done separately across the PELTIC18 and PELTIC19 surveys. Because we know there is no PCR amplification bias differences between these six small pelagic fish species, if an eDNA sample had the equivalent read proportions of 50% Atlantic horse mackerel and 50% European pilchard an EDSU with a NASC value of 101 would be partitioned to have 50.5 NASC value of Atlantic horse mackerel and European pilchard.

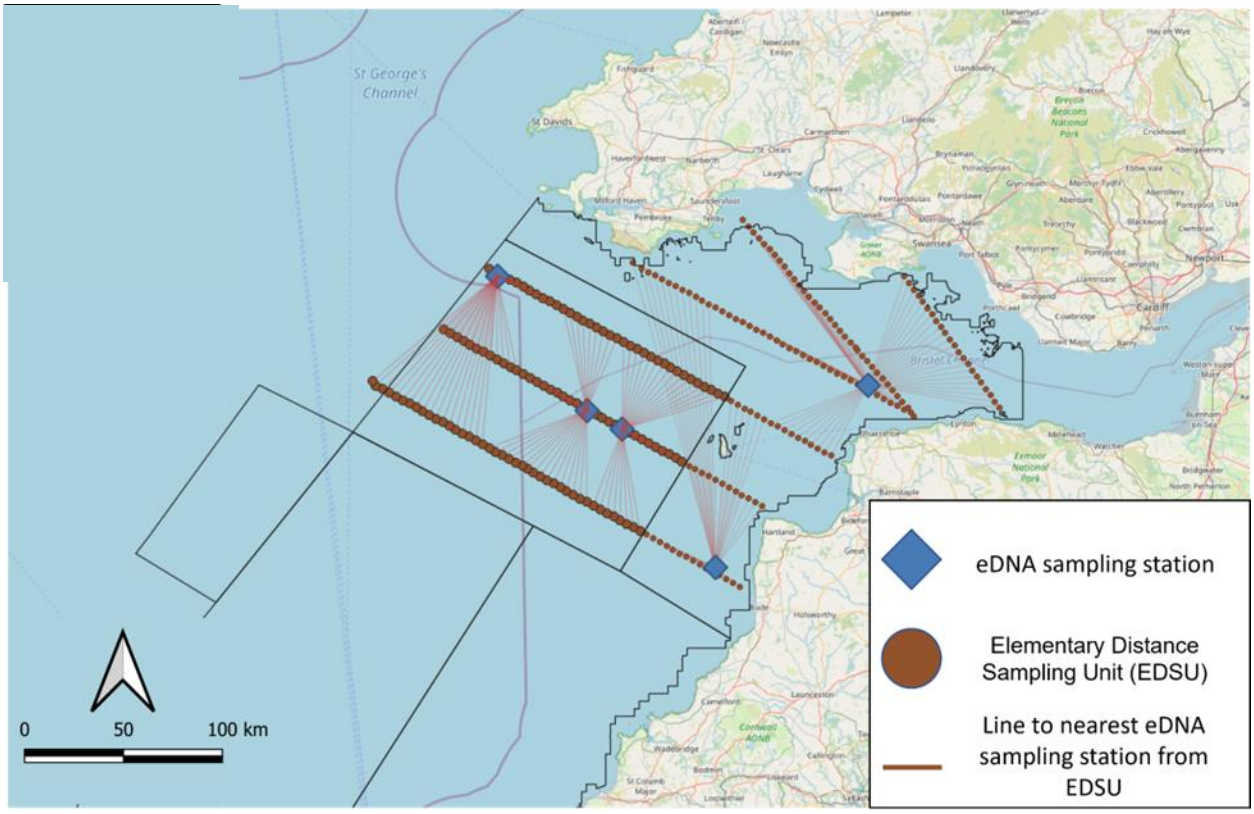


Figure 4.3 A zoomed in example of how NASC values from EDSUs were assigned to eDNA sample points at a stratum-by-stratum basis across the South-West coast of the UK. The aggregated NASC values at each EDSU were assigned to the nearest eDNA sampling point within each stratum. Small brown EDSUs indicate a different stratum zone, the brown lines from each EDSU show which eDNA sample points the NASC values of that EDSU were being partitioned by. The stratum zones are shown by the black lines. This method was repeated across all stratum zones across the South-West coast of the UK, not just this example area.

Pie charts of the cumulative eDNA-validated and trawl-validated NASC proportions across each stratum zone for European pilchard, European anchovy, Atlantic herring, Atlantic horse mackerel and boarfish were mapped for each survey year (Fig.4.4a). A heat map using the PELTIC18 and PELTIC19 eDNA-validated and trawl-validated NASC densities for sprat was generated (Fig.4.4b).

To investigate whether the difference in proportions between eDNA and trawl sampling increased with the distance (km) from an eDNA sample point the species proportions from each EDSU (PELTIC18 & PELTIC19 n=13693) was combined into one data set. The

eDNA and trawl inferred proportions (%) of each species at each EDSU were subtracted from each other, negative percentage values were converted to show absolute percentage change. This meant that the larger the variance the more dissimilar the eDNA and trawling proportions were. A generalised linear regression model was then applied to the data. The distances were then categorised into 13 different groups with 5km increments (0-5km to 65km) to find if there was a certain distance away from the eDNA sampling point where the variances between eDNA proportions and trawl proportions significantly differed. A kruskall-wallis test was then used to check for a significant difference in proportion variance between the distance categories, this was also repeated at the species level to investigate whether there were any species-specific proportional variances in the data.

To test the relationship between trawl and eDNA derived NASC values we assumed that the trawling derived values for each species is the dependent variable and the eDNA data for each species as the independent variables. For European pilchard, European sprat, European anchovy, Atlantic herring, Atlantic horse mackerel and, boarfish analysis between the trawling and eDNA derived data was investigated by means of a spearman's rank correlation in R. We then used a two-way MANCOVA to investigate the impacts of covariates on the species that had a significant correlation between trawl and eDNA derived data. The covariates included the total depth of the water column sampled at each EDSU, the average sea surface temperature (SST) and salinity (SSS) across each transect and distance between the eDNA sample station and EDSU. The normality, multicollinearity and homoscedasticity of the covariates were checked using 'sjplot' R package (v2.8.12; Lüdecke, 2021).

A Gaussian multivariate generalised linear model (GLM) was then built for each species and all species combined, using any significant covariates for each species and removing outliers that exceeded mahalanobis' distance, as well as NASC values of less than 1, to increase the accuracy of the model, and reduce the influence of bias from amplification effects. The GLM regressions were plotted on a log10 scale, and the predicted regression slope and a localised loess-smooth line were applied using the 'sjplot' and 'sjmisc' R packages (v2.8.12; Lüdecke, 2021). To investigate whether these relationships are

retained at smaller habitat ranges, the GLM methods were repeated on species data aggregated by the assigned strata zones (Supplementary Fig.4.1-4.2) and combined for an overall comparison. Pearson's correlation coefficient was calculated to measure the correlation strength between the data sets.

4.4 Results

The MiSeq sequencing run yielded a total of 7.8 million reads for PELTIC18 and 18.3 million reads for PELTIC19. From the bioinformatics and downstream removal of blanks we were left with a total of 3.98 million reads and 105 ASVs (Teleost and Chondrichthyes at the species level) from PELTIC18 and 6.72 million reads and 120 ASVs (Teleost and Chondrichthyes at the species level) from the PELTIC19. Of the 475 eDNA samples a total of 315 contained enough data to be retained after filtering procedures; this amounted to 70% of PELTIC18 samples and 59% of PELTIC19 samples. There was a total of 1.28 million reads (an average of 9300 reads per sample) from 66 fish species in the PELTIC18 data and 2.95 million reads (an average of 13720 reads per sample) from 57 fish species in the PELTIC19 data; the species accumulation curves were then plotted between the sequencing runs (Supplementary Fig.4.3).

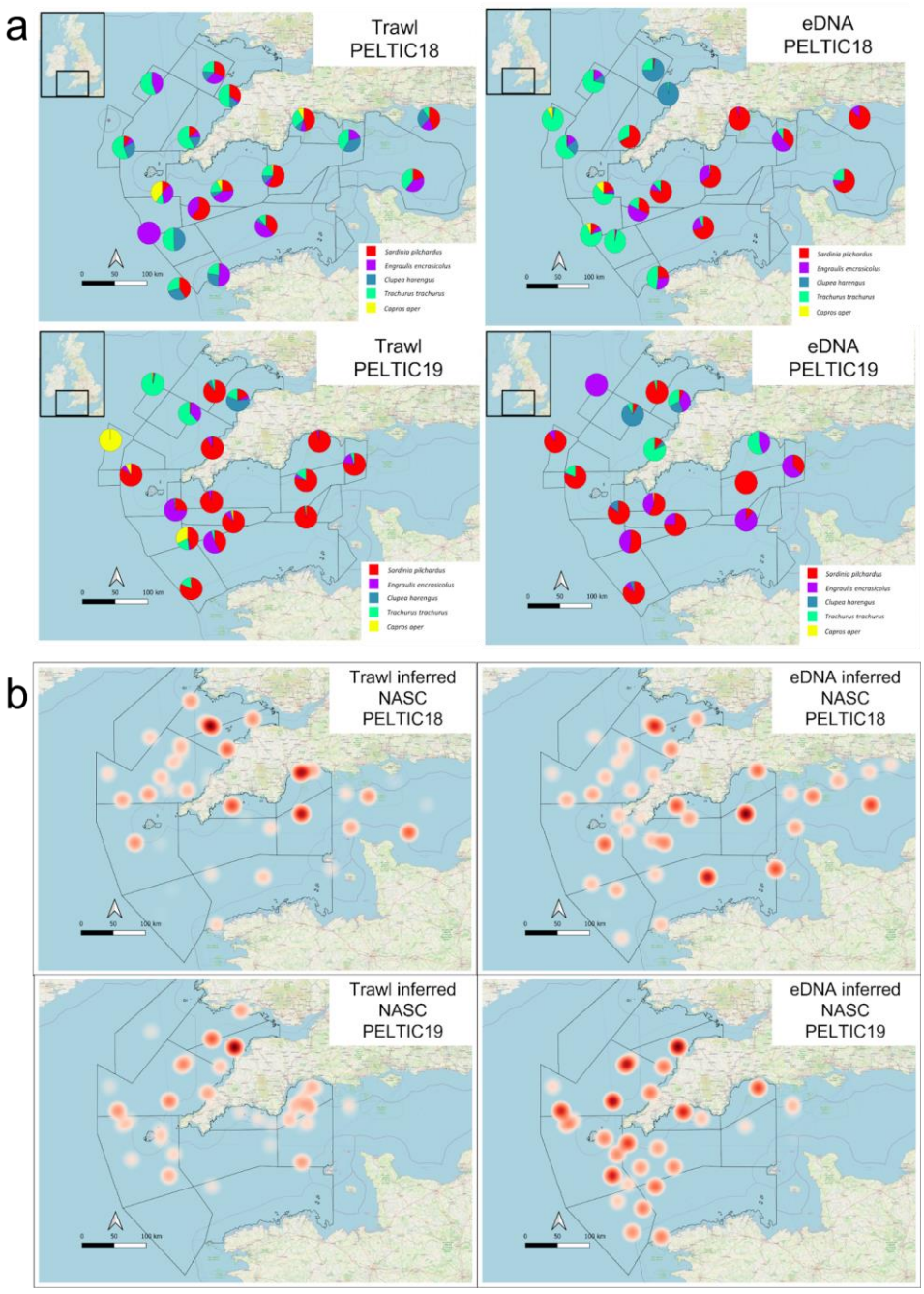


Figure 4.4 Panel plot of stratum aggregated NASC proportions of European pilchard (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), Atlantic herring (*Clupea harengus*), Atlantic horse mackerel (*Trachurus trachurus*) and boarfish (*Capros aper*) and trawl and eDNA validated NASC densities of sprat (*Sprattus sprattus*) across the South-West coast of the UK
a) NASC values were calculated by using the proportions of the fish species in the trawl (left panels) or from the ratios of amplicon reads in the eDNA (right panels).**b)** NASC values for sprat were calculated by using the proportions of sprat caught in the trawl or from the proportions of amplicon reads in the eDNA. Darker red represents higher NASC densities of sprat. Black lines represent different sprat stratum zones

The generalised linear model found a significant relationship (p= 0.001) where only 0.5% of the variance is explained (R^2=0.005) between the exact distance (km) and the difference in proportions of species between eDNA and trawl validated NASC, but it is unlikely that distance from the sample was creating this relationship. There was no significant relationship between the distance categories from all species data combined (X^2 = 2.23, df= 12 p=0.99; Fig.4.5) and neither between the distance categories when separated for each species (X^2 = 0.4-0.7, df= 12 p=0.64-0.99; Fig.4.6)

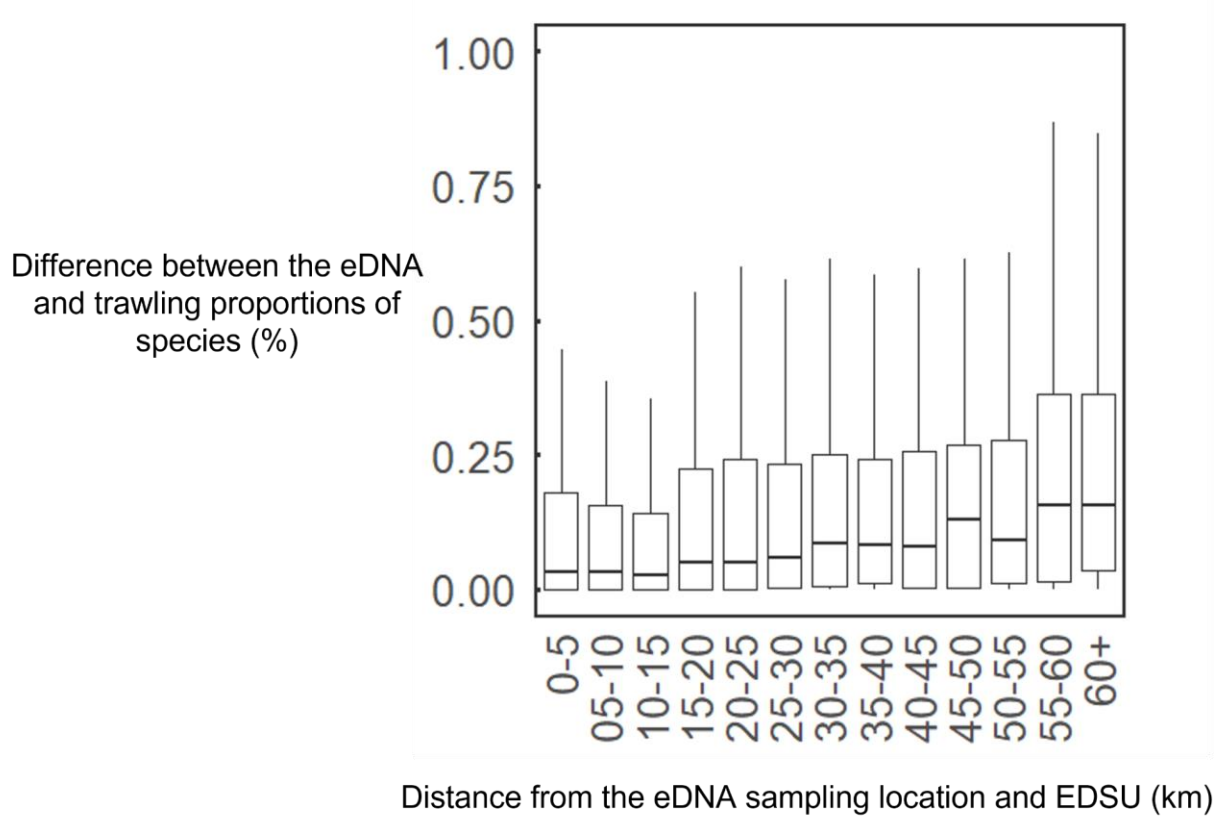


Figure 4.5 Percentage difference in the proportions of pelagic fish abundances between eDNA and trawling data and the distance from a the eDNA sample location

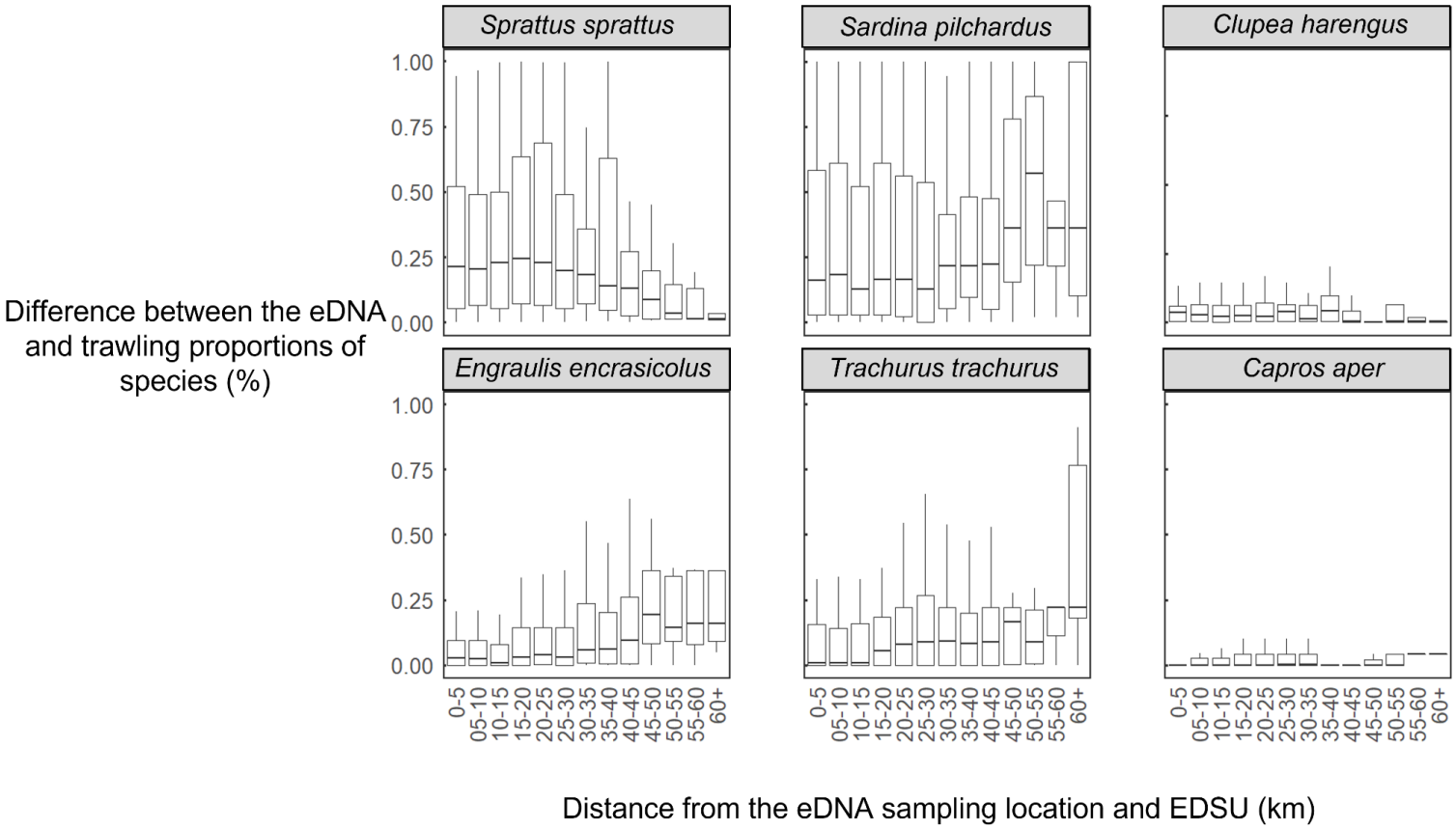


Figure 4.6 Percentage difference in the proportions of each pelagic fish between eDNA and trawling data at each EDSU against the distance from a eDNA sampling point

Only 50% of PELTIC18 and 27.8% of PELTIC19 paired trawl and eDNA EDSUs had partitioned backscatter values for pelagic fish species present in both the eDNA partitioned and trawl partitioned NASC data, which varied between species (Supplementary Table.4.1).

The sea surface temperature (SST) and salinity (SSS) covariates used in the MANCOVA were found to be significantly inversely correlated in the PELTIC18 data (df=838, $p<0.001$, cor = -0.9) and PELTIC19 data (df=638, $p<0.001$, cor= -0.8) and were treated as one covariate. The MANCOVA showed SST & SSL and total water column depth covariates were significantly ($p<0.001$) contributing to the relationship between trawl and eDNA derived NASC values for each species in both the PELTIC18 & 19 data sets and

thus SST was used as an additional variable in the multivariate GLMs for each species. When the species were combined into one data set, total water column depth was the only covariate that was significantly negatively affecting the relationship ($p<0.001$) between trawl and eDNA derived NASC values in the PELTIC18 dataset.

An overall GLM, by combining all the species from the PELTIC18 data from every sample location, found a significant positive correlation between trawl and eDNA derived NASC values ($p<0.001$, $r= 0.75$, $R^2= 0.56$, Fig.4.7a). When this data is aggregated by the corresponding species stratum zones the correlation is no longer significant and has a reduction in model strength and variance ($p=0.64$, $r= 0.06$, $R^2= 0.004$, Fig.4.7b).

When the PELTIC18 data is separated into each species, we find that the European pilchard ($p<0.001$, $r=0.83$, $R^2= 0.37$, Fig.4.8a), Atlantic herring ($p<0.001$, $r= 0.4$, $R^2= 0.7$, Fig.4.10a), boarfish ($p<0.001$, $r=0.75$, $R^2= 0.58$, Fig. 4.12a) and sprat ($p<0.001$, $r= 0.88$, $R^2= 0.8$, Fig.4.13a) also exhibit positive correlations between the trawl and eDNA derived NASC values. When these species are aggregated into their designated stratum zones there is a reduction in significance, strength and variance of the correlations for all species (Fig.4.8b, Fig.4.10b, Fig.4.13b), apart from Atlantic herring where the relationship is stronger ($p<0.001$, $r=0.7$, $R^2= 0.93$, $df=5$, Fig.4.10b), and for the boarfish where the correlation was inverted but had a significant reduction in statistical power ($p<0.001$, $r= -0.9$, $R^2= 0.95$, $df=5$, Fig.4.12).

The data from the repeated survey year (PELTIC19) also had a significant positive correlation when all species data were combined from every sample location ($p<0.001$, $r= 0.3$, $R^2 = 0.1$, Fig.4.7a). When all species were combined by stratum zone the correlation strength and variance improved and was still statistically significant ($p<0.001$, $r= 0.5$, $R^2 =0.23$, Fig.4.7b). Of the individual species from the PELTIC 19 data, European pilchard ($p< 0.001$, $r= 0.43$, $R^2 =0.17$, Fig.4.8a), Atlantic Herring ($p< 0.001$, $r= 0.14$, $R^2 =0.36$, Fig.4.10a) and sprat ($p= 0.001$, $r=0.32$, $R^2 =0.13$, Fig.4.13a) have significant positive correlations between trawl and eDNA derived NASC values. When the data for these species are aggregated over the stratum zones, European pilchards, Atlantic horse

mackerel and boarfish did not have matched data between eDNA and trawl findings to be compared. At the stratum level only the correlation between trawl and eDNA derived NASC for Atlantic herring improved in strength of the relationship and variance ($p<0.001$, $r= 0.15$, $R^2 =0.42$, Fig.4.10b).

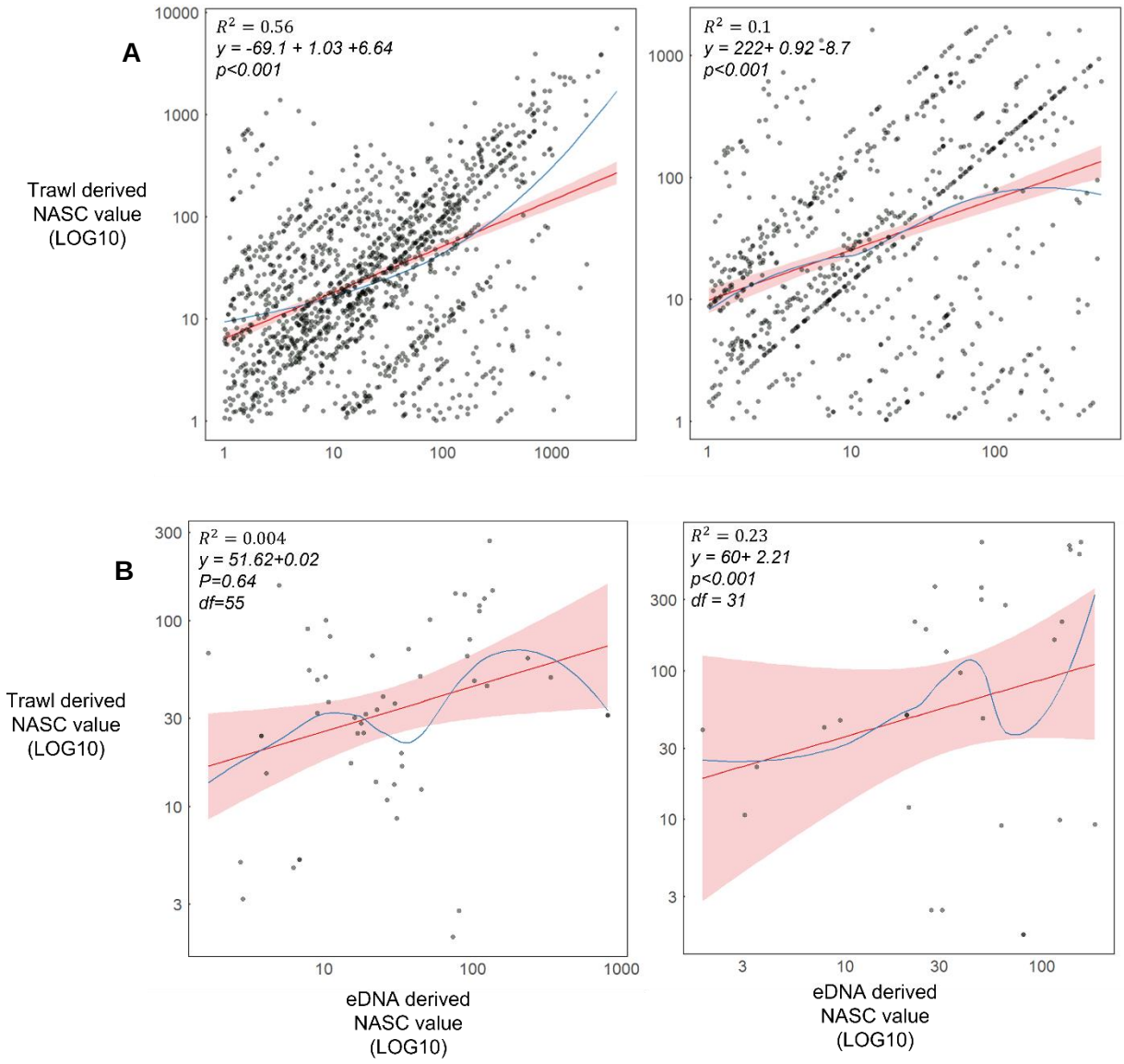


Figure 4.7 Log-log plot of trawl and eDNA derived NASC values of European pilchard, European anchovy, Atlantic herring, Atlantic horse mackerel, boarfish and sprat combined. Trawl and eDNA data from the PELTIC18 survey (left panel) and trawl and eDNA from the PELTIC19 survey (right panel), The red line is a fitted regression slope using the GLM prediction and the blue line is a localised loess-smooth line that represents the mean distribution of the data . A) data combined from all species across the entire survey in both PELTIC18 &19 b) Proportions for all fish species were aggregated by stratum

zone(Supplementary Fig.4.1-4.2) combined. The stratum zones for sprat and other species remained separate (n=39 stratum zones from PELTIC18 and n=34 stratum zones from PELTIC19).

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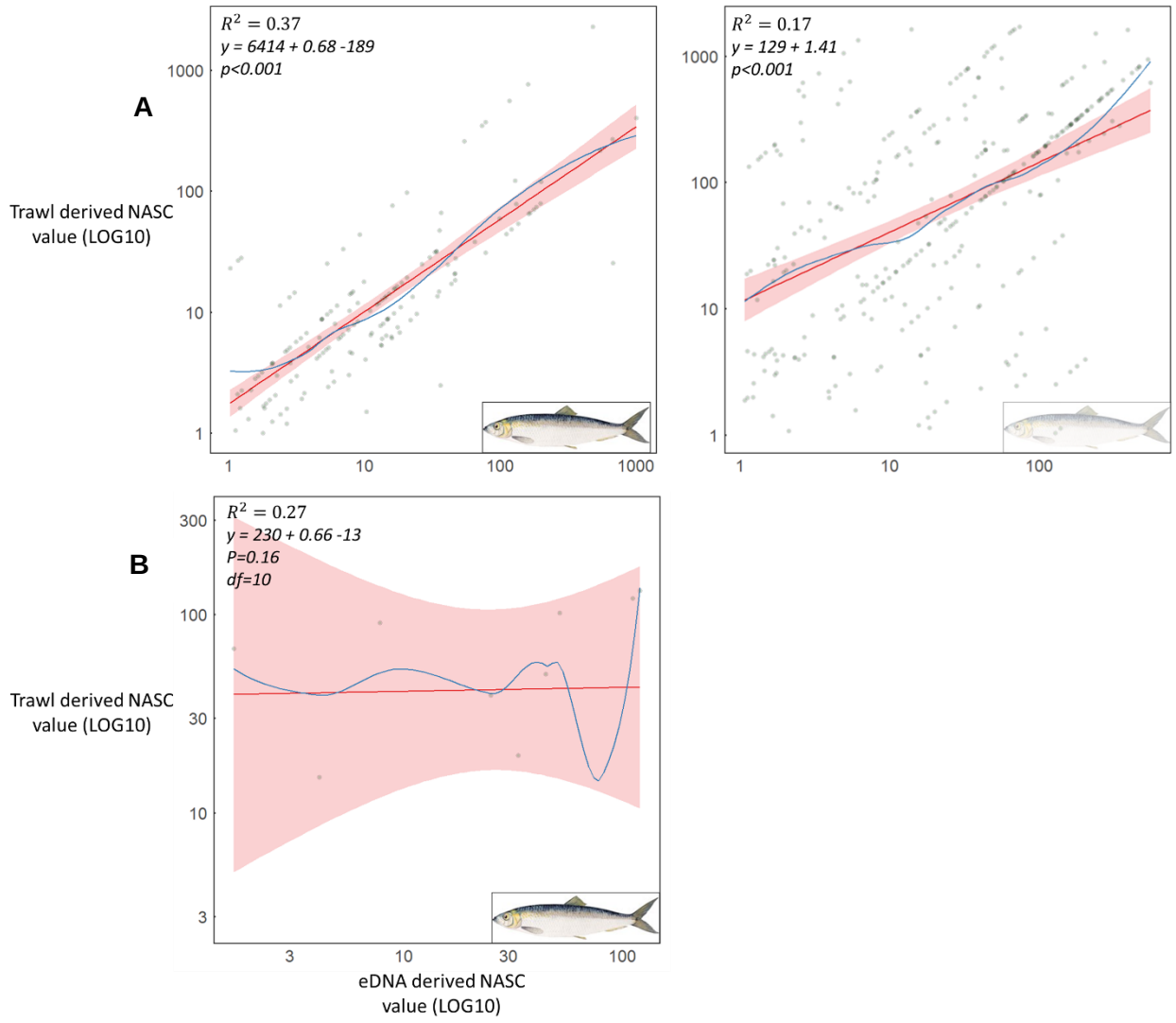


Figure 4.8 Log-log plot of trawl and eDNA derived NASC values for European pilchard (*Sardina pilchardus*). Trawling and eDNA data from the PELTIC18 survey (left panels), trawling and eDNA data from the PELTIC19 survey (right panels) There was not more than two stratum zones represented by data in the PELTIC19 data set for European pilchard. The red line is a fitted regression slope using the GLM prediction and the blue line is a localised loess-smooth line that represents the mean distribution of the data . A) data combined from all species across the entire survey in both PELTIC18 &19 b) Proportions for all fish species were aggregated by stratum zone(Supplementary Fig.4.1-4.2) combined.

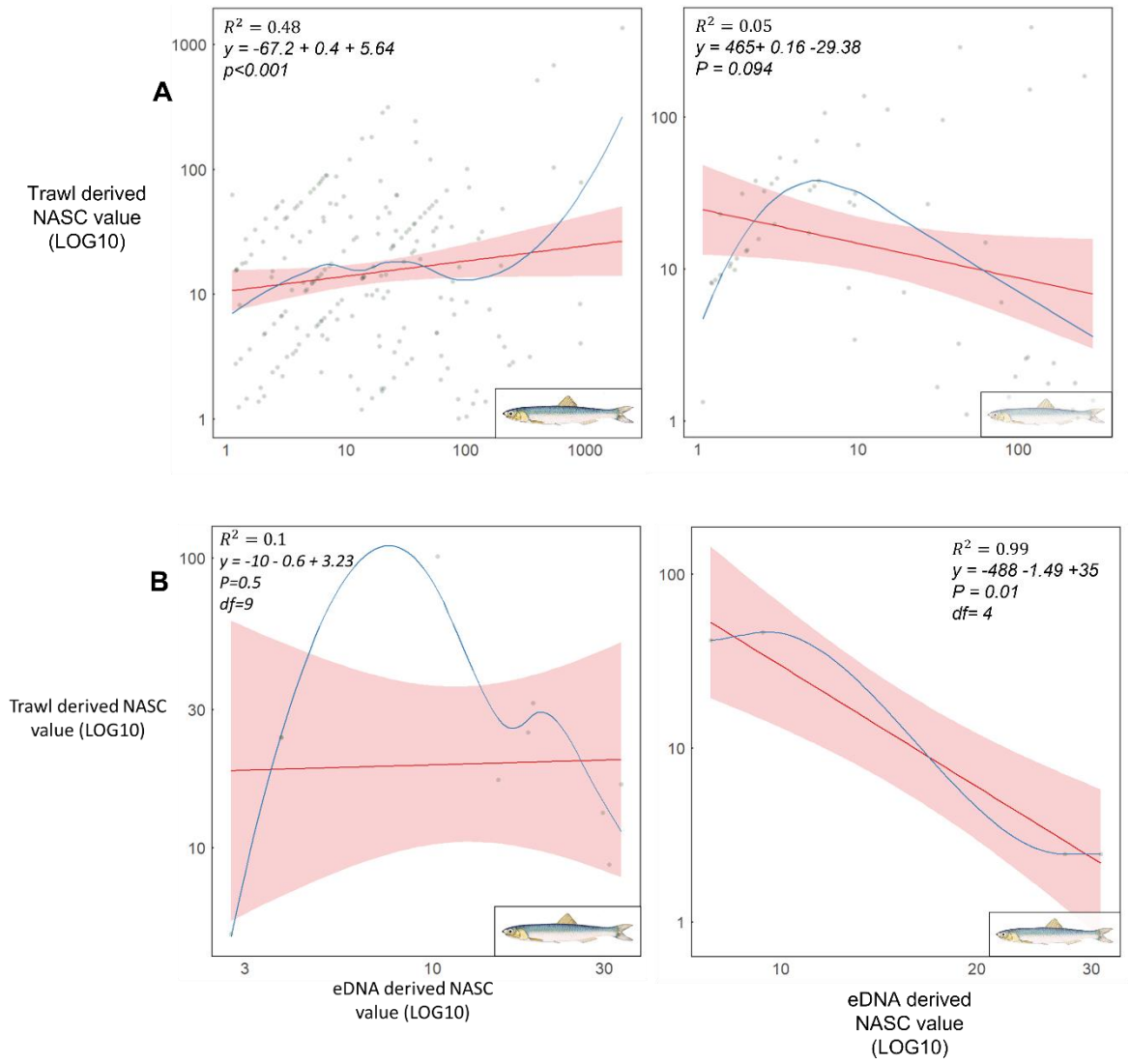


Figure 4.9 Log-log plot of trawl and eDNA derived NASC values for European anchovy (*Engraulis encrasicolus*).Trawling and eDNA data from the PELTIC18 survey (left panels), trawling and eDNA data from the PELTIC19 survey (right panels). The red line is a fitted regression slope using the GLM prediction and the blue line is a localised loess-smooth line that represents the mean distribution of the data A) data combined from all species across the entire survey in both PELTIC18 &19 B) Proportions for all fish species were aggregated by stratum zone (Supplementary Fig.4.1-4.2) combined.

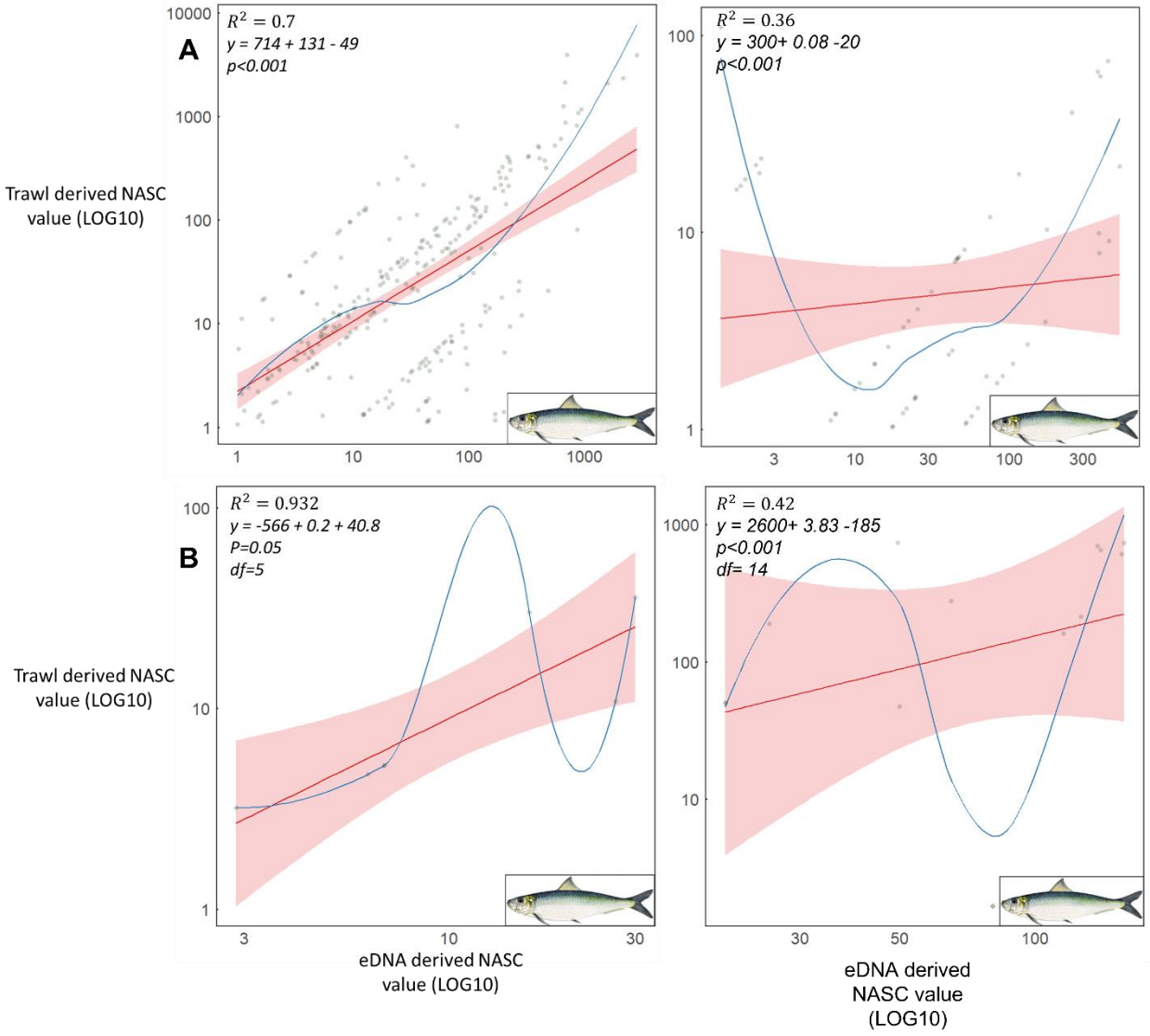


Figure 4.10 Log-log plot of trawl and eDNA derived NASC values for Atlantic herring (*Clupea harengus*). Trawling and eDNA data from the PELTIC18 survey (left panels), trawling and eDNA data from the PELTIC19 survey (right panels). The red line is a fitted regression slope using the GLM prediction and the blue line is a localised loess-smooth line that represents the mean distribution of the data . A) data combined from all species across the entire survey in both PELTIC18 &19 B) Proportions for all fish species were aggregated by stratum zone (Supplementary Fig.4.1-4.2) combined.

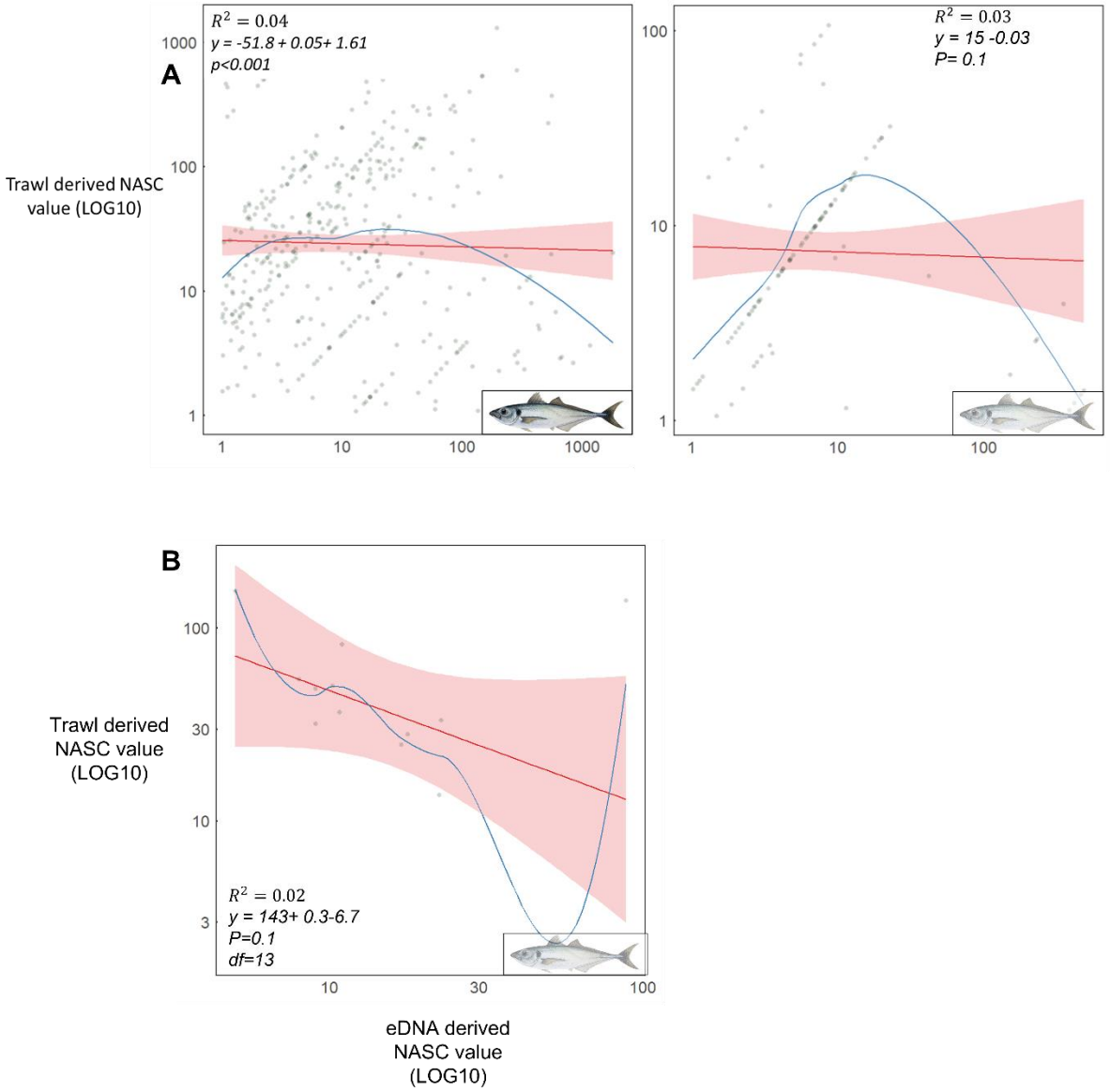


Figure 4.11 Log-log plot of trawl and eDNA derived NASC values for Atlantic horse mackerel (*Trachurus trachurus*). Trawling and eDNA data from the PELTIC18 survey (left panels), trawling and eDNA data from the PELTIC19 survey (right panels). There were not more than 2 stratum zones that contained data for the Atlantic horse mackerel from the PELTIC19 data set to statistically compare. The red line is a fitted regression slope using the GLM prediction and the blue line is a localised loess-smooth line that represents the mean distribution of the data. A) data combined from all species across the entire survey in both PELTIC18 &19 B) Proportions for all fish species were aggregated by stratum zone(Supplementary Fig.4.1-4.2) combined.

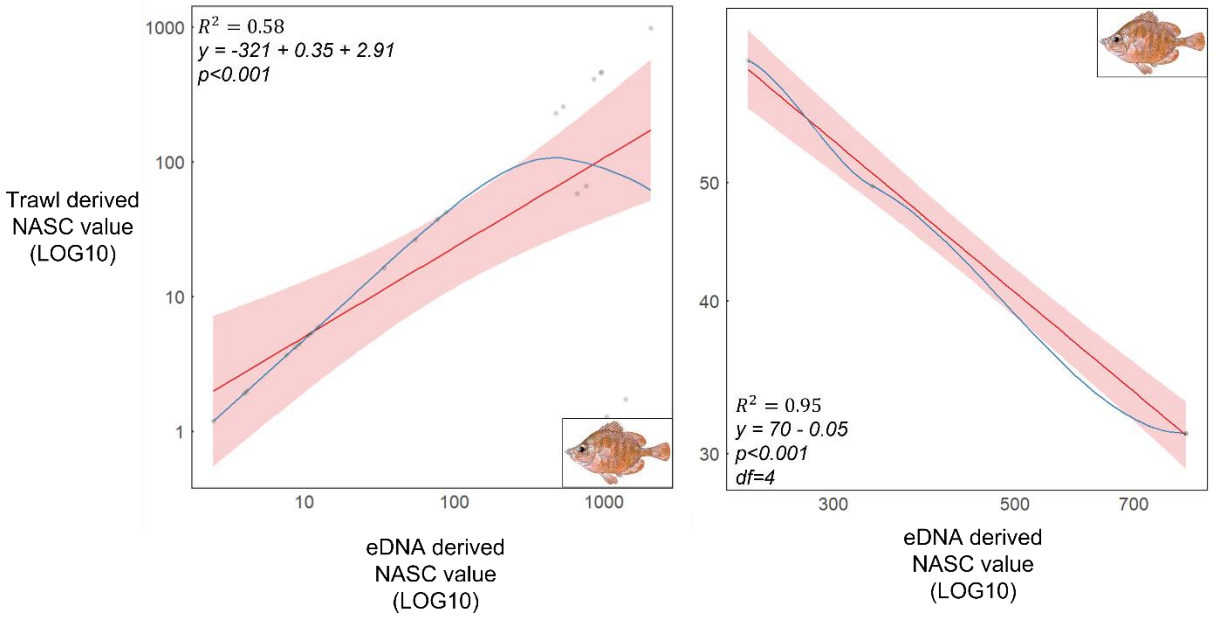


Figure 4.12 Log-log plot of trawl and eDNA derived NASC values for boarfish (*Capros aper*). Trawling and eDNA data from the PELTIC18 survey, there were no comparable data points between trawl and eDNA for the PELTIC19 survey. The red line is a fitted regression slope using the GLM prediction and the blue line is a localised loess-smooth line that represents the mean distribution of the data. Data combined from all species across the entire PELTIC18 survey (left panel). Proportions for all fish species were aggregated by stratum zone (Supplementary Fig.4.1-4.2) combined (right panel).

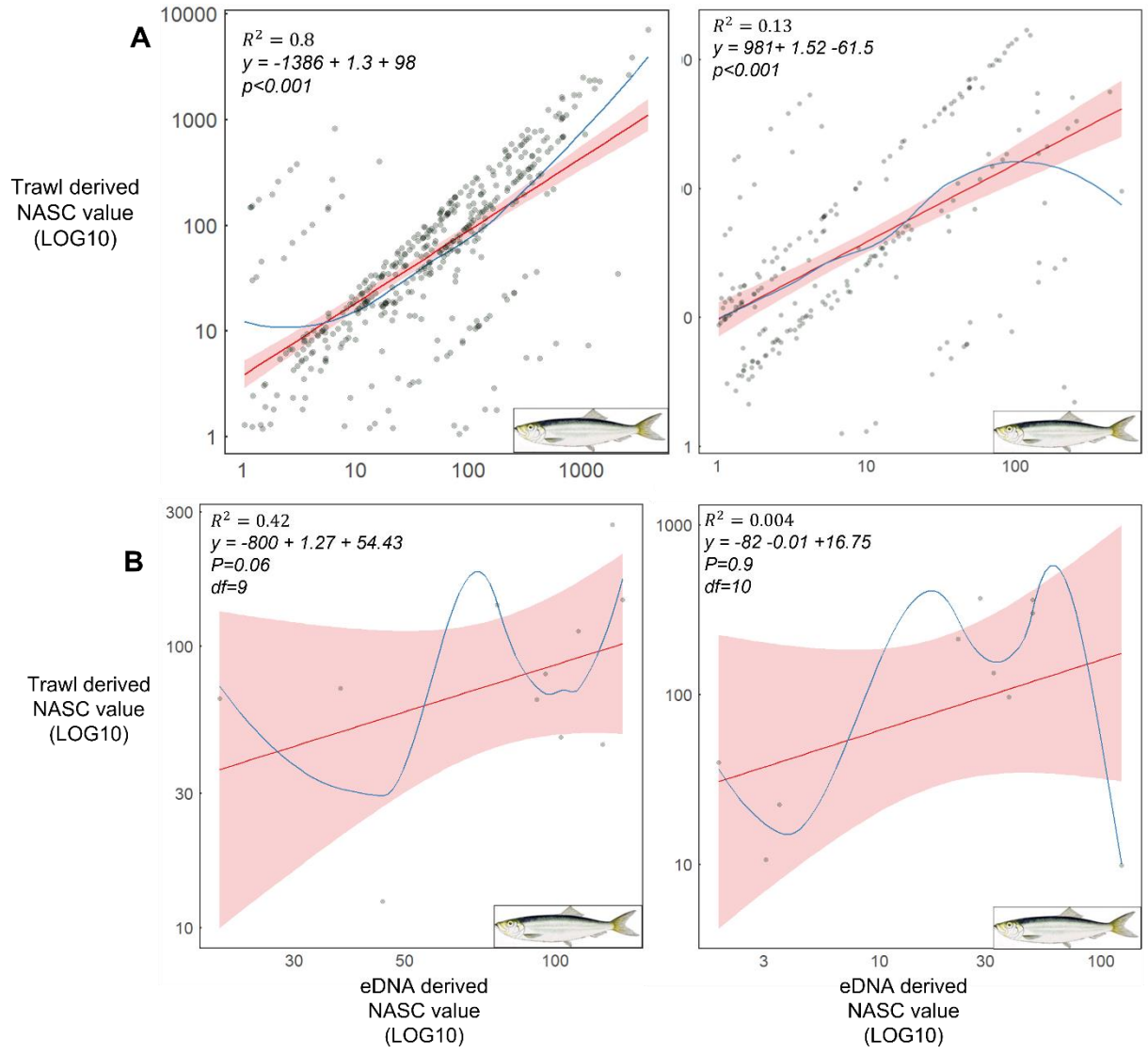


Figure 4.13 Log-log plot of trawl and eDNA derived NASC values for sprat (*Sprattus sprattus*). Trawling and eDNA data from the PELTIC18 survey (left panels), trawling and eDNA data from the PELTIC19 survey (right panels). P-value and R^2 show correlation. a) The hydroacoustic backscatter NASC values have been Log10 transformed to better visualise the correlations between eDNA, and trawl verified NASC, with a 95% CI area around the trendline. B) Proportions for sprat were aggregated by stratum zone (Supplementary Fig.4.1-4.2) and averaged.

4.5 Discussion

Our results suggest using eDNA to partition hydroacoustic backscatter values (Nautical Area Scattering Coefficient; NASC) is positively correlated with the backscatter values partitioned with midwater trawling. However, as observed in other comparative studies between eDNA methods and hydroacoustics (Li et al 2022, Berger et al., 2019), we also see fluctuations in significance level and strength of correlation, over spatial areas, between sampling rounds and between different species.

4.5.1 Species-specific correlations

Using the PELTIC18 and PELTIC19 data points we observed the following relationships between trawl and eDNA derived NASC values for each individual species. The European pilchard, Atlantic herring and sprat had similar detection rates by both trawl and eDNA as well as similar positive correlation in terms of strengths and variance. However, when the data was aggregated into their stratum zones only the strength and correlation observed in Atlantic herring were retained and variance increased for both surveys. Boarfish was detected the fewest by eDNA in both survey years and had no matched detections during the PELTIC19. When boarfish was detected by both trawl and eDNA the correlation over the entire sample area the model found a strong positive correlation, however when this data was aggregated into stratum zones this relationship was inversely correlated, albeit that the statistical power was significantly reduced to four degrees of freedom. Atlantic horse mackerel for both surveys had weak negative correlations between trawl and eDNA derived NASC across the entire survey area and when aggregated into stratum zones.

Similar to the findings by Li et al (2022), the fluctuations in significance between species could be amplified because the eDNA sampling methods are not optimised to be a direct comparison to a trawl catch. This is magnified when we observe the strength and positivity of the relationships for each species by the percentage crossover between eDNA and trawl. Variances between species could be directly affected by amplification bias from PCR methods that impacts the overall proportions in the eDNA data (Krehenwinkel et al., 2017). For example, if there is any bias towards the most abundant species; Atlantic

herring, European pilchard, sprat, European anchovy, Atlantic horse mackerel and boarfish, it may skew or mask the presence of other species changing the ratios of abundance. Conversely, the variation in amplification between other, low abundance, species can indirectly impact the proportions of the six species that we used to compare to midwater trawling.

Species that had a higher detection rate by both trawl and eDNA had stronger positive relationships with more of the variance explained by the model used. However, trends in this data for all species apart from Atlantic herring became weaker when the data was aggregated and split into stratum zones over our sample area. This suggests that there is a difference in the trawl catch or eDNA detection rates between stratum zones. The weakening of these relationships between stratum zones could be influenced by a change or lack of repeatability in the eDNA or trawling sampling between these regions or influenced by environmental covariates that were not measured in this study.

We also see that the relationships observed between survey years were significantly weaker in the strength of the correlation and the amount of variance explained by the models but each species had similar amounts of crossover detections by trawl and eDNA apart from sprat and boarfish.

4.5.2 Limitations of eDNA and midwater trawling as backscatter partitioning methods

Whilst we can only speculate on what is causing the variation between the eDNA and midwater trawl partitioning methods, the consistency of the variation between the two methods across spatial areas implies that there may be an underlying variable that consistently impacts all samples. From the findings of chapter 2, we know that this underlying variable is unlikely to be caused by differing environmental factors or from the method of eDNA sampling (flowthrough or CTD rosette). Horizontal transport of eDNA across the pelagic ecosystem could be increasing the variability of DNA read proportions in comparison to the trawling, which targets specific segments of the water column. DNA particles are transported from their source to different locations after initial release, the

rate and distance the DNA particles spread has been found to be dependent on the local weather systems, water regimes and distance from coastal areas (Jones and Davies, 2008; O'Donnell et al 2017; Harrison et al., 2019; Bonfil et al., 2021; Darling et al., 2021). Despite the consensus in research on the transportation of eDNA in aquatic environments (Deiner and Altermatt, 2014; Andruszkiewicz et al., 2019) the results of many different studies give contrasting conclusions on the ability to detect species over small and large spatial scales using eDNA (O'Donnell et al., 2017; Murakami et al., 2019; West et al., 2020; Miya, 2022; Shelton et al., 2022; West et al., 2022). The contrasted findings over spatial scales from the aforementioned studies could indicate that for taxa- and/or species that do not swim large distances across a 48 hour period or are in areas where there is little water movement have a decreased chance of detection in relation to other species because their DNA is less distributed across the water column. This may mean that for certain taxa, or within water bodies with low water displacement- such as deep water- specific sampling methods that target schools of fish or an increase in sample replicates is needed to increase the detection probability.

This species-specific variation can also be seen in our data. Boarfish was found significantly less than any other species using eDNA. Although, boarfish populations are smaller than that of other pelagic fish species in the North-East Atlantic, they are not considered a 'rare' species (Coad and Hüssy, 2012; Oskarsson et al., 2019). Species-specific variations in release of eDNA has been theorised by Murakami et al (2018) who found that the DNA of caged fish would intermittently be detected from ~30 metres. But sometimes would not be detected in samples less than 1 metre away from the caged fish. Because the tidal direction and movement did not impact the results found by Murakami et al (2018) this would suggest that DNA transport can significantly impact the detection of small schools or individual fish over multiple samples or large areas. This may also be dependent upon the morphology, school size, metabolic rates, and surface area of the specific species (Maruyama et al., 2014; Jo et al., 2019; Wood et al., 2020). Although, there could be primer bias influencing the lack of boarfish detection because we sampled at different depths across the water column, and in multiple areas and still found a lack of boarfish it is likely that their school distribution in the water column was not optimal for our sampling methods. Boarfish's slower movement and small aggregative groups means

that a sample must be taken in a specific spot which decreases the likelihood of detection in comparison to species, such as sprat, that have significantly larger schools and move around the water column a lot more. Subsequently increasing the variation between eDNA and midwater trawl partitioning methods.

Generally, the species data from PELTIC 19 tended to have weaker relationships between eDNA and trawl partitioned NASC. Because there is no difference in the way we processed the downstream analysis or conducted the sampling between PELTIC18 and PELTIC19 samples it is likely that this difference is caused by a change before sequencing took place. For example, we changed the extraction method slightly because of lack of available equipment in the lab and changed some of the reagents used to adapt to the difference in equipment. This is further supported by the significant difference in species accumulation between PELTIC18 and PELTIC19 samples that suggests a change within the sample location or post-sample collection has increased the differences between the sample years- which is unlikely to be caused by a dramatic change in species presence because of the size and ecological stability of the areas sampled.

Aside from potential variation caused from eDNA, midwater trawl proportions of the hydroacoustic densities will have some variation between trawling sites because of the behaviour and distribution of fish schools (Kaartvedt et al., 2012; Kotwicki et al, 2018). To overcome the impact of fish behaviour trawling data can be aggregated over large spatial areas and multiple trawls (Vabø et al., 2002; Gerlotto et al., 2004; De Robertis & Handegard., 2013; Brehmer et al., 2019). Therefore, on the spot comparisons between trawl and eDNA portioned hydroacoustics are likely to have variation but when aggregated over large spatial scales may decrease the impact of data skews by transport of the DNA or behaviour of the fish making the correlations between highly abundant/ commonly detected fish species more accurate.

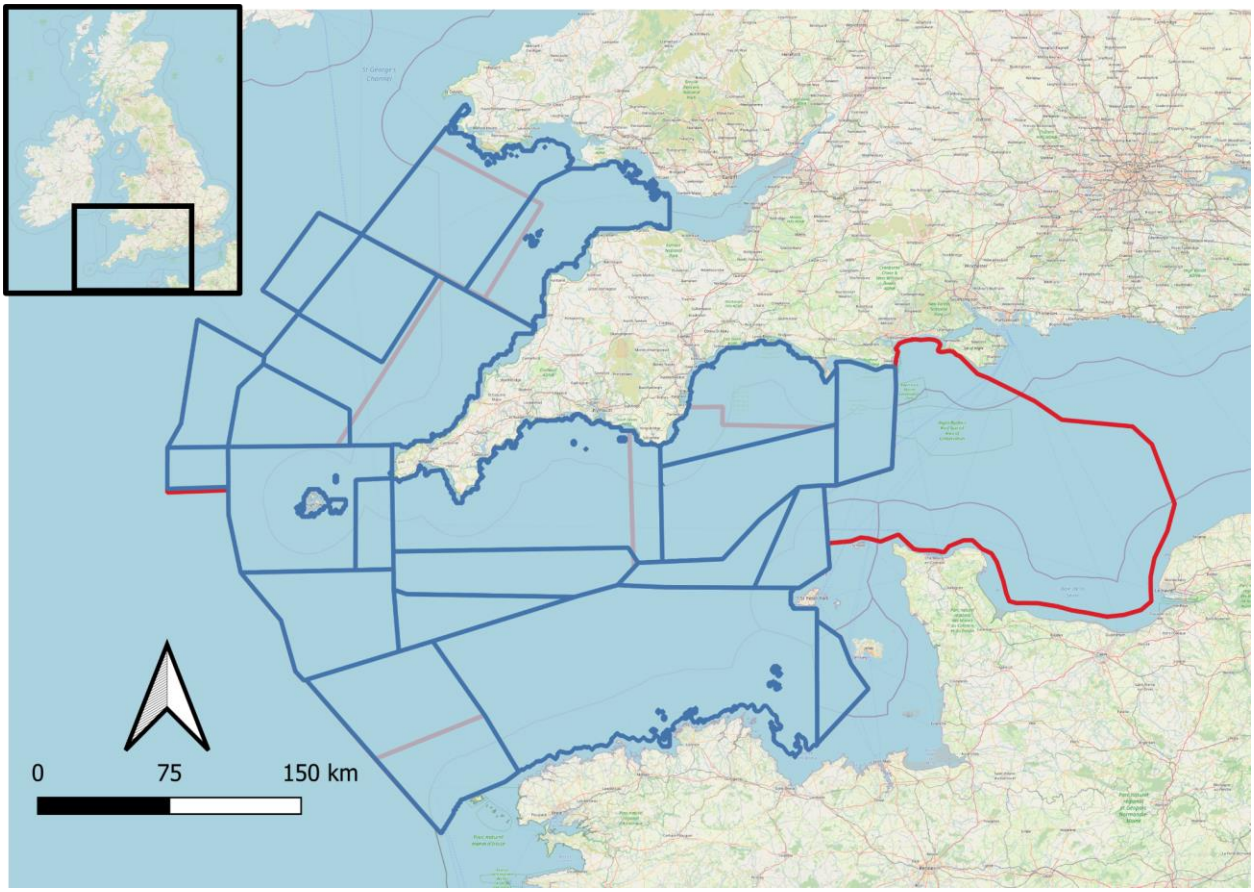
4.5.3 Further development of eDNA partitioning methods

These findings follow similar trends to other eDNA and hydroacoustic comparison studies, finding that species-specific correlations between the hydroacoustic backscatter and

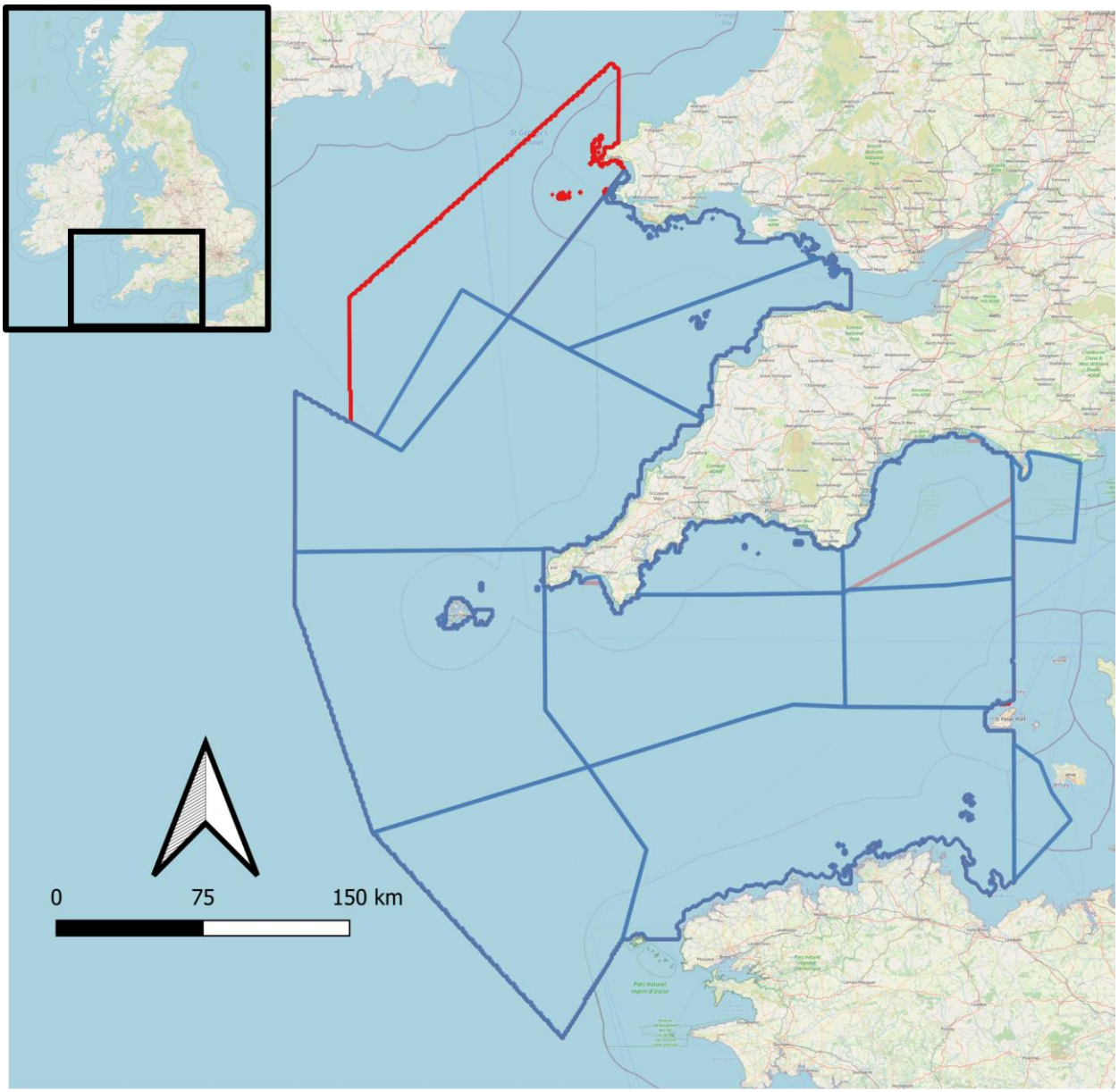
eDNA concentrations that is highly variable across spatial areas (Berger et al; 2019; Shelton et al., 2022; Li et al 2022). However, differences in the strength of trawl and eDNA correlations between species is not necessarily a discouraging sign because there are multiple species specific impacts that can affect the NASC proportions calculated from trawling and eDNA (Vabø et al., 2002; Gerlotto et al., 2004; De Robertis & Handegard., 2013; Brehmer et al., 2019). This does not mean we can assume that the eDNA partitioning is more accurate to that of the current midwater trawling procedures. There are still compounding factors, such as fish behaviour, DNA transportation and amplification biases that could impact species -specific comparisons before making a more exact correlation between the two methods. Although, we can account these issues by increasing in the number of eDNA and trawls taken place or by aggregating data sets over large spatial areas. But this would mean that small scale direct comparisons are likely to be more impacted and less reliable using this method unless more replicates and sampling takes place over smaller areas.

To better scrutinise eDNA methods of partitioning hydroacoustic densities a working understanding of eDNA movement in the marine environment will be needed. As well as, increasing the number of replicates per sampling point to increase the chances of a more normalised eDNA validated NASC values. Ensuring that species with intermittent DNA release or low abundance of DNA does not significantly impact the variances seen between eDNA and trawl validated proportions of hydroacoustic backscatter NASC.

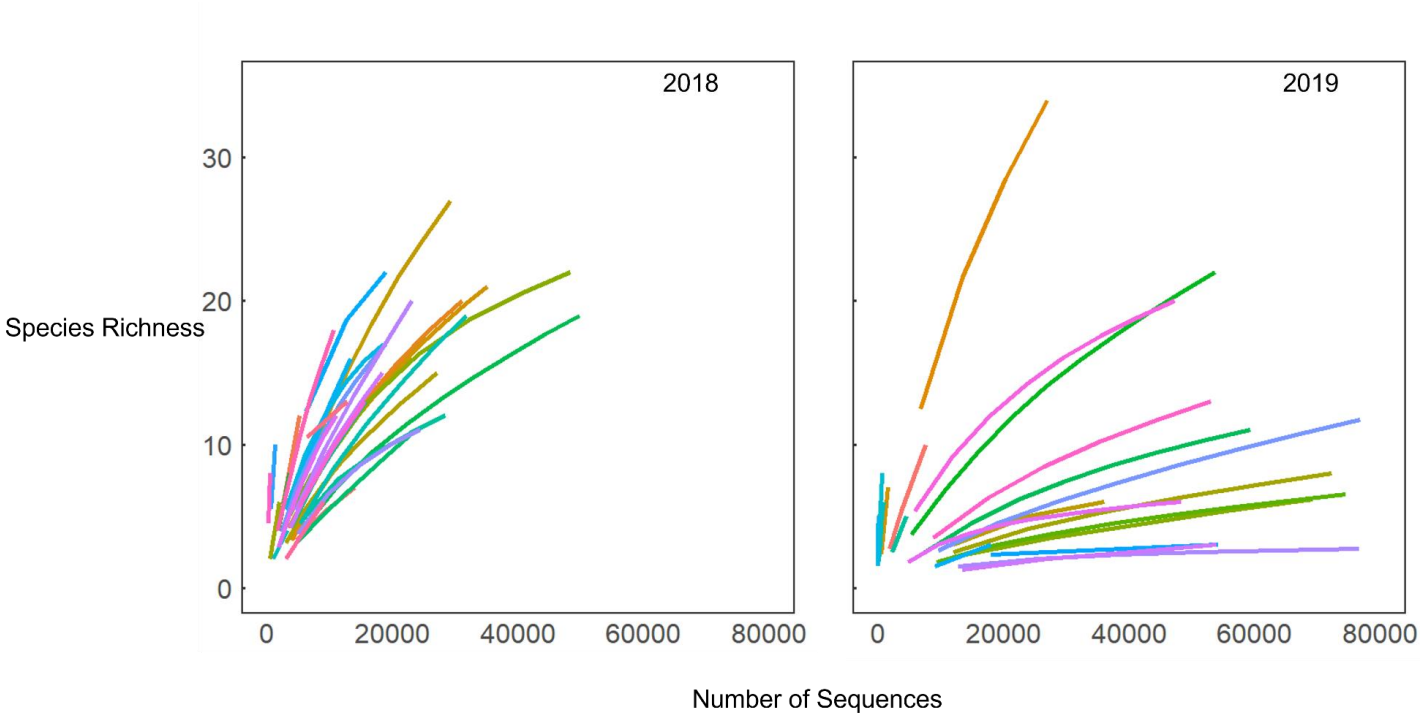
4.6 Chapter IV Supplementary Figures



Supplementary Figure 4.1 Map of the stratum zones for European pilchard (*Sardina pilchardus*), European anchovy (*Engraulis encrasicolus*), Atlantic herring (*Clupea harengus*), Atlantic horse mackerel (*Trachurus trachurus*) and boarfish (*Capros aper*) across the South-West coast of the UK. Blue lines indicate stratum zones for PELTIC18 AND PELTIC19. Red lines indicate additional stratum zones and differences in boundaries from the PELTIC18 survey.



Supplementary Figure 4.2 Map of the stratum zones for sprat (*Sprattus sprattus*) across the South-West of the UK. Blue lines indicate stratum zones for PELTIC18 AND PELTIC19. Red lines indicate additional stratum zones and differences in boundaries from the PELTIC18 survey.



Supplementary Figure 4.3 Species accumulation curves from the 2018 (PELTIC18, left panel) and 2019 (PELTIC19, right panel) sequencing data. Different colours represent different transect lines where eDNA samples were taken from.

Supplementary Table 4.1 Percentage crossover where eDNA methods and trawling methods both detected the same species at a sample location (EDSU) from both PELTIC18 & PELTIC19 surveys

	European pilchard	Sprat	European anchovy	Atlantic herring	Atlantic horse mackerel	Boarfish
PELTIC18	48%	93%	51%	61%	43%	4%
PELTIC19	52%	38%	14%	33%	30%	0%

4.7 Chapter IV Supplementary Methods

4.7.1 DNA extraction

PELTIC18 extractions followed the Mu-DNA water protocol. PELTIC19 samples, the Mu-DNA protocol was adapted to exclude the use of garnet beads, through optimisation tests, the Proteinase K was adjusted to 40 µl per extraction for optimum recovery of DNA and removal of potential contaminants and incubated on a thermomixer (Thermo-scientific) for at least 12 hours. Samples were extracted in batches of 12; for each batch of extractions a blank was taken, 20 for PELTIC18 and 24 for PELTIC19 samples. The sample extractions and blanks were then checked by loading the DNA on a 2% agarose gel stained with SYBR Safe and quantified for quality using a Nanodrop 2000 (Thermo Fisher Scientific).

4.7.2 PCR amplification

The Tele02 primers used for amplification were customised with 8bp unique indexing oligo tags on both the forward and reverse ends of the primers, and additional 4-6 randomly degenerate nucleotide positions (Ns) at the beginning of each tag to increase sequencing diversity.
Each sample was PCR-amplified in triplicate, in a total volume of 20 µl. For the PELTIC18 samples each reaction consisted of 10 µl Amplitaq Gold Master Mix (ThermoFisher Scientific), 0.16 µl BSA, 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer. This was added to a scaled ddH20-to-template input depending on the nucleic acid yield of each sample, which ranged from 0.1ng/ul to 72 ng/ul, from the Nanodrop. For the PELTIC19 samples each reaction consisted of 12µl Myfi Master Mix (Bioline), 0.04µl BSA, 1 µl of 5 µM forward primer, 1 µl of 5 µM reverse primer 5uL sample DNA and 0.96 µl ddH20.
The PELTIC18 samples were split into 3x82 sample plates and PELTIC19 samples were split into 4x86 samples with three PCR replicates per plate, PCRs were performed following the protocol in Taberlet et al. (2018) with an initial denaturation step of 94°C for 10 min, 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 1 min and a final extension step of 72°C for 5 min. The PCR products were checked on a 1.5% electrophoresis gel and pooled per plate.

4.7.3 DNA clean-up

A double-sided AMPure (Beckman Coulter) magnetic bead clean-up was used on both the PELTIC18 & 19 samples post PCR, with 0.7:1 left sided to remove fragments larger than the target and 1:1 right sided to remove fragments smaller than the target band. Each sample was then quantified on a Qubit 4.0 Fluorometer using the dsDNA high sensitivity assay (Invitrogen™).

4.7.4 Library preparation and Sequencing

These libraries were not prepared at the same time, PELTIC18 data was sequenced in 2019 and PELTIC19 was sequenced in 2021. Samples were separated and pooled for a target library concentration of 1µg total DNA which made 3 libraries of 82 from the PELTIC18 samples and 4 libraries of 86 for the PELTIC19 samples. The 3 PELTIC18 libraries and 4 PELTIC19 libraries were then pooled separately at an equimolar concentration (4nm). After end repair, a-tailing and unique dual index adapter ligation, using the KAPA hyper prep kit (Roche), the library was checked on a tapestation 4200

(Aglient). Each library was quantified using a NEBNext quantkit (Illumina) with serial dilutions of 1000, 10,000 and 100,000. The PELTIC18 pooled library quantifications were checked on the MIC RT-PCR machine (Bio Molecular Systems) and PELTIC19 pooled libraries were checked using a RotorGene Q RT-PCR machine (QIAGEN). For both PELTIC18 & 19 a diluted 20µl library was loaded onto a Illumina MiSeq platform using (v2) 2×150 cycle with a 10% PhiX control and a final sequencing molarity of 8pM.

Chapter V

General Discussion

5.1 Main Findings

This thesis aimed to optimise the use of environmental DNA (eDNA) methods for the semi-quantitative detection of pelagic fish and facilitate the integration of eDNA with current pelagic monitoring practices. Environmental DNA has quickly proven to be a powerful and versatile tool for assessing aquatic diversity in both freshwater and marine environments (Darling et al., 2017; Harper et al., 2019; Di Muri et al.,2020; Gilbey et al., 2021; Norros et al., 2022). Since its first application to marine macro-organisms a decade ago (Thomsen et al., 2012; Foote et al., 2012), we have seen substantial developments towards efficient implementations of marine eDNA such as the improvement of reference databases (Marques et al., 2021), measurements of DNA persistence (Collins et al., 2018; Wood et al., 2020; Holman et al., 2021) that have increased our ability to detect more species and define covariables that limit eDNA sampling. Despite the magnitude of progress made towards marine applications of eDNA, the pelagic environment presents unique and persisting challenges to the integration of marine eDNA into current pelagic monitoring surveys.

Marine pelagic ecosystems are a vast, continuous, and interconnected water regime that are a part of the largest ecosystem on earth (Jennings et al., 2009). Pelagic fish are able to move freely in multiple directions without the restrictions of physical barriers (Kaiser et al., 2011), thus releasing their DNA throughout the water column. One of the few restricting factors within the pelagic environment occurs when the temperature and/or salinity drastically changes creating separate distinct stratified water bodies that have different pH, salinity and temperature compositions (Fiestal et al., 2008). This often influences the movement and distribution of pelagic organisms as some are forced to

move to stay in favourable conditions, such as zooplankton, whereas as macro-organisms, e.g. fish, will follow and change their depth to match the distribution of their prey species (Johnson and Allen, 2012; Yu et al., 2014; Roman et al., 2019; Maboloc et al., 2020). For example, Jeunen et al. (2020) found distinctly different populations of fish, crustaceans, and eukaryotes in stratified water bodies from the Patea fjord (New Zealand). Whereas Closek et al (2019) found no differences in the communities of fish species assemblages above and below the point of stratification using eDNA methods off Californian continental slope. Govindarajan et al (2021) showed that communities of marine zooplankton detected using eDNA, collected in the Western Atlantic, significantly differed between shallow water (0-200 metres) and deeper mesopelagic water (200-800 metres). However, within the deeper water there was no significant difference between different depth categories of 100-200 metres, 200-500 metres, or 500-800 metres. A better understanding of how the distribution of pelagic fish, or the biochemical changes in the water, affect the molecular detection potential of pelagic fish is a prerequisite for accurate eDNA-based data collection. Understanding these factors enable more complex and encompassing models of eDNA use within the environment, which can be used to develop better sampling strategies that encompass these factors and increase the efficiency of detecting the target taxa.

Herein lies the first challenge tackled by this thesis, which was to explore whether depth, stratification or other abiotic factors impacts the detectability of pelagic fish using eDNA. In chapter 2, I specifically looked at optimising the sampling protocols for marine pelagic fish by exploring whether sampling at the surface or at set depths within the water column (20% and 80% of the total depth of the sampling area) impacted the detectability of eDNA. This was conducted when the water was both stratified or mixed, ensuring that different water bodies were sampled when both stratified and mixed. I then investigated the impact of the sampling depth (Fig.2.4) on eDNA observations including the number of species, species evenness or the number of amplicon reads differed between depths. Similar to the findings of Closek et al. (2019), the sampling depth, stratification, temperature, and salinity of the water column had no significant impact on species richness, evenness or number of reads obtained within and between sample sites. In addition, the number of species derived from eDNA sampling was the same in upper and lower water column, but

species composition was different, with surface water ($p<0.001$) and the upper water column ($p<0.05$; Fig.2.5) consisting of more pelagic fish versus lower water column dominated by demersal fish.

I then looked at whether there was a noticeable difference between the fish communities detected in stratified and mixed water bodies using both the number of reads and presence/absence data for each species. A principal coordinate analysis (PCoA) showed that Bray-Curtis indices (that used the proportion of reads as a proxy for abundance) found a significant dissimilarity in species assemblages between stratified and mixed water bodies ($p=0.001$, $R^2= 0.09$) whereas there was no such dissimilarity when Jaccard indices were used ($p= 0.07$, $R^2 =0.04$; Fig2.6a). When the species assemblages were observed using principal component analysis (PCA), that looked at only highly correlated variables, there was no significant difference between the DNA of species found in mixed water to the species in stratified water (Fig 2.6b). Thus, we consider that the difference in eDNA distribution and lack of impact on community differentiation from stratification is due to fish behaviour and ecology. But it is also likely that the relatively shallow depths of our study area on the Atlantic continental shelf results in the DNA in the water being highly mixed and homogeneous. This is because most of the fish species can move across the entire water column, where there are no extreme depths, and the oceanographic dynamism distributes particles across the water column significantly reducing the partitioning effect of stratification on the distribution of DNA. For the purposes of pelagic fish monitoring on continental shelves, the subtleties of environmental variability do not appear to introduce significant biases to eDNA analysis and interpretation. This meant that surface water sampling, where water was collected from an inflow pump 4 metres below the RV through a FerryBox system, provides a practical and robust sampling procedure for eDNA collection of the pelagic fish. However, it will be essential to continue the implementation of an appropriate CDT sampling programme to keep the confidence in eDNA results, specifically with reference to the demersal fish community. Marine eDNA also provides a vast amount of information on the presence of other species. In chapter 2 we show that eDNA detected 11 families and 46 fish species that were not sampled by traditional pelagic trawling methods. Although similar to other eDNA studies (Thomsen et al., 2012; Afzali et al., 2020; Fraija-Fernández et al., 2020), eDNA methods do not detect

all species. In our data six species from 3 families that were caught in the trawl were not found through the use of eDNA. These species included the Atlantic bonito (*Sarda sarda*) and Black seabream (*spondyliosoma cantharus*), both have been caught around the North-East Atlantic, but the populations of black seabream tend to be more spatially restricted in comparison to the bonito (Neves et al., 2020). These two species also have smaller populations in comparison to other small pelagic fish species such as European pilchard or sprat and tend to be smaller in size such as the Atlantic bonito average length is only 40cm when they have been caught in the Nort-East Atlantic (Lucena-Frédou et al., 2021). The other species that were not detected by eDNA included the Great pipefish (*Syngnathus acus*), Tub gurnard (*Chelidonichthys lucerna*), Grey gurnard (*Eutrigla gurnardus*), and the lesser weaver (*Echiichthys vipera*). These species are non-schooling, and the latter are generally demersal species that inhabit muddy sand and rocky sea bottoms, relying on camouflage to avoid predators (Mytilineou et al., 2005; Gorman et al., 2020). These species, being lower in population than other pelagic species, and/or demersal species, will significantly lower the amount of DNA output for these species making them less likely to detect from pelagic water sampling that does not target their detection.

The species inventory from chapter 2 included Atlantic bluefin tuna (ABT) in multiple locations around the South-West coast of the UK. In chapter 3, we investigated the application of eDNA to become a temporal monitoring tool for ABT in collaboration with the Thunnus UK project (https://www.thunnusuk.org; defra ref MF1247). In addition to eDNA samples collected during the 2018 and 2019 PELTIC surveys, weekly samples were collected on a fixed location off Falmouth from smaller vessels, without rosette deployment, ensuring that weekly sampling was cost efficient. This sampling was carried out to also investigate eDNA's potential to reliably capture/monitor seasonal fluctuations in wider biodiversity particularly in those species that were thought to be ABT prey. This study found that marine eDNA sampling was able to detect fluctuations of the fish community, ABT presence, and the ABT prey population dynamic over season. I also compared sighting data to eDNA, being able to detect ABT DNA within two of the known sighting areas using previous surveys. This suggests that routine use of eDNA

metabarcoding could serve as a tool for the validation of ambiguous sighting data (Di Natale et al., 2018; Horton et al., 2021), but it is uncertain that this approach could provide fine-scale granularity, given the high mobility of ABT and seeing that eDNA methods did not detect their presence in six of the sighting areas.

The ABT sequencing also under-clustered, leading to a small average of 2,934 amplicon reads per sample, and 5 sample weeks had no reads, this resulted in unrealistically low species richness and biodiversity in late August, September and early October. Nevertheless, these results indicate that continual eDNA sampling provides useful information on the presence of marine species. This is an especially important finding for coarse phenology mapping over relevant time scales and monitoring of highly migratory species like ABT, or rare, cryptic, and alien species.

In chapter 4 I investigated the integration of marine eDNA into the PELTIC and/or similar hydroacoustic survey for small pelagic fish monitoring. As a typical pelagic monitoring survey, PELTIC use a mid-water pelagic trawl in combination with hydroacoustic methods to map and quantify six dominant target fish species: European anchovy (*Engraulis encrasicolus)*, sprat (*Sprattus sprattus*), boarfish (*Capros aper*), European pilchard (*Sardina pilchardus*), Atlantic horse mackerel (*Trachurus trachurus*), and Atlantic herring *(Clupea harengus)*. The proportion of species from fish caught in a midwater trawl is used to validate, split (partition), and assign the hydroacoustic values to those species. In this chapter I explored whether eDNA could provide a rapid, non-invasive, and low effort tool for the partitioning of hydroacoustic data for the six dominant pelagic fish species rather than the use of trawling which cannot guarantee a representative catch of the fish schools. Like the findings in chapter 2, my work showed that the hydroacoustic data partitioned by eDNA are statistically positively correlated with the hydroacoustic data inferred from midwater trawling. We also found similar correlations between eDNA -and trawl-partitioned hydroacoustic data by species at the stratum level for sprat, European pilchard and Atlantic herring. This relationship was absent for European anchovy, Atlantic horse mackerel and boarfish. The results suggest that deviations between eDNA reads of these species are caused by the difference in abundances: more abundant species are more likely to be caught and more likely to be detected by eDNA. Therefore, the use of marine

eDNA for small pelagic fish could be used to provide information on the density and abundance of certain species through combination with hydroacoustics. Moreover, eDNA provides a certified avenue for the detection of high population species in comparison to pelagic trawling, where there are known to be catchability issues. This implication of this work suggests that smaller vessels that are in capable of using a trawl set up and in areas where trawling would not be viable (e.g., Sites of specific scientific interest, wind farms, shipping ports) could use eDNA and hydroacoustics to monitor specific pelagic populations. Vastly increasing the utility and ability to generate data on marine pelagic species.

5.2 eDNA sampling: Improving spatial relevance

A major limitation that is not answered by the current literature is how and where eDNA is transported and whether it significantly affects the detection between sample locations. Like looking both ways while you cross a road ensures that you do not step out into potential problems, knowing where DNA has come from is needed to ensure spatial relevance of specific locations. Spatial ambiguity of eDNA methods is well noted in many eDNA studies where eDNA can be moved in several dimensions at different spatial scales (Closek et al., 2019; Fraija-Fernández et al., 2020; Monuki et al., 2021; West et al., 2021). Although theories on particle tracking could potentially be useful in understanding where the DNA could have moved from (Harrison et al., 2019) these models are highly complex and need in-depth data on the multiple dimensions water could be travelling in within the water column (Andruszkiewicz et al., 2019). Current techniques cannot “age” the DNA to see how long the particles have moved since release from the organism. In Chapter 4, we find the dissimilarity between the proportions of species detected does not significantly increase with distance between an eDNA sampling point and a trawl-validated hydroacoustic sample station. Neither do we see a significant difference in the proportional components between different species, although some species had greater variation in the proportional similarity between eDNA and trawl-validated hydroacoustic NASC than others (Fig.4.6). This suggests that there is little variation in the species

detected across our sampling area, which is unsurprising given that the species we compared have the hight abundance in Atlantic waters and the waters samples are not significantly different in terms of their ecological parameters (temperature, salinity, prey availability etc).

Although some focal species, such as horse mackerel and boarfish, were less abundant in the eDNA data, they are not considered to be rare in our study area (Coad and Hüssy, 2012; Oskarsson et al., 2019). Therefore, the species-to-species variation in the amount and pattern of the release of DNA may be relevant in this context, with some species releasing DNA more intermittently, creating background noise (Ushio et al., 2017; Chapters 2 & 4). This means that the spatial biodiversity information gathered from eDNA could be masked by taxa that form large shoals skewing read abundance data and increasing the likelihood of PCR or sequencing amplification bias out competing species that have low mt-DNA copies in the water. This could reduce our ability to detect fluctuations in fish biodiversity at small spatial scales or using one-off sample points. Thus fine-scale distributions of rare or migratory pelagic species such as ABT, that seasonally swim through bodies of water (Walli et al., 2009; Chapman et al., 2011), could be missed from a single eDNA sample station or low read-depth per sample. Further insights into whether large numbers of other species could mask the presence of migratory ABT could be tested via judicious integration of satellite tagging data or by using qPCR to find whether there is ABT presence in samples were ABT were not in the metabarcoding data.

5.3 Future Directions of Marine eDNA

As mentioned previously in the discussion, the use of eDNA in the monitoring of pelagic fish requires further understanding of the spatial movement and displacement of DNA from a release source. This will help to align eDNA and other methods such as visual data, drone-assisted observations, and midwater trawling enabling future integrated eDNA approaches. It will also help develop further research into the combination of eDNA

with hydroacoustics for more accurate interpolation of fish species densities in the water column. Crucially the use of eDNA will provide a robust data on multiple different species from all levels of the pelagic water column, that wouldn't otherwise be detected by species and taxa specific trawling, providing additional data to environmental managers (Misund, 1990; Misund and Aglen, 1992; Fréon & Misund., 1999; Handegard et al., 2003; Jørgensen et al., 2004; Queirolo et al., 2010; Heino et al., 2011; Williams, 2013; Robert et al., 2020; De Robertis et al., 2021).

Although this thesis was not able to establish ecological or trophic relationships between the presence of species over the weekly samples (chapter 3), it can be envisioned that more tailored eDNA sampling designs can help future research in obtaining more robust species association links that may reflect trophic interactions (e.g. strong positive correlations underlying predation, and strong negative correlations possibly underlying competition). To use eDNA to better understand the ecological changes within the pelagic environment, future work will need to incorporate diet analysis from pelagic species to strengthen the ability to determine trophic relationships. Making marine eDNA a multiple faceted tool that can not only verify the abundance of pelagic fish but also infer their ecological interactions within the pelagic ecosystem.

5.4 Final Conclusions

This thesis has advanced our understanding of the applications of marine eDNA in the pelagic realm, specifically ironing out potential issues such as sampling depth and the impact of stratification on the detection of marine pelagic fish (chapter 2). I have found that eDNA can be used to detect the presence of large migratory species such as the ABT (chapter 3), which may have significant inferences for the future monitoring and management of ABT in European waters as their population recovers. The comparisons between eDNA- and trawl-partitioned hydroacoustic backscatter (chapter 4) contributes to the discourse around the potential of marine eDNA quantification and shows eDNA potential as a new supporting tool for worldwide pelagic fish monitoring. Although there

are still questions to be answered about marine eDNA sampling, I expect eDNA analysis to continue its ascent and become more commonly integrated within marine surveys to provide additional biological information for policy makers worldwide.

Chapter VI

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