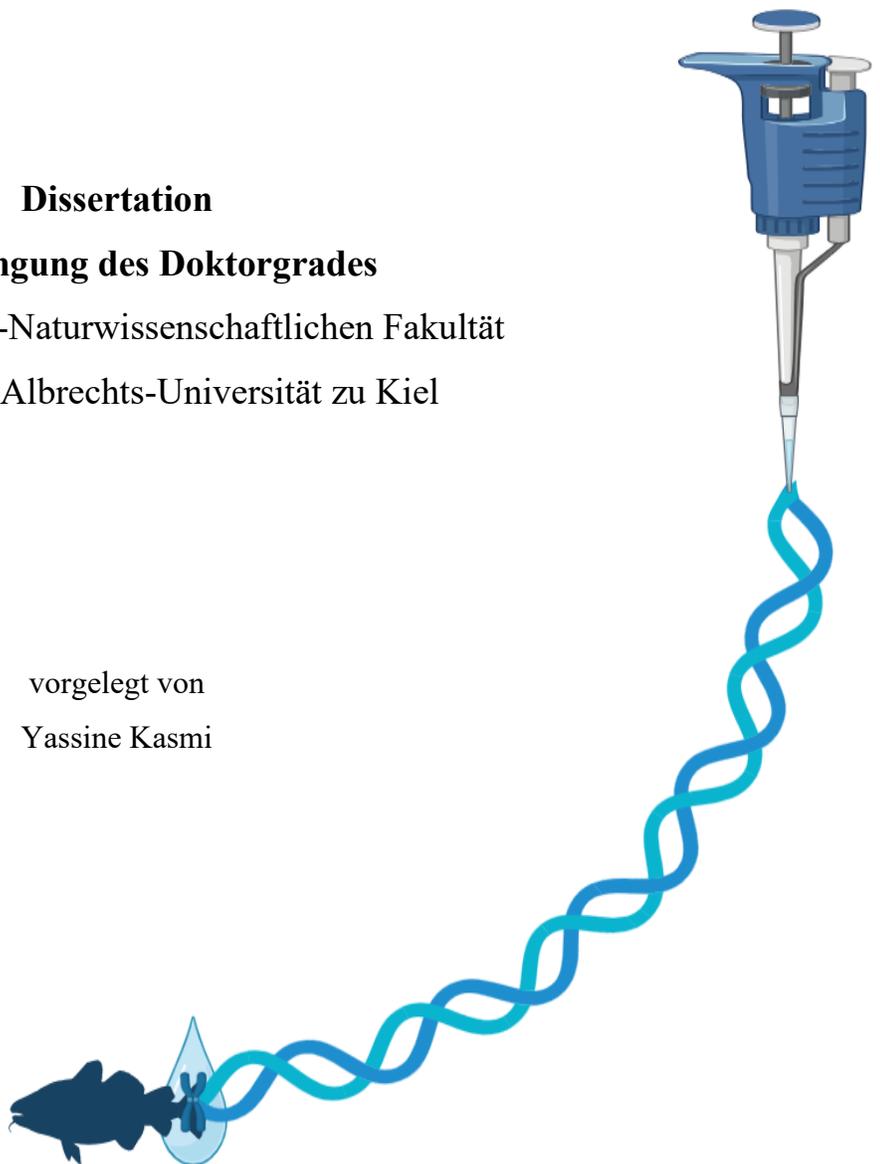


The role of eDNA in the marine biodiversity monitoring of the future

Running Title: *Advances in eDNA-based finfish community analyses and fish stock assessments in the North Sea*

Dissertation
zur Erlangung des Doktorgrades
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Summary

Sustainable fisheries management is of global interest due to rapid changes of aquatic ecosystems on a global scale. The essential element of any fisheries management is a proper assessment of target and non-target stocks. However, standard monitoring methods have shown limitations in accurately reflecting the diversity and abundance of certain species. The rapid development of environmental genomics tools provides an exciting alternative for overcoming these limitations. One of these new promising tools is the analysis of environmental DNA (eDNA).

This thesis evaluates the potential of eDNA to improve fish stock assessment and fisheries management, particularly in terms of quality. In addition, this thesis addresses the issue of the ability of eDNA analyses to provide quantitative information comparable to data from reference methods, namely trawling, using mathematical models.

The results of this thesis support the idea that eDNA-based methods have the potential to substantially add to or even replace catch-based fish biodiversity and fish stock assessments in the near future. However, they also point at the limitations of eDNA applications and the current need for a standardisation of tools, including a recommendation to move towards long-read sequencing methods for better discrimination between species. The thesis emphasises the importance of developing and adopting appropriate computational methods capable of gathering quantitative information from genomic data and discusses possible implementation scenarios and future directions.

Zusammenfassung

Nachhaltiges Fischereimanagement ist aufgrund der schnellen Veränderungen aquatischer Ökosysteme auf globaler Ebene von globalem Interesse. Das wesentliche Element jedes Fischereimanagements ist eine ordnungsgemäße Bewertung der Ziel- und Nichtzielbestände. Standardüberwachungsmethoden haben jedoch gezeigt, dass sie die Vielfalt und Häufigkeit bestimmter Arten nur begrenzt genau widerspiegeln können. Die rasche Entwicklung von Instrumenten der Umweltgenomik bietet eine spannende Alternative, um diese Einschränkungen zu überwinden. Eines dieser neuen vielversprechenden Instrumente ist die Analyse von Umwelt-DNA (eDNA).

In dieser Arbeit wird das Potenzial von eDNA zur Verbesserung der Bewertung von Fischbeständen und des Fischereimanagements, insbesondere in Bezug auf die Qualität, untersucht. Darüber hinaus befasst sich diese Arbeit mit der Frage, ob eDNA-Analysen mithilfe mathematischer Modelle quantitative Informationen liefern können, die mit Daten aus Referenzmethoden, insbesondere dem Schleppnetzfang, vergleichbar sind.

Die Ergebnisse dieser Arbeit stützen die Idee, dass eDNA-basierte Methoden das Potenzial haben, in naher Zukunft die fangbasierte Bewertung der Fischbiodiversität und der Fischbestände erheblich zu ergänzen oder sogar zu ersetzen. Sie weisen jedoch auch auf die Grenzen von eDNA-Anwendungen und die derzeitige Notwendigkeit einer Standardisierung der Instrumente hin, einschließlich der Empfehlung, auf Long-Read-Sequenzierungsmethoden umzusteigen, um eine bessere Unterscheidung zwischen den Arten zu ermöglichen. Die Dissertation betont die Bedeutung der Entwicklung und Einführung geeigneter Berechnungsmethoden, die in der Lage sind, quantitative Informationen aus Genomdaten zu gewinnen, und diskutiert mögliche Umsetzungsszenarien und zukünftige Richtungen.

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Abbreviations

DNA	: Deoxyribonucleic Acid
RNA	: Ribonucleic Acid
eDNA	: Environmental DNA
eRNA	: Environmental RNA
PCR	: Polymerase Chain Reaction
qPCR	: Real Time Quantitative PCR
NGS	: Massive parallel sequencing or Next-Generation Sequencing
EU	: European Union
CFP	: Common Fisheries Policy
MAPs	: Multi-Annual Plans
DG-MARE	: Directorate-General Maritime Affairs and Fisheries
ICES	: International Council for the Exploration of the Sea
ML	: Machine Learning
GLM	: Generalized Linear Model
EMFF	: European Maritime and Fisheries Fund
DCF	: Data Collection Framework
TI	: Thünen Institute
FAO	: Food and Agriculture Organization
UNEP	: United Nations Environment Program
UN	: United Nations

Preface:

The European Union (EU) Common Fisheries Policy (CFP) emphasizes a scientifically informed approach of management advice that is based on accurate data collected both by sampling fisheries and by scientific surveys-at-sea. Such an approach ensures that marine biodiversity and the health of ecosystems are protected to guarantee sustainable fishing. Research surveys are crucial to provide vital data of fish stocks and ecosystems, particularly in EU Member States' shared waters. The key surveys are carried out at EU level, as set out in Table 1 of Commission Decision (EU) 2021/1168, based on Regulation (EU) No 2017/1004, aiming to ensure data collection that supports the CFP goals. The annual expenditure on such marine research under the EU's Data Collection Framework (DCF) and Multi-Annual Plans (MAPs) averages around € 33 million (European commission Ares. (2018) 3154439 - 14/06/2018), while the effectiveness of these surveys varies, with some focusing only on certain species or areas. As a result, traditional methods of collecting data on fish stocks have limitations, and there is a demand for better models and information, particularly when managing mixed-stock fisheries.

In this context, the EU Commission, via its directorates responsible for maritime affairs and those concerned with the conservation of these resources, such as Directorate-General Maritime Affairs and Fisheries (DG-MARE), have announced a series of tenders and calls for projects to explore the feasibility of new genomic techniques based on high throughput DeoxyriboNucleic Acid (DNA) sequencing, especially environmental DNA (eDNA), to replace or enhance traditional fish stock assessments.

A significant part of this work was carried out within the FishGenome project funded by European tender funds EASME/EMFF/2018/015 and DCF funds as part of the North Sea eDNA pilot project (EMFF Regulation 508/2014), with the aim to explore the potential of eDNA techniques and a specific focus on their application in the North Sea to complement or replace traditional trawl surveys, thereby improving the timeliness and efficiency of fish stock assessments.

Preview of Thesis Contributions:

This thesis addresses the following major questions based on experimental methods and by means of mathematical and numerical simulations:

- 1. Exploring the Potential of Environmental DNA (eDNA) to Reveal Marine Biodiversity:**
 - a. Is eDNA a reliable indicator of marine biodiversity?
 - b. How do eDNA results compare with established methods like trawling?
 - c. Do the long-read capabilities of 3rd generation NGS align with traditional methods?
 - d. What are the limitations inherent in eDNA methodologies?
- 2. Assessing the Quantitative Capabilities of eDNA:**
 - a. Can we quantify species richness using eDNA data obtained from single and multiplex quantitative PCR assays?
 - b. Does eDNA metabarcoding provide semi / quantitative insights?
- 3. What are the possible scenarios for implementing eDNA techniques in European marine habitats and their surveys?**

Dedication

Für meine Familie

I read one day that:

*" Behind the scenes of any success,
there are many people we don't see."*

Acknowledgements

*“Nothing is difficult/impossible,
but nothing can be done alone/itself.”*

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1. General introduction

Marine ecosystems are characterized by an important biodiversity, serving as a habitat for numerous macro and microorganisms (Cronan, 2023; Peterson, 1992; Rabone et al., 2023). However, this biodiversity is currently facing the risk of a significant decline, both in terms of species number and abundance. The current extinction rates of some organisms is more than 1,000 times higher than the baseline extinction rate e.g. cichlid fishes in Africa's Lake Victoria (Pimm et al., 2014). Moreover, Boyce et al., (2022) suppose that more than two thirds of the marine species have either disappeared or are threatened with extinction (Nikolaou & Katsanevakis, 2023; Penn & Deutsch, 2022; Yarincik & O'Dor, 2005). These disturbances underline the urgent need for conservation measures to protect and preserve the biological richness of our marine ecosystems.

The term “Biodiversity” or “Biological diversity” is a vast term that goes beyond the narrow meaning that confines it to the number of species or varieties alone. It delves deeper, encompassing not just morphological variations but also the intricate tapestry of genomics. Beyond that, it encapsulates the relationships between species and the chronological evolution of living organisms (Grosberg et al., 2012; Katona et al., 2023).

1.1. Marine Ecosystems: Challenges and Preservation

Sustainable fisheries management is of global interest due to the rapid changes of aquatic ecosystems on a global scale. A considerable number of fish stocks have undergone notable declines in number as well as life cycle alterations as results of anthropogenic pressures, impacts of climate change, over-fishing and habitat destruction (Bezerra et al., 2019; Katona et al., 2023; Kумму et al., 2016; Lam et al., 2020; Leal Filho et al., 2019; Nikolaou & Katsanevakis, 2023). In the same vein, species extinction rates are currently estimated to be 10 to 1,000 times higher than they were in pre-industrial times (Ceballos et al., 2020; Pimm, 2021; Pimm et al., 2014), indicating a significant biodiversity loss. Consequently, this precipitous declines in biomass and species extinction is cited as the onset of the sixth mass extinction (Barnosky et al., 2011). It is therefore imperative that biodiversity resources are managed in sustainable manner in order to endure their

continued existence and availability for future generations. Fish are now among the most threatened vertebrates in the world, with 12-17% of species threatened (Dirzo et al., 2014; Nikolaou & Katsanevakis, 2023). Luypaert et al. (2020) estimated that marine abundance has declined by 38% between 1970 and 2007. In same line, McCauley et al., (2015) revealed that around 22% of marine vertebrates have declined in abundance during the past four decades, in which marine fishes have declined in aggregate by 38%, and certain baleen whales by 80 to 90%. Moreover, the number of marine species at risk of extinction has risen sharply, with 11% of marine species evaluated by the International Union for Conservation of Nature (IUCN) classified as critically endangered, endangered, or vulnerable; within specific marine groups, this percentage increases dramatically, with 31% of Actinopterygii and 18% of Chondrichthyes species considered threatened with extinction (Luypaert et al., 2020).

Overfishing in the North and Baltic Seas poses a clear threat to marine ecosystems and the sustainability of fisheries, leading to a domino effect of ecological and socio-economic consequences (Bluemel et al., 2022; Carruthers et al., 2014; ICES, 2022a; Jackson et al., 2001; Sendek & Bogdanov, 2019). Harvesting fish stocks beyond sustainable levels disrupts the balance of marine life, reduces biodiversity and affects trophic interactions (Bluemel et al., 2022; Carruthers et al., 2014; Sendek & Bogdanov, 2019). Many fish species in the North Sea suffer significant declines, which may lead to overpopulation of their prey, thus altering the ecological balance and biodiversity (ICES, 2022b, 2022a). From a structural standpoint, the North Sea cod and dab serve as quintessential and focal species for studying regime shifts, given their current state of decline resulting also from excessive fishing practices (Sguotti et al., 2019). Numerous studies reveal a significant fall in the spawning stock biomass (SSB) of Baltic cod from its historical peak to less than 100,000 tonnes in the early 1990s, as documented in Bagge et al. (1994). In recent years, Baltic cod has also suffered from unfavourable environmental conditions (Receveur et al., 2022). Therefore, the substantial levels of bycatch and the resultant discarding of flatfish species is a major issue, which is particularly pronounced for species such as dab and plaice, as highlighted in ICES (2023).

Addressing overfishing in these regions requires a comprehensive strategy: enforcing strict fishing quotas, introducing progressive fishery management methods, and promoting the recovery of dwindling stocks through protected marine zones (ICES, 2022c). Embracing cooperative,

multinational initiatives and integrating scientific insights into decision-making are crucial for effectively managing marine ecosystems and ensuring the longevity of the fishing industry.

1.2.Importance of monitoring biodiversity and managing fish stocks

It becomes clear that marine ecosystems require urgent action to ensure the sustainable use of their biological resources and the maintenance of the health of natural habitats. It is therefore imperative to amplify efforts to preserve and maintain marine diversity and its associated benefits (Frelat et al., 2018; Lam et al., 2020).

To address these challenges facing aquatic ecosystems and understanding the way in which the organisms interact with environmental stresses, and adapt management and fishing practices, the monitoring and the assessment of fish stocks are crucial tools (Groeneveld, 2000; Tesfaye & Getahun, 2021). The continuous monitoring and assessment of aquatic ecosystems allow the implementation of management measures to conserve and restore fish stocks, protect threatened species and maintain overall marine ecosystems health. Furthermore, an effective monitoring and assessment system also plays key role in crisis response to environmental disasters, by helping to assess the extent of damage and direct recovery efforts (Heiskanen et al., 2016), as well as by anticipating future problems and taking suitable preventive or mitigating measures.

Fish stock assessment is defined as the scientific process of collecting, analysing and communicating the status of fish stocks and estimating their sustainable productivity (Rodríguez-Mendoza & Saborido-Rey, 2023). It draws its conclusions from different types of data, including, but not restricted to, catches, landings, biological information recorded by on-board observers and fishery-independent data from research surveys (Heiskanen et al., 2016; Tesfaye & Getahun, 2021).

The assessment of fish stocks is therefore a scientific exercise of the first order (Rodríguez-Mendoza & Saborido-Rey, 2023). In addition to providing information on the abundance and distribution of different marine species, it helps researchers to better understand the evolution of marine ecosystems (Sparre & Venema, 1998). Stock assessment also reveals evolutionary trends, such as adaptation to new environmental conditions, the impact of climate change on marine species and the impact of introduced invasive species on ecosystem health and stability (Lam et al., 2020). Nevertheless, it is not exclusive to the scientific community, but is rather an area where

science converges directly with public policy. Data on the abundance and distribution of fish and other biological resources are essential for developing sustainable and equitable fisheries policies (Campling et al., 2012; Greenstreet, 2008; Orach & Schlüter, 2021). The relationship between science and policy in the field of fish stock assessment and monitoring is not unidirectional. Science itself benefits from the results of sustainable management of fisheries resources. For instance, the improvement in the health and stability of the fish stocks that results from the management measures put in place to preserve them makes it easier to assess these stocks. While the outputs and outcomes of fisheries assessment are often subject of heated debate and controversy, the monitoring and assessment of fisheries and marine resources remains extremely important and absolutely necessary for a proper management of marine resources and the development of effective public policies on fisheries and their conservation (Begg et al., 1999; Y. Chen et al., 2003; Leonart & Maynou, 2003; Vivekanandan, 2017).

1.3. Global assessment of fisheries resources

Since frontiers between nations do not restrict the movement of marine species, the conservation of natural resources and the assessment of fish stocks are becoming an international priority and necessitate concerted action at a global level (McQueen et al., 2020; Smith et al., 2011; Tesfaye & Getahun, 2021; Vivekanandan, 2017).

Over-exploitation, habitat degradation, global climate change and marine pollution are threatening marine ecosystems and their natural resources (Nikolaou & Katsanevakis, 2023; Vethaak et al., 1992; Webb & Mindel, 2015). As a result, a worldwide collective awareness has emerged of the seriousness of the current environmental situation, notably the significant and continuing decline in marine life in general and fish in particular, and an international awareness of the need to manage fishing sustainably and preserve marine biodiversity (Haas et al., 2019; Rodríguez-Mendoza & Saborido-Rey, 2023). This growing awareness has been translated into a series of measures at global level through the adaptation/conclusion of a number of multilateral and bilateral agreements, as well as the development of an extensive series of reform measures and the adoption of a precautionary approach, e.g., International Convention for the Regulation of Whaling, 1946 (Iliff, 2008; Jordan, 2011; Kobayashi, 2005; Maffei, 1997; Martin & Brennan, 1988; Rose & Crane, 2014; Spencer, 1991); Committee on Fisheries (COFI), 1965 (Aguilar-Manjarrez, 2018; Sainsbury, 2009; Session, 2005; Sullivan & Lem, 2017; Swan & Satia, 2001); Agreement on the Conservation

of Albatrosses and Petrels (ACAP), 2001 (Beer, 2011; COOPER et al., 2006; Cooper & Ryan, 2001; Phillips et al., 2016; Wolfaardt et al., 2010); Convention on Biological Diversity (CBD) - informally called Biodiversity Convention, 1993 (Chandra & Idrisova, 2011; De Oliveira et al., 2011; Diversity, 2001; Dobson, 2005; Essl et al., 2020); United Nations Fish Stocks Agreement (UNFSA), 1995 (Balton & Koehler, 2010; W. C. Burns, 2006; W. C. G. Burns, 2008; Munro, 2000).

An important measure is the adoption of the Sustainable Development Goals (SDGs) in 2015. Especially Goal 14 which concerns the protection of the marine environment and its resources and ensuring their sustainability, more specifically, it aims to establish rules to "preserve the oceans, seas and aquatic ecosystems and the sustainable use of their resources". Sustainable fisheries management, an end to overfishing, the restoration of fish stocks and the elimination of harmful subsidies to fishing are specific sub-objectives (Biermann et al., 2017; Haas et al., 2019; Kirkfeldt & Frazão Santos, 2021; Rickels et al., 2019). In view of the central role of the United Nations in raising collective awareness, the United Nations Convention on the Law of the Sea (UNCLOS), or the so-called "Constitution of the Oceans", in conjunction with the United Nations Fish Stocks Agreement, has been instrumental in fostering a collective understanding of the necessity to monitor and evaluate marine resources (Nordquist, 2011; UN General Assembly, 1982).

In the same spirit, and to complement the roles of the United Nations, the Food and Agriculture Organization of the United Nations (FAO) is actively involved in the conservation of fisheries resources and the assessment of fish stocks. This is done via a range of regulatory programs and agreements, such as the 1995 "Code of Conduct for Responsible Fisheries" (FAO, 1995). FAO also seeks to enshrine the importance of fisheries monitoring by publishing reports on the "State of World Fisheries and Aquaculture", which provide a detailed analysis of the state of fish stocks and fishing effort around the world (FAO, 2000; Stankus, 2021). FAO and other UN organizations consider stock assessment to be an essential component of efforts to achieve Goal 14 of the United Nations Sustainable Development Goals and the Code of Conduct for Responsible Fisheries. They consider it to be the most effective tool for evaluating current efforts and determining their effectiveness in achieving sustainable fisheries, or for reconsidering and evaluating them, if necessary, in line with established objectives. Furthermore, the United Nations Environment Program (UNEP) has encouraged the creation of marine protected areas (MPAs), which, if properly

designed and managed, can be essential for preserving fish breeding habitats and enabling the recovery of overexploited fish stocks (Desai, 2017; Gray, 1990; Petsonk, 1989).

These efforts are not limited at the global international organization level, but are also undertaken at the regional level by organizations such as the Indian Ocean Tuna Commission (IOTC) and the International Council for the Exploration of the Seas (ICES), which contribute to the monitoring, assessment and management of fish stocks in their regions, complementing international efforts (Andersens, 2009; Edeson, 2007; ICES, 2020; Sinan et al., 2021).

1.4. Assessment of fisheries resources in the European Union

European countries have played and continue to play an important role in international and global initiatives to conserve fisheries resources and assess fish stocks. The European Union (EU) is a key stakeholder and official supporter of the implementation of the United Nations Fish Stocks Convention, which aims to ensure the conservation and sustainable management of trans-boundary migratory fish and migratory fish stocks (Rickels et al., 2019). Moreover, the EU has established its own fisheries policy, the Common Fisheries Policy (CFP), focusing on sustainable fisheries and based on accurate assessments of fish stocks, as stipulated in Regulation (EU) No 1380/2013. This is based on fisheries data provided by member states. According to the CFP, member states are required to provide information on the state of marine resources, the impact of fishing on these resources and ecosystems, and the economic health of the fishing and aquaculture sectors. To centralize these data, the EU has set up the EU Data Collection Framework (DCF) as defined in Regulation (EU) 2017/1004 and Delegated Decision (EU) 2021/1167, which launches a multi-annual program for the collection and management of these data in the fisheries and aquaculture sectors from 2022.

Pursuant to EU Regulation No 508/2014 Article 21 and Commission Executive Decision (EU) 2021/1168, a list of 51 mandatory research surveys has been specified, covering the Baltic Sea, the North Sea, the Eastern Arctic, the North Atlantic Ocean, the Mediterranean Sea and the Black Sea. These include the Baltic International Trawl Survey (BITS-Q1) in February-March and BITS-Q4 in November. The two demersal BITS trawl surveys mentioned above are coordinated by ICES' WGBIFS, and aim to monitor the spatial distribution and age-class abundance of demersal species, namely Atlantic cod (*Gadus morhua*), European flounder (*Platichthys flesus*), other flatfish (plaice

- *Pleuronectes platessa*, turbot - *Scophthalmus maximus*, Common dab - Limanda limanda , brill - *Scophthalmus rhombus*) and to some extent clupeids (European sprat - *Sprattus sprattus* and Atlantic herring - *Clupea harengus*) in the Baltic Sea. In the North Sea, the International Bottom Trawl Survey (IBTS) is charged to monitor and assess the stocks. It is coordinated by the International Bottom Trawl Survey Working Group (IBTSWG) in ICES, and it mainly aims to monitor the fish abundance and biomass of demersal species such as Atlantic cod (*Gadus morhua*), European flounder (*Platichthys flesus*) and other fish in the North Sea at different times of the year (quarter 1 and 3).

1.5. Assessment methods and tools for fish stocks

The need to understand ecosystems, track species and monitor their deterioration and dynamics relies on reliable and reproducible methods that gather as much qualitative and quantitative information as possible. The reference methods adopted by international institutions are mainly based on the visual identification of fish, which requires sound taxonomic knowledge, and depends on long hours in the field to collect enough data (Marandel et al., 2016; Rodríguez-Mendoza & Saborido-Rey, 2023). These methods vary according to the nature of the environment studied and the organisms that inhabit the area. These methods are divided into:

1.5.1. Fisheries-based methods

Fishers and local communities possess invaluable spatial knowledge, offering unique insights into the condition of fish stocks. Data derived from both commercial and recreational fishing, gathered through reports or sea-based sampling, as well as observations on docks and within fishing communities, is instrumental. Primarily relying on commercial data, catch and effort metrics (including the number of trips, duration at sea, exclusive capacity, and more) are utilized to estimate survival and mortality rates, which subsequently inform assessment models (Cotter et al., 2004). "Catch data" pertains to the information directly provided by fishers, detailing the species, quantity, location, and timing of their catches. These data are crucial for tracking the shifts in fish populations over time (Armstrong et al., 1987; Heiskanen et al., 2016; ICES, 2022c; Marandel et al., 2016). However, these data are intrinsically biased due to the reaction of fishers to alterations (like weather, commercial reasons, new rules... etc).

1.5.2. Fishery-Independent Data Sources

Data derived from fishery-independent sources pertains to research conducted externally from the realm of standard fisheries. This type of research typically takes the form of surveys, designed according to strict protocols to ensure randomized and reproducible data collection. As the name suggests, fishery-independent data arises from means completely unconnected to any fishing activities. The methodologies and equipment used to gather this data are diverse: trawls, hydroacoustics, underwater video footage, and autonomous underwater vehicles (AUVs) (Liu et al., 2023; Meyer et al., 2019), and biological samples (Aguzzi et al., 2020; Heiskanen et al., 2016; ICES, 2020; Jourdain et al., 2020; Schmitt et al., 2002; van Helmond et al., 2020). These biological samples often encapsulate, within others, age-, size- and sex-composition as well as extrapolations on survival rates, all of which significantly contribute to fish stock models.

Marine surveys stand out as one of the primary tools for fisheries assessment. To extract meaningful information, data collected through these surveys must be rigorously analysed with fish assessment models. These mathematical tools estimate fish stock sizes and track their trajectories over time. Importantly, fish stock evaluations aren't solely dependent on scientific survey data; they often incorporate a blend of data, including both admissible and commercial sources (Hilborn & Walters, 1992; Höhne et al., 2023). Fish stock models offer evaluations of current fish stock health and advise on sustainable fishing limits. Models vary in complexity, from straightforward overproduction models to intricate integrated assessment models that ingest a plethora of data sources (Maunder & Punt, 2013; Pons et al., 2020).

It's worth mentioning that the scope of these surveys extends beyond just fisheries assessment. They also address the imperative to comprehend varying ecosystem facets - like hydrography, seawater and sediment chemistry, benthic fauna, zooplankton, and phytoplankton. Furthermore, they delve into areas like marine litter, as highlighted by ICES (ICES, 2020, 2022c; Rodríguez-Mendoza & Saborido-Rey, 2023).

1.6. Shortcomings of conventional assessment methods

Traditional approaches to assessing the status of fish stocks, often based on scientific research, have made limited progress and now show clear shortcomings (Maunder & Piner, 2015). In

particular, they are characterized by prohibitive financial costs, complicated logistics, spatially and temporally widely distributed data, and significant delays in processing and analysing the collected information (Cadrin, 2020).

Two major problems are associated with these traditional methods:

- Firstly, the mismatch between the scale of biological populations and appropriate management units, which seriously compromises the sustainability of resources and hinders the achievement (Ouréns et al., 2015; Reiss et al., 2009; Reissmann et al., 2009).
- Secondly, errors in estimating fish intake and other factors needed to manage the population, due to the scarcity of data (Maunder & Piner, 2015; Punt et al., 2021).

The use of bottom trawling can cause physical damage to the seabed and benthic habitats (Golpour et al., 2022; McLaverty et al., 2021; Viana et al., 2021). Hence it can alter the structure of habitats, affecting the distribution and behaviour of fish species and other benthic organisms. Such changes may affect the accuracy of stock assessments, as changes in habitat can lead to changes in the abundance and distribution of fish (McLaverty et al., 2021). In addition, trawl fishing is not suitable for catching all species of fish, because its success varies according to the species targeted (Rodríguez-Mendoza & Saborido-Rey, 2023). Trawls are generally considered to be an ineffective means of monitoring in both rocky and muddy coastal areas, thereby limiting their effectiveness (Day et al., 2020; Hixon & Tissot, 2007; McLaverty et al., 2023; Wedemeyer et al., 1983). There, the main sources of information are divers, recreational or commercial fishers. Furthermore, in these areas, periodic monitoring is practically impossible due to oceanographic conditions and the availability of divers or fishers. Though modern technological facilities have allowed the exploration of depths in excess of 50 meters, there are still obstacles in the way of these non-invasive techniques, such as the limit of vision at depth and the importance of the clarity of the water (Campanella & Taylor, 2016; Connolly et al., 2021; D. Zhang et al., 2022). Another outstanding issue is the extent of the impact of light pollution emanating from cameras on the animal habitat and the possibility to monitor it. As far as acoustic systems are concerned, although they have overcome the aforementioned obstacles, they are still not always able to distinguish between species, especially in multispecies aggregations of similar sizes. In addition, differences in target strength can have a negative impact on quantitative estimates. The uncertainty surrounding these parameters therefore significantly affects the outcome of fish stock assessments and

subsequent scientific advice on the state of exploited resources and ecosystems (Bayracki & Klingelhofer, 2024; Minello et al., 2021; Sato et al., 2021; Todd et al., 2015).

1.7.Environmental DNA

The rapid technological advances in high-throughput sequencing and bioinformatics have raised questions about the ongoing suitability of invasive methods for biodiversity and stock assessment of single species in aquatic habitats and about the extent to which newly developed genomic methods can provide useful inputs to improve the cost-effectiveness of conventional methods while increasing the speed and accuracy of fish stock assessments.

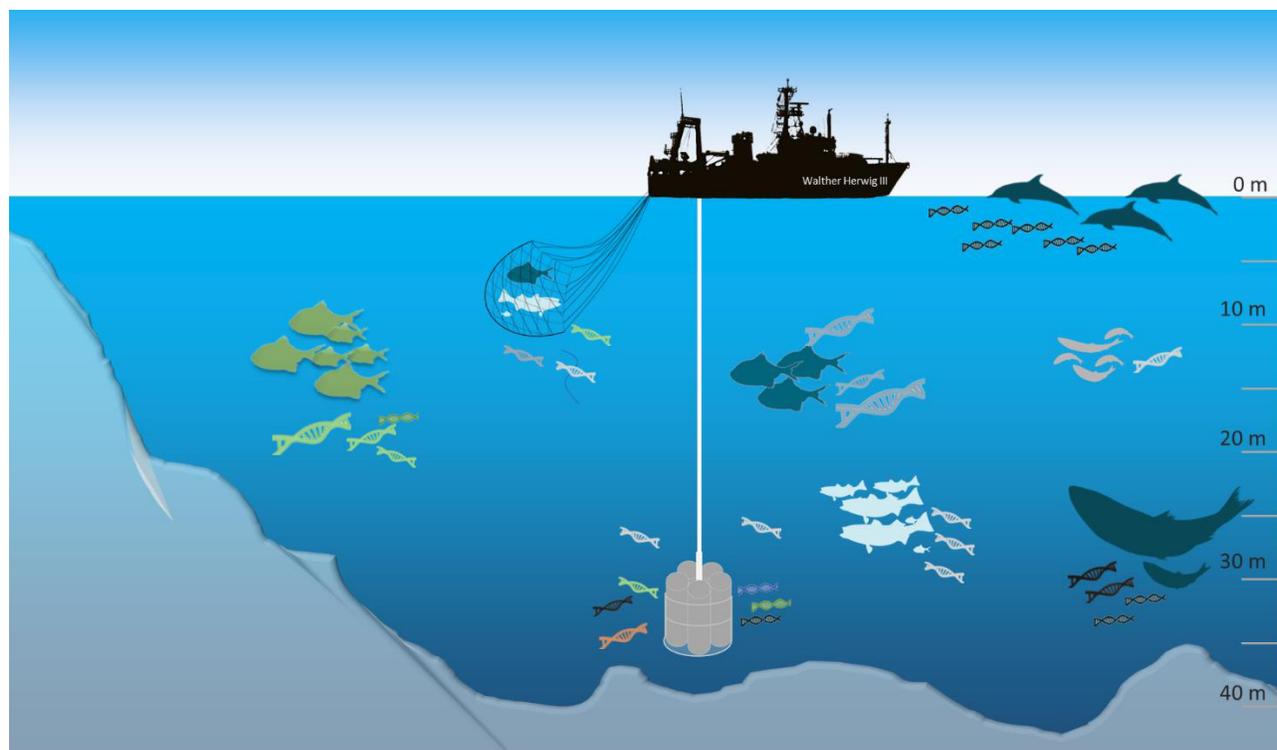


Figure 1-1: eDNA availability in the aquatic ecosystems.

Assuming that all organisms “contaminate” their environment with their DNA via the contact between water and the shed skin and scales, excretion, reproductive material, tissues decomposing, secretions and ingested food (Andersen et al., 2012; Barnes et al., 2021; Bienert et al., 2012; Willerslev et al., 2003), it should be possible to collect this DNA, to amplify it via PCR barcode tags and to sequence subsequent amplicons, using NGS, which is the so-called environmental DNA (eDNA). It is therefore considered a non-invasive method. Given that eDNA is collected from

aquatic or environmental sources, it is possible to identify the species that have lived in the studied area over a period of time (Figure 1-1).

1.7.1. eDNA: Terminology and Historical Overview

Environmental DNA (eDNA) is DNA extracted from an environmental sample (e.g. soil, water, air), and can be extracellular (exDNA) or intracellular (inDNA) (Alawi et al., 2014; Barnes & Turner, 2016; Pawlowski et al., 2020; Rodriguez-Ezpeleta et al., 2020). exDNA (environmental extracellular DNA) refers to the extracellular genetic material that organisms release into the environment. It is made up of DNA fragments from different sources. inDNA represents the DNA preserved inside a cell. But scientists are still debating the terminology of eDNA' keywords (Pawlowski et al., 2020, 2021; Rodriguez-Ezpeleta et al., 2021). A major concern is whether eDNA includes both macro-organisms and micro-organisms, or whether it is specific to macro-organisms that are not entirely embedded in the sample. In the same line: does eDNA contain DNA from both intracellular and extracellular. Thus, some suggest that eDNA should refer to any DNA extracted from an environmental matrix. (Pawlowski et al., 2020; Rodriguez-Ezpeleta et al., 2020). In this thesis, we used eDNA to refer to any DNA being extracted from an environmental matrix.

One of the most important characteristics of eDNA analysis is that it is a non-invasive tool, which satisfies the requirements of sustainability in monitoring and assessment, particularly in programs primarily aiming at protecting marine resources in MPAs, as well as contributing to the development of non-invasive solutions for preserving biodiversity in general and fish in particular (Ardura & Planes, 2017; Barnes et al., 2021; Mathon et al., 2022; Specchia et al., 2022). eDNA analysis provides a more comprehensive assessment of biodiversity in the surveyed area, allowing the detection of several species simultaneously from a single sample without the requirement for continuous iteration (Andruszkiewicz, Starks, et al., 2017; Cilleros et al., 2019; Closek et al., 2019; Jerde et al., 2011).

The earliest documented identification of DNA in seawater can be attributed to Holm-Hansen (1969) and Holm-Hansen et al. (1968), who demonstrated a correlation between the amount of isolated DNA and the depth of water sampling. We assume that this work is the first work signalling the importance of studying eDNA in marine environments and highlighting its potentialities. It has inspired subsequent research on a variety of questions, mainly focusing on the detection of species.

The complexity of the questions posed evolved in tandem with the development of molecular techniques over time. The concept of eDNA as an environmental tool was first employed in microbiology by Paul & Carlson (1984) and Paul & Myers (1982) to demonstrate the utility of DNA analysis in revealing bacterial diversity in water environments and signalling the presence of a correlation between DNA extracted from environmental matrix and the amount of bacteria. However, many published studies refer to Ogram et al. (1987), who presented a method to extract intra- and extra-cellular DNA from sediment as the first work done on eDNA (Bandara et al., 2021; Barnes & Turner, 2016; Bohmann et al., 2014; Cristescu & Hebert, 2018; Pawlowski et al., 2020, 2022; Taberlet et al., 2018; Thomsen & Willerslev, 2015).

Probably the first nekton related eDNA study was published by Amos et al. (1992) to detect species such as the humpback whale (*Megaptera novaeangliae*) and sperm whale (*Physeter macrocephalus*). The term “eDNA” was established by Suzuki et al. (1997), who investigated bacterial biodiversity in seawater. However, the specific application of the term “environmental DNA” for macro-organisms or metazoan in aquatic biology was introduced in 2008 by Ficetola et al. (2008), who used it to detect tadpole diversity in a natural alpine spring. This terminology was further popularized by Jerde et al. (2011).

1.7.2. eDNA: Dynamics of Persistence and Degradation

One of the potential advantages of eDNA biodiversity monitoring is its temporally integrative nature, given the average half-life of DNA of up to 24 hours in water (Collins et al., 2018; Lance et al., 2017; MBARI et al., 2021), e.g. compared to average trawl times of 15 and 30 minutes in many fisheries surveys (ICES, 2020; Jourdain et al., 2020). eDNA has an exponential decay in copies per litre of seawater over time, seasons and species (Collins et al., 2018), however this rate could be influenced by various environmental and ecological parameters, including water temperature and salinity (Andruszkiewicz, Sassoubre, et al., 2017; Collins et al., 2018; Dejean et al., 2011; Merkes et al., 2014; Salter, 2018). Barnes et al. (2014) and Maruyama et al. (2014) demonstrated that eDNA from fish in ambient water has a relatively slow degradation rate of 10% per hour and 90% per day, allowing for a longer-term view of fish diversity compared to short-duration bottom trawl surveys. Govindarajan et al. (2021) found that eDNA-derived fish diversity estimates remain stable for up to 16 hours, making them a sensitive and robust monitoring tool. This highlights the advantage of eDNA in providing meaningful information and a comprehensive

overview of species richness integrating longer timeframes compared to fisheries-based reference methods.

1.7.3. eDNA, Water Currents, and Their Effects on Dispersal

Important aspects in interpreting eDNA outputs for stock assessment are the persistence of eDNA in the water column and the influence of hydrodynamic characteristics, such as currents or sedimentation rates, on eDNA dispersal. Some methods have been developed to relate observed eDNA levels to the specific location and timing of eDNA origin (Deiner et al., 2016; Jerde et al., 2016; Sansom & Sassoubre, 2017; Wilcox et al., 2013). In this sense, the work conducted by Sansom and Sassoubre (2017) introduced an adaptation of a one-dimensional plug flow reactor that considers a primary decay constant. Other models in freshwater environments consider factors such as sedimentation rates and the rate at which organisms release eDNA (Carraro et al., 2023; Wilcox et al., 2013). Another study in Monterey Bay found that eDNA is transported over tens of kilometres southwards, however this transport is related to the rate of DNA degradation in the sea (Andruszkiewicz et al., 2019; Andruszkiewicz, Starks, et al., 2017; Wood et al., 2021).

1.7.4. Key Genetic Markers in eDNA Analysis

The purpose of the eDNA studies is either to (i) qualitatively detect marine biodiversity in terms the presence/absence of species or even populations (Andruszkiewicz, Starks, et al., 2017); or (ii) quantitatively estimate the abundance of a particular population or species in a given area (Karlsson et al., 2021). To answer each of these two questions, two key methods are applied: metabarcoding via Next Generation Sequencing (NGS) or quantitative PCR (qPCR).

Most eDNA studies target mitochondrial DNA fragments or genes because of their large copy number per cell compared to genomic DNA or genomic genes (Ragot & Villemur, 2022). In this regard, a reasonable proportion of the scientific work published on eDNA focused on the development and selection of new primers capable of distinguishing between marine species. The genes used for primer development include 12S, COI, CytB, 16S and 18S (Table 1-1) (Arif & Khan, 2009; Milan et al., 2020) . Recent studies found that primers targeting the 12S rRNA gene generally detected a greater diversity of fish than those targeting the 16S rRNA or COI genes, while primers targeting the cytochrome b gene were least effective (Polanco F. et al., 2021; S. Zhang et al., 2020).

Table 1-1: eDNA primers specifically developed for targeting fish diversity

Primer	Target gene	Target species	References
12SF1/R1	12S	Vertebrata	(Coissac et al., 2012)
12SV5	12S	Vertebrata	(Coissac et al., 2012)
Ac12S	12S	Actinopterygii	(Evans et al., 2016)
MiFish-U	12S	Teleostei	(Miya et al., 2015)
Tele02	12S	Teleostei	(Taberlet et al., 2018)
Teleo	12S	Teleostei	(Valentini et al., 2016)
Ac16S	16S	Actinopterygii	(Evans & Lamberti, 2018)
Fish16s/D16s2	16S	Fish	(Berry et al., 2017)
L2513/H2714	16S	Vertebrata	(Kitano et al., 2007)
Ve16S	16S	Vertebrata	(Evans & Lamberti, 2018)
Vert-16S	16S	Vertebrata	(Vences et al., 2016)
Fish_18S	18S	Fish	(MacDonald et al., 2014)
coi.175f/coi.226r	COI	Fish	(Collins et al., 2019)
FishF1/2	COI	Fish	(Ward et al., 2005)
PS1-F/R	COI	Fish	(Shu et al., 2021)
Fish2bCBR/CBL	Cytb	Fish	(Thomsen et al., 2012)
Fish2degCBL/CBR	Cytb	Fish	(Thomsen et al., 2012)
FishCBL/CBR	Cytb	Fish	(Thomsen et al., 2012)

Over the past years, the outcomes of investigations on the best target gene varied (Evans & Lamberti, 2018; Polanco F. et al., 2021; Shu et al., 2021; Valsecchi et al., 2019; S. Zhang et al., 2020). Most of these studies were characterized by being confined to single species or families, while studies that targeted multiple species showed their inability to distinguish between closely related species. For example, Mifish primers (Miya et al., 2020) are considered ineffective for distinguishing between certain genera of sailfishes (Istiophoridae) and Hairyfish (Mirapinnidae), as the difference between the species does not exceed at best three mutations in a 200-nucleotide sequence. Incomplete DNA reference datasets are also still affecting the accuracy of eDNA-analyses and limit the degree of confidence in certain universal primers (Claver et al., 2021). This has stimulated the search for new genetic and molecular markers, proposing functional genes and mitochondrial genes such as COI and 12S rRNA in combination with D-Loop, 16S or CytB

(Garrido-Sanz et al., 2016). Such combinations increase specificity and offer a solution to the problem of discriminating between closely related species (Yoshitake et al., 2019, 2021, 2021; Zanolello et al., 2023). In many cases, one or more ribosomal RNA regions are selected prior to sequencing (16S or 18S rRNA for prokaryotes/archaea and 12S, COI, ITS, 23S or 28S rRNA for eukaryotes) so that most of the community composition can be characterized and quantified (Zhang et al., 2020).

1.7.5. eDNA and its application for detecting marine fauna

Since the beginning of the last decade, the use of eDNA has become increasingly popular for the detection of fisheries resources (Foote et al., 2012). Several studies have demonstrated the potential of eDNA to reveal fish richness in freshwater and marine habitats with similar results as compared to trawl fisheries (Feng et al., 2023; Stoeckle et al., 2021; Maiello et al., 2022). Mathon et al. (2022) detected 16% more species using eDNA than visual underwater surveys in the central and south-west Pacific and the western Indian Ocean by analysing more than 1,224 stations. Zou et al. (2020) demonstrated that eDNA was able to show the presence of 32% more of species than trawl methods in coastal wetlands of the Pearl River Estuary. Wurz et al. (2021) found that for marine biodiversity assessments eDNA analyses reveal results comparable to those of trawl catches in the same area.

In the framework of NATURA2000 project, the application of the eDNA metabarcoding to assess the fish biodiversity in Mediterranean “*Aquatina di Frigole*” Lagoon has shown promising results. The researchers were enabled to reveal the a large amount of valuable biodiversity data in the studied area using a low sampling effort and rapid laboratory practices which highlighted the potential of eDNA in diverse environments but also the limits of applications due mainly to the lack of specific primers for certain species or absence of reference data (Specchia et al., 2022).

One controversially discussed aspect is the capacity and effectiveness of eDNA methods for surveillance and monitoring of rare and endangered species. Boussarie et al. (2018) used eDNA metabarcoding to detect shark species that are no longer detected by traditional surveys in habitats where they formerly occurred in New Caledonia, demonstrating the ability of eDNA to detect rare species. Zhang et al. (2023) used eDNA to detect the presence of an individual Bryde’s whale (*Balaenoptera edeni edeni*), nicknamed Xiaobu, in Dapeng Bay in China and tracked its traces throughout the coastal region. Furthermore, Feng et al. (2023) could proof the presence of the

endangered Naked schizothoracin (*Oxygymnocypris stewartii*) and two non-native Asian swamp eel and Mandarin fish (*Monopterus albus* and *Siniperca chuatsi*) in the Yarlung Zangbo River. On the other hand, other authors proofed the current methods still ineffective to reveal the presence of rare species (Bessey et al., 2020), e.g. with only 13% of samples tested positive in a study aimed to detect and monitor the threatened marine cryptic Red handfish (*Thymichthys politus*) using eDNA (Bessell et al., 2023). One reason for conflicting experiences in the use of eDNA for the detection of rare fish species could be habitat-specific differences in environmental parameters influencing the detection limits and, as a consequence, the sensitivity of the method (Hatzenbuehler et al., 2017). Therefore, the interpretation of presence/absence and relative abundance data requires caution unless metabarcoding methods are optimized for precision and accuracy (Evans et al., 2017).

The determination of the proportion of mixing and originality of stocks are crucial parameters for the optimal stock assessment and management (McGill et al., 2023; Weist et al., 2019). Yoshitake et al. (2019) put forth a novel approach, entitled "haplotype count from eDNA by sequencing" (HaCeD-Seq), which sought to ascertain the haplotype diversity of a given species from eDNA. The method has been demonstrated to be applicable to the European eel and tuna populations (Halvorsen et al., 2023; Yoshitake et al., 2019, 2021, 2023). These findings have the potential to significantly alter the utility of eDNA by enabling the estimation of population size using the HaCeD-Seq method, obviating the need for direct capture (Andres et al., 2023). Azarian et al. (2021) proposed an analytical framework for the detection of genetic differences among populations, utilizing the AMOVA test to estimate haplotypes from eDNA. In a recent study, conducted by Wakimura et al. (2023), the potential of eDNA as a tool for evaluating population structure and determining the different haplotypes of the Japanese freshwater gudgeon *Gnathopogon caerulescens* was investigated. This was achieved by analysing the number of reads for each Operating Taxon Unity (OTU) and Amplicon Sequence Variant (ASV). These results illustrate that eDNA haplotyping can precisely ascertain the genetic diversity of a whale shark population, offering a minimally invasive alternative to traditional tissue biopsy methods for the collection of genetic data essential to the conservation and management of marine megafauna.

A recent study revealed the possibility of examining methylation in eDNA to predict certain important parameters such as age and population structure (Zaiko et al., 2018; Zhao, B, 2023). Therefore, further studies are needed to explore this hidden face of eDNA.

1.7.6. eDNA for fish abundance estimation

eDNA is increasingly used to assess fish abundance quantitatively using species-specific method (Dickie et al., 2018; Jerde, 2019; Jerde et al., 2011; Lacoursière-Roussel et al., 2016). Most studies using eDNA for quantitative assessments were mainly targeting economically important species such as Atlantic cod (*Gadus morhua*), Atlantic mackerel (*Scomber scombrus*) and common sole (*Solea solea*) or threatened species (Jourdain et al., 2020).

Knudsen et al. (2019) demonstrated the potential of eDNA to provide accurate quantitative information via a quantitative polymerase chain reaction (qPCR) assay. However, the designed assay is characterized by a high limit of quantification, more than 665 DNA copies per reaction. Furthermore, Salter et al. (2019) showed significantly positive correlations between regional integrals of Atlantic cod biomass (in kg) and eDNA quantities (copy numbers), in which the R² was in order of 0.79 with an error (p) of 0.003. These findings suggest that eDNA has the potential to represent the biomass and abundance of fish species under certain conditions, thereby enhancing its role as a complementary tool for monitoring fish stocks.

At the metabarcoding level, a positive relationship was found between the number of eDNA reads (read = NGS DNA copies) per Miseq and the biomass of whitespotted char (*Salvelinus leucomaenis*) (Nakagawa et al., 2022). The biomass of Chinese minnow (*Rhynchocypris oxycephalus*), Japanese fluvial sculpin (*Cottus pollux*) and masu salmon *Oncorhynchus masou* were positivity correlated with eDNA read to the biomass according to an GLM model that included two variables eDNA concentration and water temperature. On the other hand, Skelton et al. (2022) demonstrated a strong correlation between the number of reads and fish abundance, while the detection power for non-dominant species was low.

1.7.7. The limitations of eDNA

Behind every technology there are advantages and disadvantages, and eDNA is no exception. However, its limitations do not depend directly on the method itself, but are largely due to

constraints in obtaining the necessary components to complete the complex ecological puzzle. It is therefore essential to adopt a holistic approach to understanding the role of diverse environmental factors and biological interactions on the bioavailability of eDNA in ecosystems, to fully exploit the potential of eDNA in biodiversity studies and conservation efforts.

One of the major limitations of eDNA is the lack of genetic reference information for many marine species, whose mitochondrial genomes are either unavailable in public databases or have not yet been sequenced (Li et al., 2022; Zaiko et al., 2018). Of the more of 35,700 known fish species worldwide, until Oct. 1st, 2024 (Froese et al., 2019), databases such as Mitofish currently include complete mitochondrial genomes for only around 4,211 species (Zhu et al., 2023). This emphasises the substantial deficit in genetic data available for a considerable number of fish species. Furthermore, in 2020, among more than 1800 taxa of marine macrofauna species, including more than 220 known fish in the North Sea, only 50% of species have their COI genes available in public databases, and this number drops to 36% for 18S (Hestetun et al., 2020). This issue is particularly pertinent with regard to other genes, such as the 12S gene, despite the efforts undertaken by the scientific community as well as local and regional support programs for the protection and conservation of biodiversity to develop curated and complete databases. Therefore, more efforts are required to complete reference databases to a point that would make metabarcoding truly reliable for biodiversity assessments.

Incomplete reference sequences also result in an increase in the percentage of unknown reads during metabarcoding and render qPCR analyses questionable whenever results are obtained if they contain false positives, notably because it is impossible to validate primers and probes on all known species.

The output of any NGS machine run, whether Nanopore, Miseq or Novaseq, can generate millions to trillions of reads and a few gigabits to terabits of data. This represents an enormous mine of information and an inexhaustible variety of sequences, but it is useless for obtaining quantitative (absolute) information comparable with reference methods for assessing fish stocks, and so its use is limited to the qualitative side (presence/absence). With the exception of certain studies, these data are not even used for the investigation of sub-population and genotyping (Holman et al., 2019; Uchii et al., 2016). This is due to the lack of appropriate computational methods and pipelines to use them.

1.8. The North Sea and Baltic ecosystems

The North Sea is an epicontinental sea, which means that its depth is relatively shallow compared with other marine areas. It reaches an average depth of only 30 to 40 meters at its southern end, while in the north it can reach 60 to 90 meters.

The North Sea is home to a wide range of species, with more than 230 varieties of fish, in which the most economically significant species are Atlantic cod (*Gadus morhua*), Common dab (*Limanda limanda*), Atlantic herring (*Clupea harengus*), plaice (*Pleuronectes platessa*), Atlantic mackerel (*Scomber scombrus*) and common sole (*Solea solea*) (ICES, 2022c). It is also including various marine mammals, such as seals, porpoises and, occasionally, whales. In 2022, more than 6600 fishing vessels harvested almost 2 million tons of fish in the North Sea (ICES, 2022b). But the region has experienced fluctuations in fishing activity, notably a significant decline in cod stocks during the 1980s and 1990s (ICES, 2022b). In response to these challenges, multilateral approaches, supported by the International Council for the Exploration of the Sea, have been adopted. Natura 2000 has also set up protected areas to conserve certain habitats.

Effective monitoring tools to assess the state of the fishery resources are vital for sustainable fisheries. But technical and logistical challenges persist, especially in shallower coastal areas where access is difficult. These areas remain insufficiently monitored, since the current technology is limited. Any ship-based trawling activities are more complicated in the presence of coastal structures, like sandbanks, reefs and rock formations and pose a risk of increased mortality of non-target species as well as damage and disruption of fragile ecosystems. The diversity of the seabed, from muddy areas to rocky regions, makes conventional trawling methods imperfect, omitting or under-representing certain habitats in sampling.

2. Aims and objective of the thesis:

2.1. Preview of thesis contributions

The aim of this thesis was:

1. to assess the ability of eDNA to provide accurate qualitative information on the diversity of fish in the North and Baltic Seas compared to fisheries-based reference methods.
2. to evaluate and develop quantitative models capable of forecasting the biomass of the main fish targets from eDNA data.
3. to discuss possible scenarios of implementing eDNA-based assessment tools in marine monitoring.

2.2. Context and Outlines of the Thesis

This thesis was carried out within the frame of the EU-funded international research project FishGenome (Improving the cost-efficiency of fisheries research surveys and fish stocks assessment using next-generation genetic sequencing methods) in response to the EC call for tenders EASME/EMFF/2018/015, which aimed at providing scientific advice on improving cost-efficiency of fisheries research and fish stock assessments using next generation sequencing methods.

To ensure a sustainable use of marine natural resources requires continuous assessments. Classical techniques for monitoring fish stocks have seen limited advancements and come with certain drawbacks. These include significant financial burdens, intricate logistical requirements, limited spatiotemporal data, and a prolonged data analysis phase. The rapid technological progress in high-throughput sequencing and bioinformatics has raised questions about the necessity of continuing with invasive methods for biodiversity and single species stock assessments in aquatic habitats. In this context, this thesis concentrates on evaluating the performance of eDNA to provide qualitative and quantitative information to assess fish stocks in the North Sea.

These research questions were addressed in five chapters, as described below:

1. Mare-MAGE curated reference database of fish mitochondrial genes (published).

eDNA has revolutionized marine biodiversity research by enabling the analysis of DNA fragments in water samples. However, optimizing this technique requires a comprehensive genetic database for accurate species identification. Public databases, while covering many species, often contain inaccuracies and outdated names. Non-reference sequences may include misclassifications, and the slow performance of Blast algorithms with numerous non-target species increases the risk of false annotations. Streamlining the sequence pool for matching could save time and resources, reducing the likelihood of false positive annotations related to non-fish phyla.

In this respect, the objective of this paper was the development of a fish-specific database that includes the most frequently used genes, i.e., 12S and COI, which have undergone a specific processing to eliminate false annotated sequences, as well as a taxonomical revision. The development of this work was inspired by the SILVA database (Quast et al., 2013) targeting microbial genome protocol.

2. Comparative analysis of bottom trawl and nanopore sequencing in fish biodiversity assessment: the Sylt outer reef example (submitted)

Throughout history, surveys of marine fauna have been carried out using physical methods such as bottom trawling. However, with the advent of modern genetic sequencing technologies, innovative methods such as eDNA analysis have emerged. In particular, third-generation sequencers, such as Nanopore, promise to be cost-effective compared with second-generation sequencers, as well as allowing the sequencing of long reads of up to 20 kb with an optimum of 2 kb, making them a solution for overcoming the constraints of short-read sequencers.

This raises the question: how do these modern long reads techniques compare with traditional inventory methods? How do species inventories based on eDNA, using third-generation sequencers such as Nanopore, collected from the seabed compare with reference methods such as bottom trawling?

3. Fish diversity and semi-quantitative stock assessment through metabarcoding of environmental DNA in the North Sea (submitted).

Marine biodiversity is currently undergoing a significant reduction in both quality and quantity, a phenomenon often described as the sixth mass extinction. Faced with this ecological emergency/disaster, it is imperative to implement measures on a global and regional scale. One of the predominant strategies for conserving this biodiversity is based on oceanographic studies and missions, which seek to better understand and exploit these vitally important resources. However, conventional methods have shown certain technical limitations. At the same time, developments in genetic techniques are raising questions about their ability to provide reliable, non-invasive data on marine biodiversity.

In this respect, the study targeted two major questions:

- Can eDNA-based tools provide a highly accurate picture of marine diversity?
- Is it possible to develop a computer model capable of providing quantitative or semi-quantitative information about the main key fish species from NGS data?

4. Atlantic cod (*Gadus morhua*) assessment approaches in the North and Baltic Sea: a comparison of environmental DNA analysis versus bottom trawl sampling (published).

In fisheries management, accurate monitoring of stocks is crucial for sustainable exploitation, whereas quantitative analysis of eDNA from water samples may provide an appropriate alternative to traditional assessment methods. In particular, Atlantic cod (*Gadus morhua*), which is an economically and ecologically important fish, increasingly came under scrutiny due to declining stocks in the North and Baltic Seas. Existing assessment procedures, such as bottom trawl sampling, are both invasive and costly. Against this background, it is imperative to consider whether eDNA analysis could serve as an effective and accurate tool for detecting and quantifying Atlantic cod stocks, offering both economic and ecological benefits.

The main research question was, whether a quantitative analysis of eDNA from water samples, using real-time PCR, can be a viable alternative to invasive and costly methods, such as bottom

trawl sampling, for detecting and quantifying stocks of Atlantic cod (*Gadus morhua*) in the North and Baltic Seas?

5. Detection and quantification of *Limanda limanda* and *Pleuronectes platessa* in the Sylt outer reef: Development of a qPCR assay and forecast model based on eDNA copies. (submitted)

Fish stock assessment generally requires the simultaneous detection of several species, either because of their ecological and economic importance, or to understand the relevant factors and intra- and interspecific interactions. For these reasons, multi-target genetic methods such as multiplex real-time PCR are required.

The objective of this chapter is to evaluate the efficacy of eDNA analysis, particularly multiplex quantitative PCR (qPCR) assays, in assessing fish diversity and abundance relative to conventional methods such as bottom trawling, with a specific focus on *Limanda limanda* and *Pleuronectes platessa* populations in the North Sea. Furthermore, this chapter aims to investigate the influence of abiotic variables on the correlation with eDNA copy numbers and fish abundance estimates.

3. Chapter III: Mare-MAGE curated reference database of fish mitochondrial genes.

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3.1. Abstract

Biodiversity assessment approaches based on molecular biology techniques such as metabarcoding, RAD-seq, or SnaPshot sequencing are increasingly applied in assessing marine and aquatic ecosystems. Here we present a new reference database for fish meta-barcoding based on mitochondrial genes. The *Mare-MAGE* database contains quality-checked sequences of the mitochondrial 12S ribosomal RNA and Cytochrome c Oxidase I gene. All sequences were obtained from the National Center for Biotechnology Information- GenBank (NCBI-GenBank), the European Nucleotide Archive (ENA), AquaGene Database and BOLD database, and have undergone intensive processing. They were checked for false annotations and non-target anomalies, according to the Integrated Taxonomic Information System (ITIS) and FishBase. The dataset is compiled in ARB-Home, FASTA and Qiime2 formats, and is publicly available from the *Mare-MAGE* database website (<http://mare-mage.weebly.com/>). It includes altogether 231,333 COI and 12S rRNA gene sequences of fish, covering 19,506 species of 4,058 genera and 586 families.

Keywords: Mare-MAGE, mitochondrial database, fish, metabarcoding, eDNA

DATABASE

Open Access



Mare-MAGE curated reference database of fish mitochondrial genes

Yassine Kasmi^{*} , Erik Eschbach and Reinhold Hanel

Abstract

Biodiversity assessment approaches based on molecular biology techniques such as metabarcoding, RAD-seq, or SnaPshot sequencing are increasingly applied in assessing marine and aquatic ecosystems. Here we present a new reference database for fish meta-barcoding based on mitochondrial genes. The **Mare-MAGE** database contains quality-checked sequences of the mitochondrial 12S ribosomal RNA and Cytochrome c Oxidase I gene. All sequences were obtained from the National Center for Biotechnology Information- GenBank (NCBI-GenBank), the European Nucleotide Archive (ENA), AquaGene Database and BOLD database, and have undergone intensive processing. They were checked for false annotations and non-target anomalies, according to the Integrated Taxonomic Information System (ITIS) and FishBase. The dataset is compiled in ARB-Home, FASTA and Qiime2 formats, and is publicly available from the **Mare-MAGE** database website (<http://mare-mage.weebly.com/>). It includes altogether 231,333 COI and 12S rRNA gene sequences of fish, covering 19,506 species of 4,058 genera and 586 families.

Keywords Mare-MAGE, Mitochondrial database, Fish, Metabarcoding, eDNA

Background

Monitoring and surveillance of marine biodiversity is crucial for ecosystem conservation since it can provide decision makers appropriate arguments for the preservation and sustainable use of natural resources. Conventional biodiversity assessment tools are limited in terms of flexibility and costs, and they can be subject to bias and uncertainty [1, 2]. Thus, new molecular genetics-based approaches such as Metabarcoding, Restriction site-associated DNA sequencing (RADseq) markers, SNaPshot sequencing and Quantitative Polymerase Chain Reaction (qPCR) are increasingly used as qualitative and semi-quantitative methods, especially in the marine studies domain [3, 4].

All living organism shed DNA in their environment, and it is possible to collect this DNA, amplify it via PCR using primer sets targeting DNA marker regions and sequence the resulting amplicons using NGS [5–7]. While the use of environmental DNA (eDNA) is a manageable and almost “routine” method in microbiology, it remains a challenge in marine biology and ichthyodiversity monitoring, despite first publications dating back to 2011 (Fig. 1). Such studies face various challenges with respect to bioinformatic analyses [8, 9]. Among these challenges are: (1) The need for a fairly good knowledge in the field of programming and coding, since almost all analysis platforms operate in a Unix environment. Some pipelines have proven to be very useful in microbiological eDNA-metagenomics and meta-taxonomics, with easier interfaces for a larger user community. These pipelines are less suited to eDNA studies, due to (2) a lack of availability of a comprehensive reference database covering a sufficient number of species and (3) the incompatibility with analytical tools. The first paper was published in 2011 and since then more than 300 articles have been

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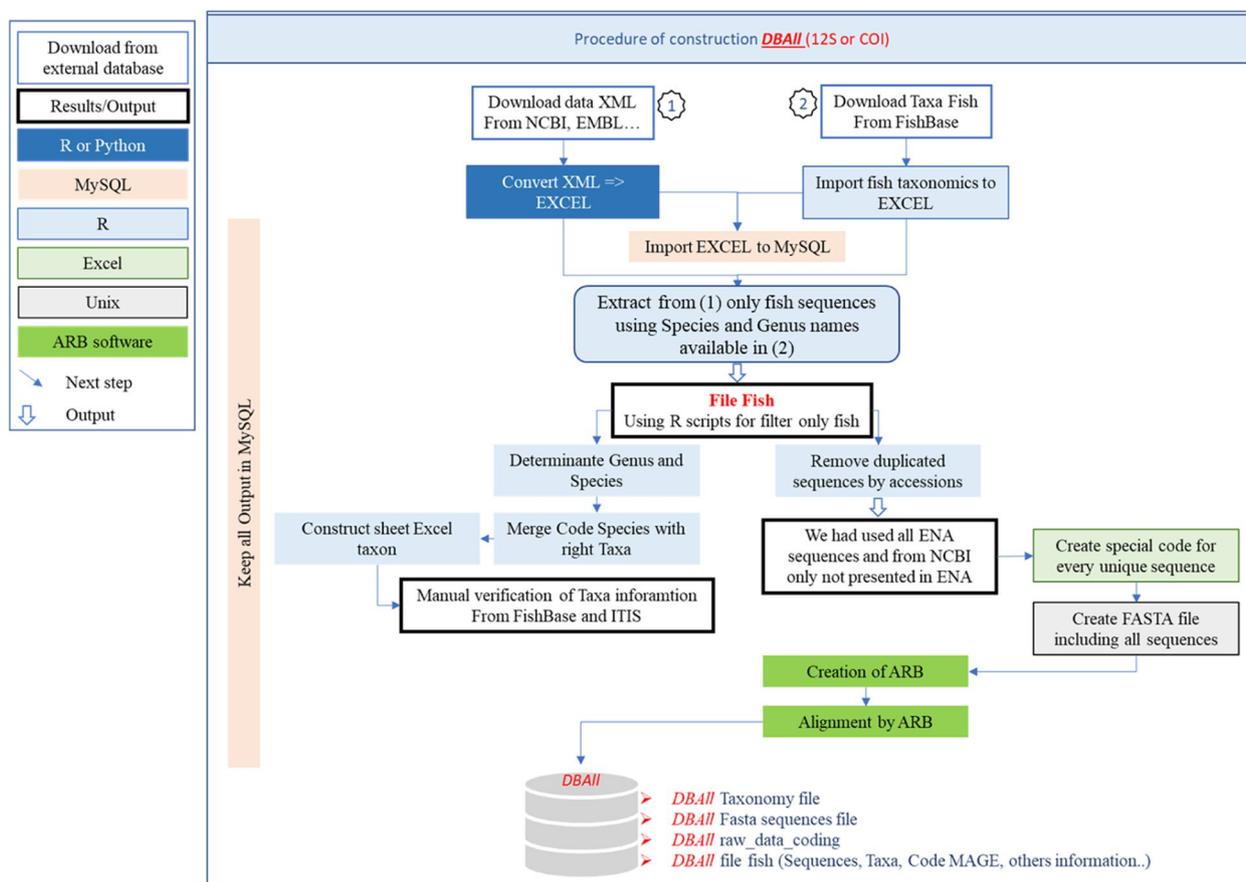


Fig. 1 Detailed schematic view of the *Mare-MAGE-DBAll* development process. The process mentioned here is the same for the both COI and 12S genes. The sub-database "Mare-MAGE-DBAll" contains raw-data collected from various public databases (ENA, AquaGene, GenBank, NCBI, BOL), consisting of all fish derived 12S or COI sequences available without any correction or validation, except for some taxonomic revisions according to the new nomenclature published by ITIS and FishBase. The MS Excel file "Other-Info" includes all sequence information (length, taxonomy and remarks) together with the relevant code in *Mare-MAGE*, the source of the database (NCBI, BOLD,..) and the author's name for the original data

published and indexed in Scopus. Compson et al. [10] in their systematic review article showed that the number of articles and papers published on eDNA on various organisms and in multiple domains has been increasing significantly during the last 5 years. The authors also found that the study of eDNA is no longer just an academic research topic, but is beginning to be touched by industry and decision makers, which explains its importance and dynamics [10].

For eDNA studies, many mitochondrial genes have been used, such as the 16S small subunit ribosomal RNA gene (16S rRNA), the 12S small subunit ribosomal RNA gene (12S rRNA), as well as the Cytochrome c Oxidase I (COI) and Cytochrome b (MT-CYB) genes. Like all mitochondrial genes, the 12S rRNA and COI genes are present in many more copies per cell compared to nuclear DNA regions. This high frequency in the number of mitochondrial sequences per cell facilitates detection and lowers the detection limit even of low-abundance

species. The mitochondrial 12S rRNA is one of the ribosomal RNAs encoded by the mitochondrial genome of eukaryotes, a component of the small subunit of the mitochondrial ribosome and of particular interest in phylogenetic studies [11, 12]. COI encodes subunit I of the mitochondrial Cytochrome c Oxidase, and is involved in the respiratory chain of mitochondria. Moreover, these genes have a particularly high sequence variability for the distinction between species, which predestined them for the use in the barcode of life project [13].

To our knowledge, there is currently no global (worldwide), specific, quality checked and taxonomy-revised 12S rRNA database for fish. The most commonly used COI database for fish identification is BOLD Systems [13], which includes FISH-iBOL [14] as a sub-database for fish. However, in spite of the number of species covered, the BOLD Systems database requires complicated pre-processing before being compatible with many common bioinformatics pipelines (Obitools, Qiime2, vsearch,

dada2), limiting its use in eDNA studies. 16S and 18S rRNA genes are represented in the Silva database [15], which also includes 23S rRNA gene information. While the Silva database is compatible with most pipelines, it is more focused on microbiota and Archaea, with very limited information on eukaryote taxa and almost none on fish. MitoFish database is an original NCBI database that essentially contains about 2800 complete mitochondrial sequences of about 600 fish species, covering only 1.7% of all known fish species (35,100 species according to Fishbase [23]) [16]. FishCARD database is a new 12S gene database specific for the coast of California and it covers 600 species [17].

In this paper, we present the Mare-Marine-Gene (*Mare-MAGE*) database as a comprehensive quality controlled and taxonomically reviewed reference database for 12S rRNA and COI sequences of fish to be ready for use by convenient analysis pipelines.

Construction and content

Data sources and content

The *Mare-MAGE* database contains mitochondrial 12S rRNA and COI gene sequences of fish, collected from The National Center for Biotechnology Information-GenBank (NCBI) [18], AquaGene [19], BOLD [13] and the European Nucleotide Archive (ENA) [20] databases as of January 21th, 2022 for the 12S rRNA gene and January 26th, 2022 for the COI gene. NCBI and ENA data were downloaded as XML (Extensible Markup Language) files, using the following keywords: “12S RNA”, “12 s RNA”, “12S ribosomal RNA”, “12 s rRNA” and “12 s” for 12S rRNA gene, and “COI RNA”, “COI”, “CO1”, “CO1”, “MT-COI”, “COX1” and “Cytochrome c oxidase I” for COI gene. We used the filter “species=animal” in GenBank database. The XML files were imported and transformed into tables via the Data-XML function in Microsoft Excel. The data from AquaGene [19], BOLD [21] databases were downloaded as Excel files.

Data processing

All sequences were initially filtered by their accession number to eliminate duplicates in the same file via R and Excel, then revised according to their accessions to keep only those sequences annotated by the authors as 12 s rRNA or COI. A second filter step to remove duplicates between GenBank and ENA was performed via an R script. A fish taxonomy file of all ranks (class, order, family, genus and species) was obtained from FishBase and included 35,100 species [22, 23]. The fish taxonomy file was used as reference list of known fish species. Using a custom R-script and the fish taxonomy file as a reference, all data were filtered to include only accessions where the

original authors identified the sequence as originating from a distinct fish species.

Each accession received a new identification code in the *Mare-MAGE Database*, 12sDBxxxx for 12 s rRNA and CO1DBxxx for COI genes. The original accessions with all information and the new codes in the *Mare-MAGE database* were saved in a table in MySQL, which includes the following fields: the original accession numbers, the new code in *Mare-MAGE*, the original database, and the original genus and species names published by the original authors (Fig. 1). All sequences and related data extracted from GenBank, AquaGene, BOLD and ENA databases have been stored locally in a relational database system (MySQL). The sequences were saved in FASTA format with the new *Mare-MAGE* codes, and formed the seed for a new ARB-home database consisting of aligned and unaligned sub-databases.

Curation of taxonomy

Each sequence in the *Mare-MAGE-DB* has the taxonomy assignments of either AquaGene, BOLD, ENA or GenBank databases, depending on its origin. The taxonomy (genus and species) was retrieved together with the sequence in the XML files. Subsequently, all organism names were synchronized and manually reviewed to revise, correct and complete the sequence taxonomy based on information provided by The Integrated Taxonomic Information System (ITIS) [24] and FishBase [22, 23] to remain consistent with the most up to date scientific names according to published recommendations, i.e. domain, class, order and family. All former names and taxa are stored in the database, as well as in the corresponding field in the ARB databases.

Quality filtration and rating

All sequences underwent a global alignment by MAFFT to confirm the 12S and COI gene region. Subsequently each family underwent an alignment by ARB-home, aiming at finding ambiguities in the sequences and the global relationship via a distance matrix generated by ARB-home [25]. The data of each family were uploaded to the ARB-home environment, to build a database, and standardized by aligning the unique sequences for each family. Finally, a *Sq* score—“Sequences Quality score”—was calculated according to Eq. 1, as published by Pruesse et al. [15].

Sequences with a *Sq* criterion < 30% were rejected, as mentioned by Pruesse et al. [15]. Since the quality of the final datasets is critically dependent on the quality of the sequence alignments, the datasets were checked during the database construction. In this process, all sequences that could not be unambiguously aligned were removed from the database.

$$Sq = 1 - \left(\frac{\left(\frac{A}{A_{max}} \right) + \left(\frac{H}{H_{max}} \right) + \left(\frac{V}{V_{max}} \right)}{3} \right) * 100, \quad (1)$$

where Sq corresponds to sequence quality score, A to ambiguities, H to homopolymer and V to vector identity. A second global alignment of all sequences took place to build the phylogenetic tree. Conspecific sequences from the same genus were kept only at phylogenetically very close distance. The phylogenetic trees were examined at the level of each genus and family; if the sequences were as expected, they were retained, if not, the discrepancies were examined.

The ARB alignment is based on two different parameters (consensus and by reference) using **zebrafish** (*Danio rerio*) reference sequences (accession numbers KM244705 and NC_002333), followed by a global phylogenetic tree produced for all species using the “neighbor joining algorithm” NJ incorporated in the ARB program.

Database composition

The **Mare-MAGE database** includes 3 sub-databases for each gene:

Mare-MAGE-DBAll: includes all fish-specific 12S or COI sequences available in the NCBI and ENA databases, without further cleaning or validation, except for a nomenclature revision (Fig. 1). For each gene we provide a sub-database **Mare-MAGE-DBAll** with taxa files, FASTA files, and ARB files for each gene sub-database. In addition, we provide the dada2 and Qiime2 format for each gene on our website [26].

Mare-MAGE-90DB includes only entries of species represented by more than 10 individual sequences with a similarity higher than 98%. **Mare-MAGE-90DB** is derived from **Mare-MAGE-DBAll**. It was processed and filtered by organism name and sequence frequency via an R script. Only sequences belonging to taxa with at least 10 sequences from 2 different authors in the **Mare-MAGE-DBAll** were retained. Every family then underwent an alignment by ARB to produce a similarity matrix. By combining the output of the similarity matrix and the Sq score calculated by ARB, the sequences with high similarity were selected as recommended by Pruesse et al. [15] (Fig. 2a). For each gene we provide a sub-database **Mare-MAGE-90DB** with a taxa file, a FASTA file, and an ARB file for each gene sub-database. The other files are available by request from the corresponding author.

Mare-MAGE-DBc is a sub-database containing only the sequences that cover the complete 12S rRNA and

COI genes in **Mare-MAGE-90DB**. In order to construct this sub-database, we created a seed for each family from the data issued from complete mitochondrial genome sequences published in NCBI, before aligning all sequences of **Mare-MAGE-90DB** from this seed. Only the sequences that cover the whole 12S rRNA or COI gene were collected. Subsequently, **Mare-MAGE-DBc** was processed and filtered by the name of the species and the number of sequences representing it via an R script (Fig. 2b). For each gene we provide a sub-database **Mare-MAGE-DBc** with a taxa file, a FASTA file, and an ARB file for each gene sub-database. The other files are available by request from the corresponding author.

Mare-MAGE-DBAll includes all collected sequence information, 92–18,000 bp and 100–17 000 bp for 12S rRNA and COI genes, respectively. The large size span is due to the presence of whole mitochondrial genomes together with only partial gene sequences as applied by many researchers. Complete gene sizes of 12S rRNA and COI are around 1000 bp and 1500 bp, respectively. Since **Mare-MAGE-DBAll** contains only the raw data, single gene sequences were not extracted from the whole mitochondrial genomes.

To be present in **Mare-MAGE-90DB** sequences require minimum lengths of 100 bp and 500 bp for 12S and COI genes, respectively. However, for **Mare-MAGE-DBAll** only sequences longer than 900 bp were kept.

Database validation

Taxonomic assignment is a crucial step in each eDNA analysis. Thus, the choice of the reference database may affect post-analyses and subsequent interpretation of the results. Therefore, we performed a comparison between the output of **Mare-MAGE** and NCBI with regard to taxonomic assignment, in order to evaluate the performance and demonstrate the utility of the **Mare-MAGE** database and its three sub-databases.

To accomplish this task for COI, we used a raw data set published by Günther et al. [27] as the outcome of an eDNA study on North Sea water samples based on universal COI primers (SRA accession: SRR7661161, Project accession: PRJNA485040). For the 12S rRNA gene, a seawater sample (5L) was collected during a from the North Sea fisheries research survey on board FV Walther Herwig III (mission number WH428) from 9 July to 6 August 2019. Immediately after sampling, the water was filtered through a 0.45 μ M membrane and stored at -20 °C, until DNA extraction within 4 weeks. The eDNA extraction was performed via the Phenol–chloroform method, followed by centrifugal column purification with Monarch™ PCR & DNA Cleanup Kit (New England

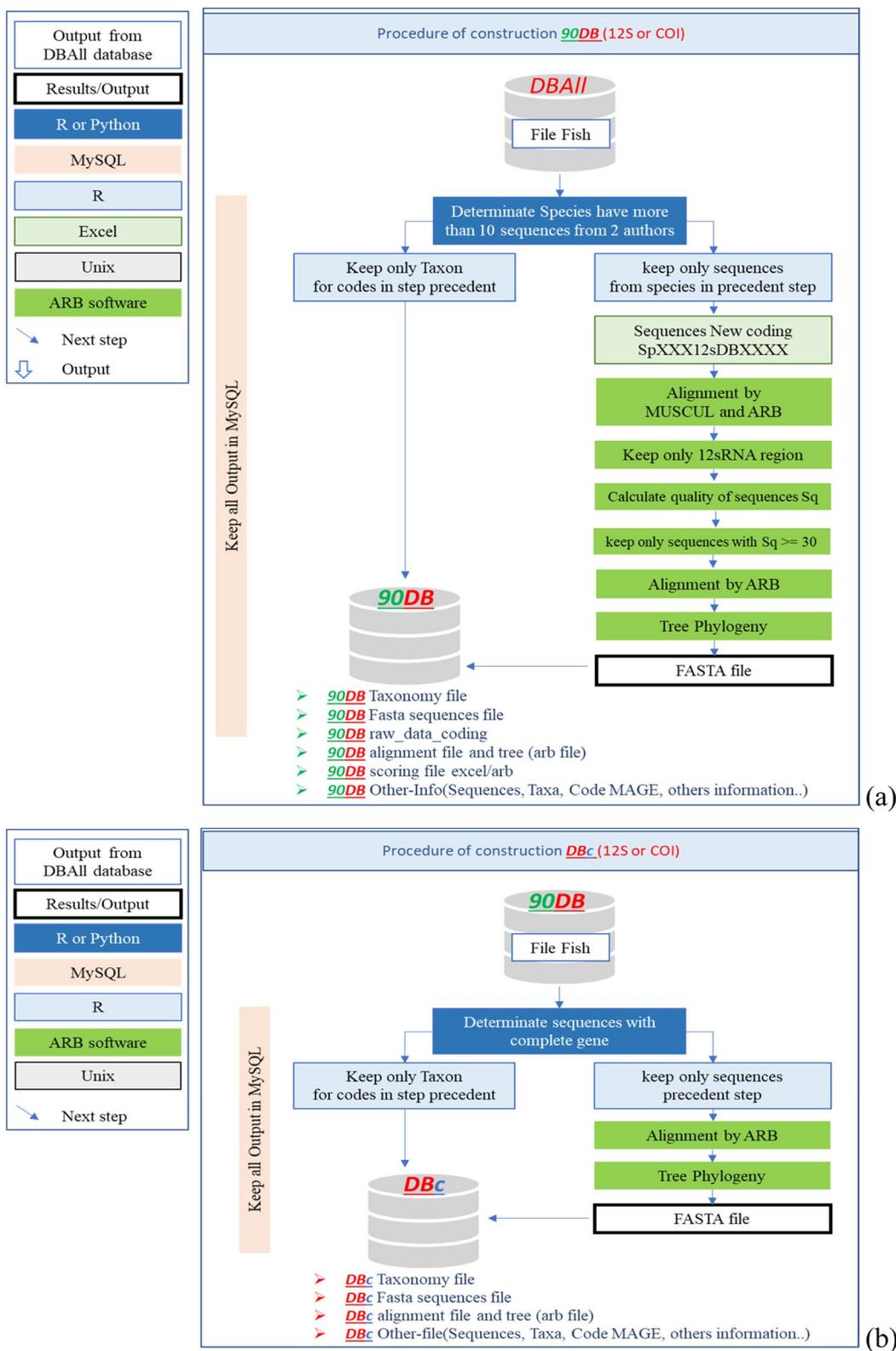


Fig. 2 Description of the procedure followed during the development of Mare-MAGE-90DB (a), the complete gene sub-database Mare-MAGE-DBc (b) and the filtering of sequences. This process is identical for both genes

Biolabs) in a dedicated room at the genetics laboratory of the Thünen Institute of Fisheries Ecology, Bremerhaven. MiSeq (NGS) library preparation and sequencing were performed according to the protocol published by Miya [28] using MiFish primers targeting the 12S rRNA gene (the Fastq file is available in *figshare* [26]-validation data/12S-).

For taxonomic assignment, the Vsearch pipeline was used as the reference pipeline of NCBI followed by BLASTN. The Fastq files initially underwent a treatment by Vsearch to assemble the forward and reverse sequences, then the primers and the adaptors were removed by Cutadapt (version 3.4). Subsequently, the Vsearch pipeline was used for the filtering the steps, to remove duplicates and cluster the OTUs. Thereafter, the BLASTN pipeline was used to assign the OTUs against NCBI and *Mare-MAGE* databases with identical parameters. Only if an OTU had a similarity of more than 97% with a database entry and query around 100% it was assigned as a species. R and Excel were used to generate the figures.

Data records

The *Mare-MAGE* databases are available via the *Mare-MAGE* interface at <http://mare-mage.weebly.com/>. The web interface is divided into 5 sections: Home, Databases, search, tutorials, Contact. It is possible to download datasets in ARB, FASTA and txt formats. It is also possible to download files in Qiime2 and Mothur formats. The tutorial includes the use of the *Mare-MAGE* databases with Qiime2. Every sub-database includes a file entitled "Search-DB.csv" which includes the raw information about the origin of every sequence.

It is also possible to have access from *figshare* [26], through the link <https://doi.org/10.6084/m9.figshare.c.5410161.v1>. Each of the 12S and COI folders includes 3 subfolders, the full or raw database (*Mare-MAGE-DBAll*), the 90% confidence database

(*Mare-MAGE-90DB*) and the complete gene database (*Mare-MAGE-DBc*). All R and Python scripts used in this work are available in GitHub at <https://github.com/kasmiyassin/Mare-MaGe-Database>.

Utility and discussion

First database validation

The *Mare-MAGE-12sDBAll* database contains the mitochondrial 12S rRNA sequence information of 3,236 genera and 11,236 species of fish with sequence lengths between 92 and 18,000 Bp (Table 1). The *Mare-MAGE-COI-DBAll* contains the mitochondrial COI gene sequence information of 3,841 genera and more than 20,657 species of fish with sequence lengths between 92 and 17,000 Bp (Table 2).

The *Mare-MAGE-12 s-90DB* database covers 10,591 sequences (27.5% of *Mare-MAGE-12 s-DBAll*), of 242 genera and 393 species of fish with sequence lengths between 100 and 1,300 Bp (Table 1 and Fig. 3). More than 150,000 sequences of 1,741 genera and more than 4,400 species remain in the *Mare-MAGE-COI-90DB* (Table 2 and Fig. 3).

The *Mare-MAGE-12 s-DBc* database contains 6,487 sequences (17.1% of *Mare-MAGE-12 s-DBAll*), of 204 genera and 309 species of fish with sequence lengths between 900 and 1,200 Bp (Table 1). More than 4,639 sequences of 429 genera and more than 1,304 species are included in *Mare-MAGE-COI-DBc* (Table 2 and Fig. 3).

The current alignment for the *Mare-MAGE-DBAll* is based on 37,888 and 150,000 positions for 12S and COI genes, respectively. The reasons for the large number of alignment positions are either (1) large insertions and/or (2) sequencing errors, such as additional artificial bases often found in the stretching of homopolymer sequences. As presented in section 3 (Database composition), this sub-database reflects the raw-data collected from various public databases (GenBank, NCBI, AquaGene, BOL), typically containing all 12S or COI sequences available

Table 1 Statistics for 12sDB

Name	<i>Mare-MAGE-12sDBAll</i>	<i>Mare-MAGE-12 s-90DB</i>	<i>Mare-MAGE-12 s-DBc</i>
Total number of sequences	37 888	10 591	6487
Species	11,236	393	309
Genera	3236	242	204
Families	535	254	94
Lengths	92–18,000 Bp	100–1300 Bp	900–1200 Bp
Origin	ENA + NCBI	12sDBAll	12 s-90DB
Alignment	No	Yes	Yes
Database files	ARB, MySQL, Excel, FASTA	ARB, MySQL, Excel, FASTA, aln	ARB, MySQL, Excel, FASTA, aln
Taxonomy revision	Yes (FishBase.org)	According 12sDBAll	According 12sDBAll

Table 2 Statistics for COI-DB

Name	<i>Mare-MAGE-COI-DBAll</i>	<i>Mare-MAGE-COI-90DB</i>	<i>Mare-MAGE-COI-DBc</i>
Total number of sequences	193,445	153,704	4639
Species	20,657	4495	1304
Genera	3841	1741	446
Families	553	553	102
Lengths	100–17 000 Bp	500–1000 Bp	900–1600 Bp
Origin	ENA + NCBI	COI-DBAll	COI-90DB
Alignment	No	Yes	
Database files	ARB, MySQL, Excel, FASTA	ARB, MySQL, Excel, FASTA, aln	ARB, MySQL, Excel, FASTA, aln
Taxonomy revision	Yes (FishBase.org)	According COI-DBAll	According COI-DBAll

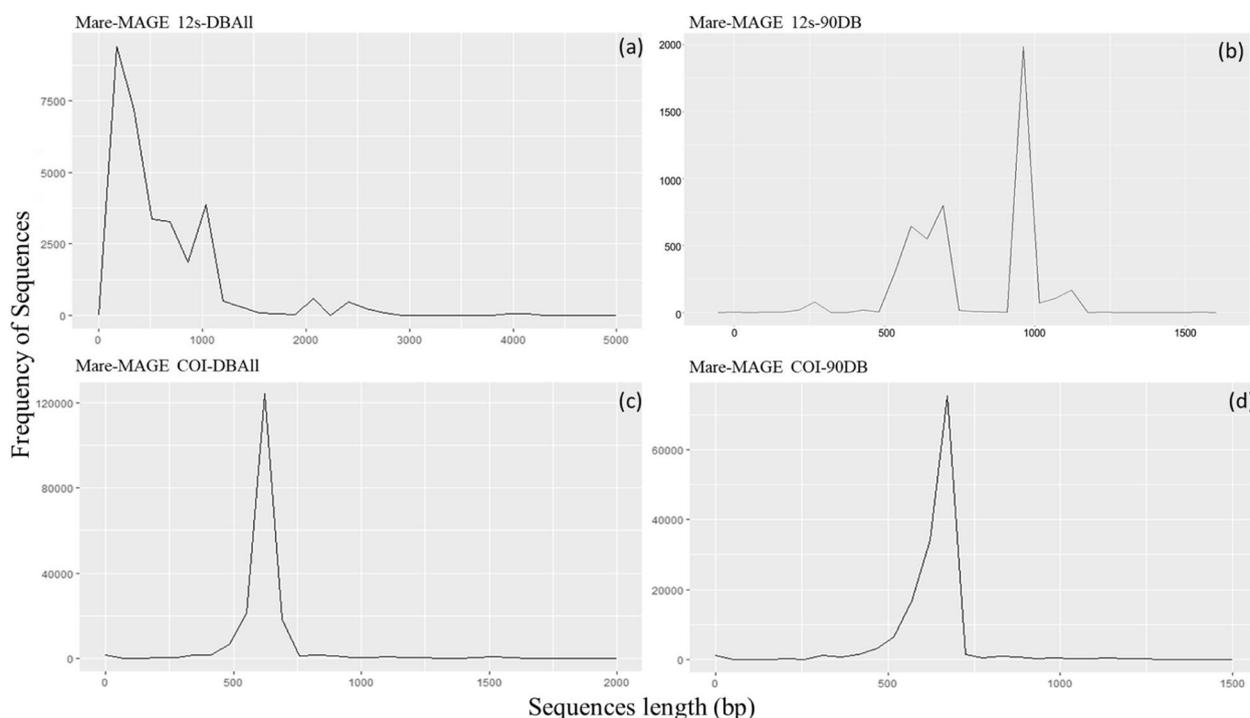


Fig. 3 Sequence lengths distribution in *Mare-MAGE* Database, for 12S rRNA and COI genes in the DB Full and 90% DB database. **a** *Mare-MAGE Database 12S-DBAll*, **b** *Mare-MAGE Database 12S-90DB*, **c** *Mare-MAGE Database COI-DBAll*, **d** *Mare-MAGE Database COI-90DB*. The solid line represents the sequence distribution directly after import. For *12S-DBAll*, most of the sequences are between 100 and 250 Bp

for fish without further correction or validation, except for some taxonomic revisions according to the new nomenclature published by ITIS and FishBase. So, users need to be aware that *Mare-MAGE DBAll* contains some false annotations of genes or species.

While the total alignment output for all sequences of *Mare-MAGE-90DB* constitutes 10,591 and 6,370 positions for 12S and COI genes, respectively, for species of the same family it is restricted to ~2500 and ~2000 positions. The quality and consistency of all

seed alignments in *Mare-MAGE-90DB* are excellent, thanks to the smaller number of alignments position. Some minor inconsistencies that we could not resolve mainly for the 12S gene in *Mare-MAGE-90DB*, were: (1) a lack of resolution between closely related congeneric species, as for the Scombridae genus *Thunnus*, and (2) editing problems: despite manual revision, we sometimes cannot ensure that species information is provided by at least two different authors due to some misspellings of author’s names. However, these two minor inconsistencies do not significantly influence the

performance and the accuracy of the two sub-databases *Mare-MAGE-90DB* and *Mare-MAGE-DBc*.

Second database validation: taxonomic assignment

COI and 12S rRNA raw dataset analyses yielded 1,613,759, and 178,419 sequence reads, respectively, of which 104,077 merged sequence reads (58.33%) could be assembled from the 12S rRNA file, and 1,283,078 sequence reads (79.50%) from COI file. With *Mare-MAGE* as reference database, 96.3% of all 12S rRNA sequence reads were assigned to fish taxa with more than 97% similarity, while with NCBI only 89.4%. For COI, 8,737 sequences reads (0.68%) could be successfully assigned to fish taxa with *Mare-MAGE* as reference database with a similarity higher than 97%, while only 1,080 (0.08%) with NCBI (Fig. 4a). The number of fish reads assigned with *Mare-MAGE* was significantly higher than with NCBI (Mann–Whitney–Wilcoxon Test, $p < 0.05$).

For 12S rRNA, 21 fish species out of 8 families, while for COI, 32 species out of 20 families were detected by both databases (Fig. 5). Only about 40% of the OTUs could be assigned to a distinct species with NCBI, while about 90% of the OTUs were assigned to a distinct species with *Mare-MAGE*. The remaining OTUs were assigned either to a genus (at similarities between 97 and 92%) or to a family (at similarities between 92 and 80%). For all other datasets obtained (query coverage less than 160), 32 and 134 fish species (cartilaginous and bony fish) were recorded for 12S rRNA and COI respectively (Supporting file S1). The comparison shown in Fig. 4b–e and Tables S1 and S2 clearly shows a significant difference between *Mare-MAGE* versus NCBI as reference for taxonomic assignment. From the COI dataset, 13 species were solely detected with the *Mare-MAGE* database compared to only a single species with NCBI. For the 12S rRNA gene, 6 species were detected solely with *Mare-MAGE* compared to a single species with NCBI. The reason why some species were not detected by *Mare-MAGE* even though they were detected by NCBI is that *Mare-MAGE* database only covers NCBI until January 2022. An update is planned for December 2022, so any shortcomings will be regularly overcome. Despite the observed differences in the detection of OTUs between the two databases, there was also considerable overlap. For COI, 17 of the 19 species assigned by NCBI were also assigned by *Mare-MAGE*, while for 12S rRNA, 14 of the 15 species assigned by NCBI were also present in the *Mare-MAGE* output. Interestingly, the number of reads assigned based on NCBI taxonomy was significantly lower than those based on *Mare-MAGE* for both genes (Fig. 4a).

In addition, the use of *Mare-MAGE* significantly improved the taxonomic assignment of fish species, by reducing the false assignment noise from microbial or viral sequences in NCBI. *Mare-MAGE* also includes certain species from various databases, that are still not included or synchronized with NCBI. The use of *Mare-MAGE* also reduced processing times, because all non-fish reads were automatically assigned as “unknown”. The *Mare-MAGE* database is compatible with almost all the above cited pipelines, allowing an easy and user-friendly data presentation in different graphical formats, depending on research question and pre-processing steps.

Figure 5 and Tables S1 and S2 present the percentage of reads assigned to species depending on the used reference database. The use of *Mare-MAGE* resulted in an increase in species number for both genes, because more OTUs were assigned to fish with a similarity above 97% and a complete coverage of the query sequenced fragment. Using NCBI for the same datasets, a considerable number of OTUs was assigned to microbial and virus at fragment lengths of less than 70 bp, negatively affecting the taxonomic assignment and rendering many fish species invisible in the output.

On the other hand, NCBI is not compatible to be used with both pipelines DADA2 and Qiime2, without extra pre-processing steps. Prior to any analysis, NCBI requires a rather complex creation of output files to reach compatibility with the pipelines. The taxonomic output is limited to the species level, preventing the possibility to get a visualization of the results at different taxonomic hierarchy levels. However, *Mare-MAGE* database, for both genes, was fully compatible to be uploaded into Qiime2 and DADA2 pipelines, using the command “qiime feature-classifier classify-consensus-blast” or “qiime feature-classifier classify-sklearn” for Qiime2 and “assignTaxonomy” in dada2 pipeline without any error. Another disadvantage of using NCBI on eDNA metabarcoding data is the presence of virus, bacterial and phytoplankton OTUs in the final results, which requires a post-filtering of non-fish OTUs, presuming a decent knowledge on fish taxonomy.

While non-fish OTUs (bacteria, viruses ...) quite regularly occur in eDNA outputs, the availability of a database containing only fish taxa will help reducing a bias of false assignment as well as time and effort imposed on users to eliminate all non-fish sequences from the final results. In this context, the validation results clearly showed that *Mare-MAGE* significantly reduces the noise of non-fish OTUs compared to NCBI. Non-fish OTUs represent between 10 and 90% of the results obtained from NCBI, depending on the fish sequence diversity and the primer specificity. This noise of non-fish OTUs sometimes exceeds 100 OTUs per sample as

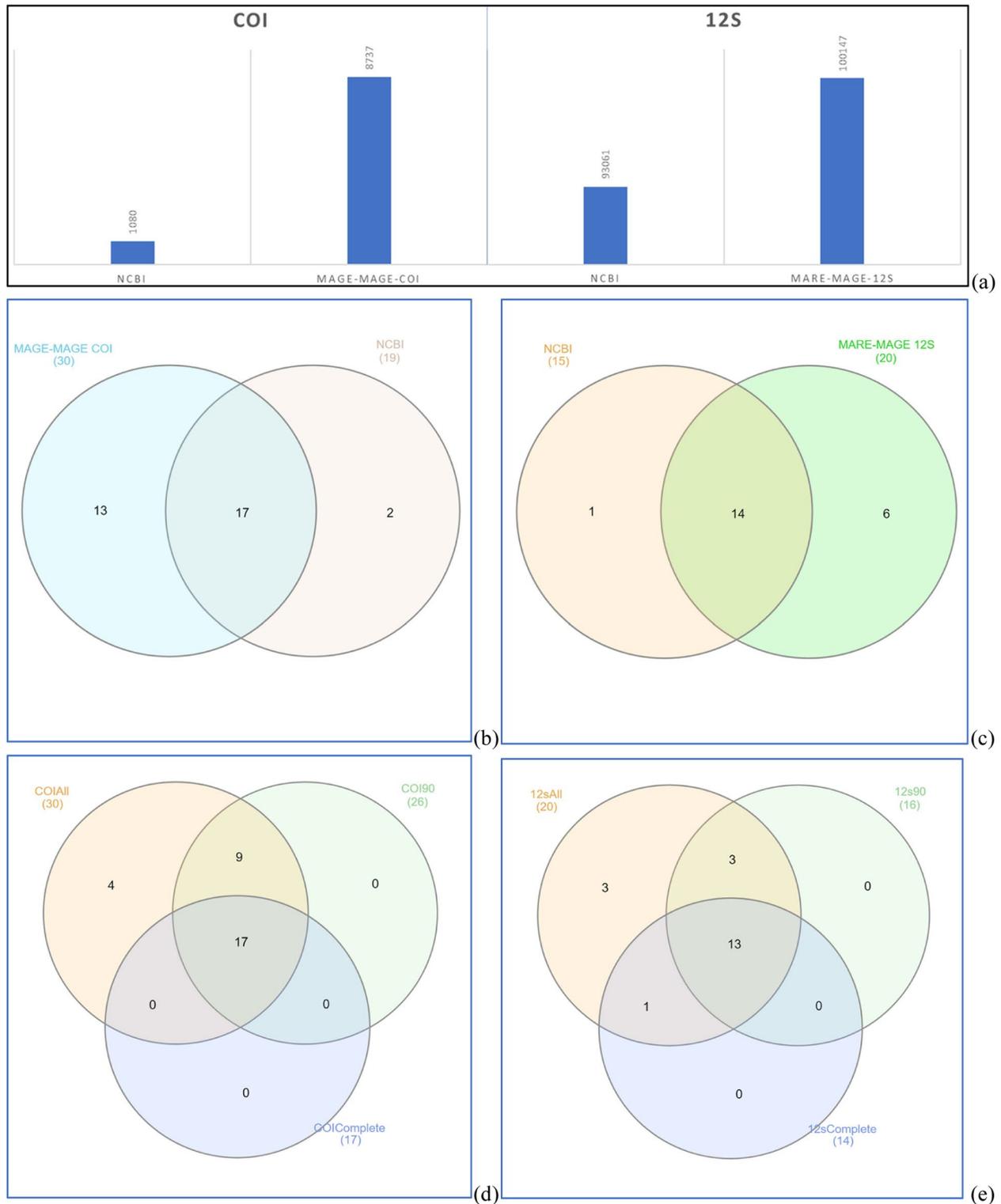


Fig. 4 Comparison between based on OTUs assigned to a distinct species. **a** Comparison of assigned read numbers. **b** Comparison of the number of identified species using NCBI and *MARE-MAGE* based on the COI gene. **c** Comparison of the number identified of species using NCBI and *MARE-MAGE* based on the 12S rRNA gene. **d** Comparison of the number identified species using COI databases, **e** Comparison of number of species identified 12S databases

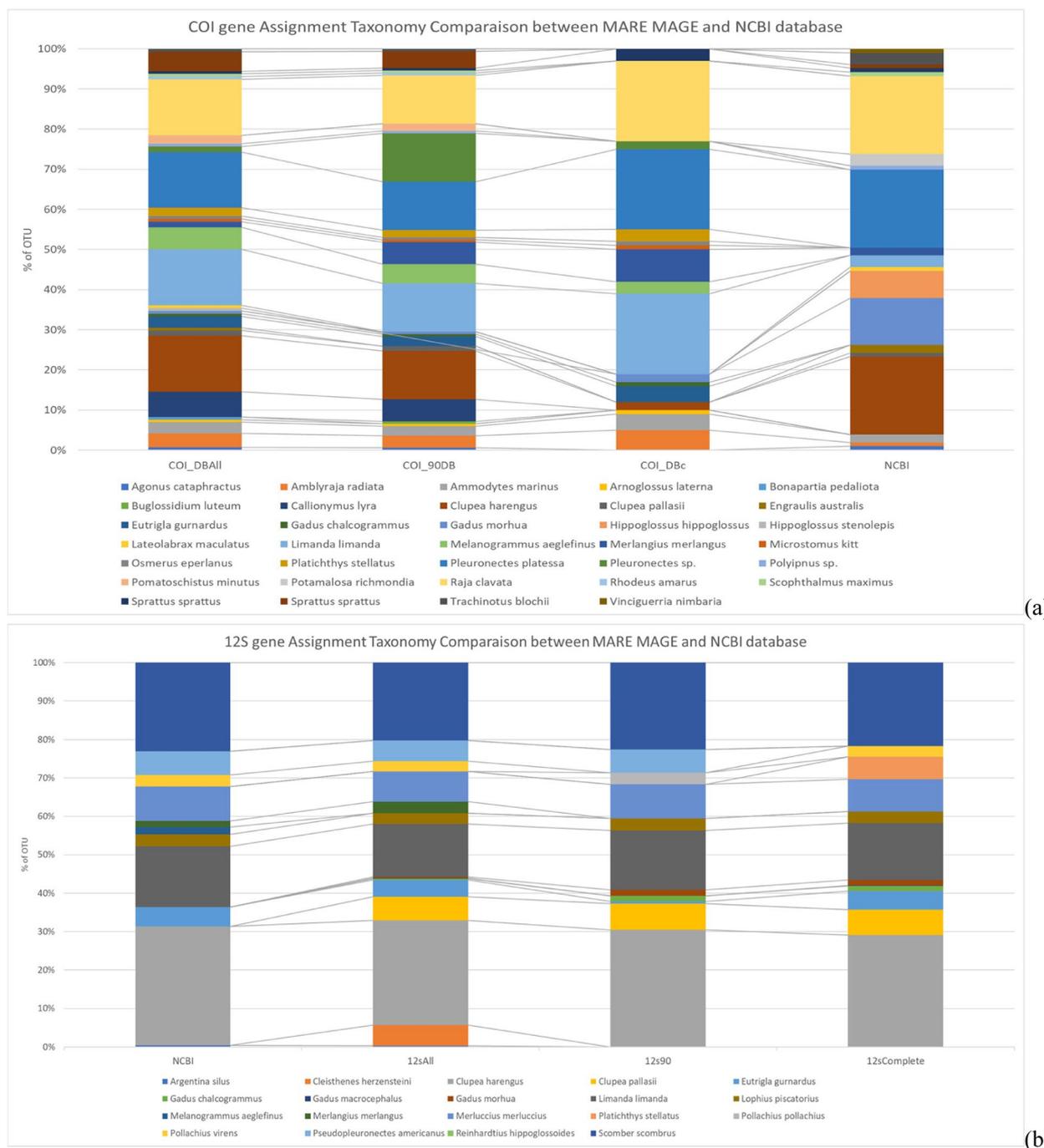


Fig. 5 Comparison of the OTU assignment between *Mare-MAGE* and NCBI database based on datasets from the mitochondrial (a) the COI and (b) 12S rRNA genes

an NCBI output, which need to be removed with considerable effort. Therefore, *Mare-MAGE* represents a highly reliable alternative to other public databases for taxonomic assignment of fish eDNA.

Conclusion

In summary, the *Mare-MAGE* database [26] contains all 12S rRNA and COI fish sequences available at ENA and NCBI databases (last download: 21 January 2022 for 12S rRNA gene and 26 January 2022 for COI

gene) with a revised and updated taxonomy based on FishBase. It contains additional sub-databases: *Mare-MAGE-90DB* for 90% confidence sequences belonging to a species. It is recommended to be used for fish eDNA analyses, *Mare-MAGE-AIUDB* can be also used as *Mare-MAGE-90DB* for the fish environmental DNA analysis, but users should be careful during use, as it may contain assignment taxonomic. *Mare-MAGE-DBc* for sequences that cover the complete gene, it is suitable to be used for the development and validation of new primers or when the primers cover area not covered with the MiFish primer and L14912 or L14841 primers for 12S rRNA and COI genes, respectively.

Mare-MAGE database is compatible for use with various pipelines, like Vsearch, Qiime2, Mothur, ObiTools. In particular allows the new database the export of ARB and FASTA files of sequences and txt files of names of taxa. Moreover, point-and-click access allows for a simple and fast import and export of sequence records and searching and mapping is provided via Qiime2.

Mare-MAGE [26] will be updated yearly in December, with the second update scheduled for 2022. In the same context of improving the database we in the future aim to include also the NADH-ubiquinone oxidoreductase (ND) gene family, mainly NADH-ubiquinone oxidoreductase chain 5 (ND5).

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12863-023-01119-4>.

Additional file 1: Table S1. Comparison table of fish taxonomic assignments and reads between *Mare-MAGE* and NCBI, and the 3 sub-databases for the COI gene. **Table S2.** Comparison table of number of Fish taxonomic assignments and reads between *Mare-MAGE* and NCBI, and the 3 sub-databases for the 12s rRNA gene.

Authors' contributions

YK: Conceptualization, Investigation, Taxonomy Revision and Database Construction, Phylogenetic Analyses, Draft writing, Visualisation. EE: Supervision, Draft correction, Resources from Aquagene database. RH: Supervision, Conceptualization, Writing, Review & Editing. The author(s) read and approved the final manuscript.

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Availability of data and materials

All codes and scripts used in this work are available in Github: <https://github.com/kasmiyassin/Mare-MaGe-Database>.

Code Unix XML NCBI was used to download the raw data under xml format. The codes R XML to csv and Python XML to csv were used to transfer XML to MS Excel format.

Code R sequences from NCBI by R were used to download sequences and also the raw data species.

DBAll analysis.R were used for treatment of DBAll data.

R code for 90DB were used for 90DB database.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The information and views set out in this manuscript are based on scientific data and information collected under Service Contract "Improving cost-efficiency of fisheries research surveys and fish stocks assessments using next-generation genetic sequencing methods [EMFF/2018/015]" signed with the European Climate, Infrastructure and Environment Executive Agency (CINEA) and funded by the European Union. The information and views set out in this publication are those of the author(s) and do not necessarily reflect the official opinion of CINEA or of the European Commission. Neither CINEA nor the European Commission can guarantee the accuracy of the scientific data/information collected under the above Specific Contract or the data/information included in this publication. Neither CINEA nor the European Commission or any person acting on their behalf may be held responsible for the use which may be made of the information contained therein.

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Supplementary Data

Table S1: Comparison table of fish taxonomic assignments and reads between *Mare-MAGE* and NCBI, and the 3 sub-databases for the COI gene.

Species	COI_DBAll	COI_90DB	COI_DBc	NCBI
<i>Agonus cataphractus</i>	11	10	0	10
<i>Amblyraja radiata</i>	50	50	25	15
<i>Ammodytes marinus</i>	4	4	4	2
<i>Arnoglossus laterna</i>	12	12	12	0
<i>Bonapartia pedaliota</i>	15	0	0	0
<i>Buglossidium luteum</i>	0	19	0	0
<i>Callionymus lyra</i>	54	32	0	0
<i>Clupea harengus</i>	1236	162	2	20
<i>Clupea pallasii</i>	21	18	0	19
<i>Engraulis australis</i>	10	0	0	2
<i>Eutrigla gurnardus</i>	4	4	4	0
<i>Gadus chalcogrammus</i>	6	1	1	0
<i>Gadus morhua</i>	10	1	2	12
<i>Hippoglossus hippoglossus</i>	0	0	0	7
<i>Hippoglossus stenolepis</i>	10	1	0	0
<i>Lateolabrax maculatus</i>	10	0	0	15
<i>Limanda limanda</i>	2680	260	250	30
<i>Melanogrammus aeglefinus</i>	120	23	16	0
<i>Merlangius merlangus</i>	280	125	10	210
<i>Microstomus kitt</i>	12	12	10	0
<i>Osmerus eperlanus</i>	15	16	12	0
<i>Platichthys stellatus</i>	3	3	3	0
<i>Pleuronectes platessa</i>	20	20	20	20
<i>Pleuronectes sp.</i>	2	20	2	0
<i>Polyipnus sp.</i>	10	10	0	10
<i>Pomatoschistus minutus</i>	30	30	0	0

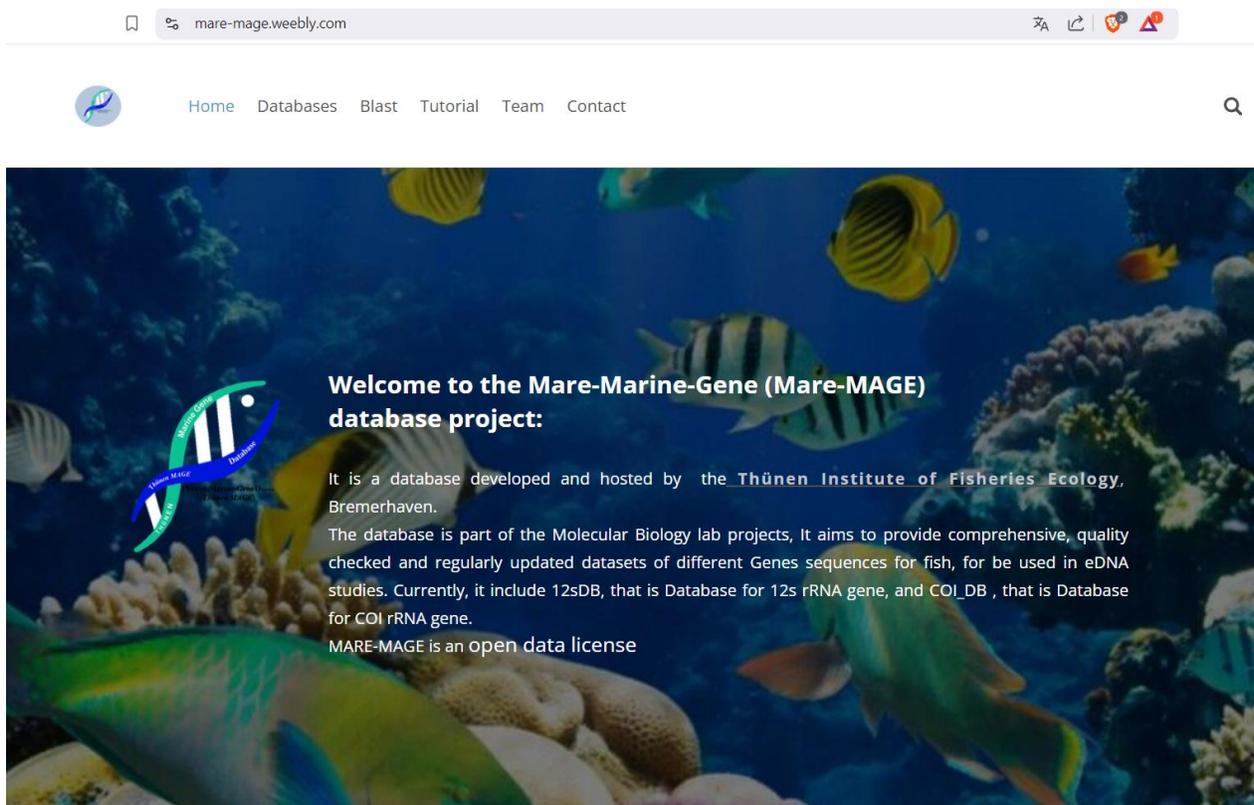
<i>Potamalosa richmondia</i>	0	0	0	3
<i>Raja clavata</i>	20	20	20	20
<i>Rhodeus amarus</i>	10	10	0	0
<i>Scophthalmus maximus</i>	10	10	0	10
<i>Sprattus sp.</i>	10	10	30	10
<i>Sprattus sprattus</i>	70	70	0	10
<i>Trachinotus blochii</i>	10	10	0	3
<i>Vinciguerria nimbaria</i>	0	0	0	10

Table S2: Comparison table of number of Fish taxonomic assignments and reads between *Mare-MAGE* and NCBI, and the 3 sub-databases for the 12s rRNA gene.

Species	NCBI	12sAllDB	12s90DB	12sDBc
<i>Argentina silus</i>	325	325	0	0
<i>Cleisthenes herzensteini</i>	0	5307	0	0
<i>Clupea harengus</i>	26846	26839	26844	26845
<i>Clupea pallasii</i>	16	6090	6090	6093
<i>Eutrigla gurnardus</i>	4407	4407	407	4407
<i>Gadus chalcogrammus</i>	0	320	1320	1320
<i>Gadus macrocephalus</i>	0	61	61	61
<i>Gadus morhua</i>	0	357	1357	1357
<i>Limanda limanda</i>	13617	13618	13616	13618
<i>Lophius piscatorius</i>	2784	2784	2784	2784
<i>Melanogrammus aeglefinus</i>	1585	0	0	0
<i>Merlangius merlangus</i>	1372	2957	0	0
<i>Merluccius merluccius</i>	7784	7784	7784	7784
<i>Platichthys stellatus</i>	0	30	20	5301
<i>Pollachius pollachius</i>	12	10	2652	0
<i>Pollachius virens</i>	2649	2650	0	2652
<i>Pseudopleuronectes americanus</i>	5309	5310	5310	0
<i>Reinhardtius hippoglossoides</i>	0	10	10	10
<i>Scomber scombrus</i>	19990	19990	19990	19990

<i>Sprattus sprattus</i>	6074	70	70	80
<i>Trisopterus luscus</i>	301	1336	1336	0

Siteweb:



4. Chapter IV:

Comparative analysis of bottom trawl and nanopore sequencing in fish biodiversity assessment: The Sylt outer reef example

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4.1. Abstract:

The assessment of fish diversity is crucial for effective conservation and management strategies, especially in ecologically sensitive regions such as marine protected areas. This study contrasts the effectiveness of environmental DNA (eDNA) metabarcoding analysis employing Nanopore technology with compare beam trawl surveys at the Sylt Outer Reef, a Natura 2000 site in the North Sea, Germany.

Out of the 17 fish species caught in a bottom trawl (using a 3m beam trawl), 14 were also identified through eDNA extracted from water samples. The three species not detected in the eDNA results were absent because they lacked representation in public DNA databases. The eDNA method detected twice as many fish species as the beam trawl, totalling 36 species, of which 14 were also detected by the trawl. Additionally, the selection of primers (Mifish) facilitated the identification of one marine mammal species, the harbour porpoise.

In conclusion, the findings underscore the potential of eDNA coupled with MinION sequencing (Long read technology) as a robust tool for biodiversity assessment, surpassing traditional methods in detecting species richness.

Keywords: Environmental DNA (eDNA), Trawl, fish biodiversity, Natura 2000, Sylt Outer Reef (SOR), metabarcoding.



Comparative analysis of bottom trawl and nanopore sequencing in fish biodiversity assessment: The sylt outer reef example

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ABSTRACT

The assessment of fish diversity is crucial for effective conservation and management strategies, especially in ecologically sensitive regions such as marine protected areas. This study contrasts the effectiveness of environmental DNA (eDNA) metabarcoding analysis employing Nanopore technology with compare beam trawl surveys at the Sylt Outer Reef, a Natura 2000 site in the North Sea, Germany.

Out of the 17 fish species caught in a bottom trawl (using a 3m beam trawl), 14 were also identified through eDNA extracted from water samples. The three species not detected in the eDNA results were absent because they lacked representation in public DNA databases. The eDNA method detected twice as many fish species as the beam trawl, totalling 36 species, of which 14 were also detected by the trawl. Additionally, the selection of primers (Mifish) facilitated the identification of one marine mammal species, the harbour porpoise.

In conclusion, the findings underscore the potential of eDNA coupled with MinION sequencing (Long read technology) as a robust tool for biodiversity assessment, surpassing traditional methods in detecting species richness.

1. Introduction

Preserving biodiversity and protecting key habitats and species are at the heart of the European Natura 2000 network of protected areas. The Natura 2000 network comprises Special Protection Areas (SPAs) and Special Areas of Conservation (SACs) under the Birds and Habitats Directive and aims to safeguard and ensure the long-term survival of the most valuable and threatened species and ecosystems (European Commission. (n.d.). Natura 2000). The Sylt Outer Reef (SOR) is one of Germany's Natura 2000 sites in the Exclusive Economic Zone (EEZ) of the North Sea and harbours a rich marine fauna, including a diverse fish community. In order to effectively conserve and manage these resources, a comprehensive understanding of fish diversity within the SOR is of vital importance.

To achieve the objectives outlined in the Natura 2000 initiative, a regular monitoring of these protected areas is essential to understand and safeguard their diversity. Trawl sampling, while being a classical tool to perform marine fish biodiversity monitoring, can inflict harm on sensitive habitats due to its invasive and possibly destructive nature.

Furthermore, trawling comes with a number of other disadvantages, namely its relatively high costs, labor-intensive nature, time-consuming processing, and limited spatial and temporal coverage (Greenstreet, 2008; Rodríguez-Mendoza and Saborido-Rey, 2023). This underlines the need to adopt non-invasive monitoring techniques that are in line with the Natura 2000 objectives of conserving threatened species and ecosystems without causing damage or disturbance.

Indeed, the use of remotely operated underwater vehicles (ROVs), satellite tracking, and sonar systems are among the non-invasive techniques that allow monitoring and assessment of marine biodiversity without disturbing its natural habitat (Busnel and Fish, 1980; Connolly et al., 2021). These tools, while effective, have shown certain limitations in their application, such as the inability to detect smaller or less mobile species, or the difficulty in covering large areas in a short amount of time.

To address these limitations, the emerging field of environmental DNA (eDNA) analysis offers a promising and innovative approach (Adams et al., 2019; Petit-Marty et al., 2023; Schwentner et al., 2021). By analysing genetic material in water samples, eDNA allows for the identification of species presences, even when traditional survey

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Glossary

eDNA	: Environmental DNA
Long-reads	Refer in this paper to the Technology allowing providing long-reads sequences, mainly MinION
Short reads	Refer in this paper to Illumina sequencing technologies

methods may be challenging or impractical (Maiello et al., 2022; Petit-Marty et al., 2023; Stoeckle et al., 2021). Therefore, it doesn't only circumvent invasive sampling methods, but even has the potential to provide a more comprehensive biodiversity assessment (Dickie et al., 2018; Schwentner et al., 2021).

Since its first application by Ficetola et al. (2008), eDNA has revolutionised the way marine biodiversity can be studied in a wide range of aquatic ecosystems (Adams et al., 2019; Coble et al., 2019; Rourke et al., 2021). This breakthrough has been made possible by the rapid development of molecular biology and sequencing tools and techniques. However, the majority of eDNA applications have been carried out using short-read technologies, particularly MiSeq, due to their low error rate (Stoler and Nekrutenko, 2021).

On the other hand, long-read sequencing technologies are still in very limited use (Doorenspleet et al., 2021; van der Reis et al., 2023). While these techniques promise potential benefits such as the ability to resolve complex genomic regions and provide a more complete picture of genetic diversity, they are still in their infancy in the eDNA field. Studies have shown that in addition to their ability to sequence long reads, these technologies are able to achieve a read quality of up to 99% (Banerjee et al., 2024; P. Chen et al., 2023). As a result, entire genes or even the mitochondrial genomes can be sequenced in a single read without the need for assemblies (Davidov et al., 2020; Doorenspleet et al., 2021; Latorre-Pérez et al., 2020). This makes it possible to improve the sensitivity of eDNA to distinguish between genetically closely related species. For example, MiFish primers are unable to distinguish among species of the scombrid genus *Thunnus*, the freshwater eel genus *Anguilla*, the salmonid genus *Oncorhynchus*, the puffer fish genus *Takifugu*, and the rockfish genus *Sebastes*, due to their genetic proximity (Miya, 2022). Long-read sequencing technologies can play a crucial role here, allowing more accurate species or even stock identification.

The objective of this study was to compare the efficacy of eDNA metabarcoding using MinION sequencer (Nanopore Technology) against traditional survey methods, specifically bottom trawling (i.e., beam trawling), in terms of species detection and diversity assessment. By providing a comprehensive assessment of fish diversity, the study aimed at showcasing the potential of eDNA as an additional tool for future monitoring in European Commission sites. By employing cutting-edge eDNA metabarcoding methods, the study aimed at providing a comprehensive and accurate assessment of fish diversity at the sampling sites.

2. Method

2.1. Trawl and catch

In the Natura 2000 site Sylt Outer Reef (SOR) 12 stations (Fig. 1) were sampled between the 30th June to the July 11, 2022 with a 3 m beam trawl, rigged with a sole net and five tickler chains. The mesh size in the cod end was 40 mm. Haul duration was fixed to 15 min with a towing speed of approximately 3 kn. Total catches were sorted and caught fish species were determined to the highest possible taxonomic level.

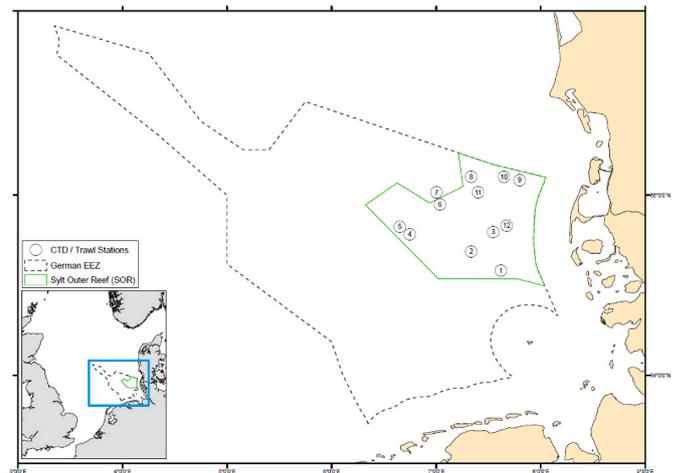


Fig. 1. Sampling sites in the Sylt Outer Reef (SOR; green line), German Bight North Sea. Dashed line is the German Exclusive Economic Zone.

2.2. Environmental DNA sampling

24 water samples from the 12 fishing stations (Table 1) plus 2 negative control samples were taken. Two 5-Liter samples were collected from each station. Water was collected in Niskin bottles and immediately filtered through 0.45 μm PES membranes in sterile funnel units (Sartorius, Göttingen, Germany) with a 2-port filtration system. Typically, 4 to 5 filters were used per sample, depending on the degree of filtration saturation. The filters were then stored in clean, sterile tubes at -20°C until DNA extraction. All filters from a single water sample were combined and processed as a single unit during eDNA extraction.

All Niskin bottles were thoroughly cleaned prior to water sample collection by rinsing first with fresh water and then with ultrapure water. Negative controls for all Niskin bottles used were performed by filtration of 0.5 L of ultrapure water rinsed through the cleaned bottles prior to the first sample collection. All filtration steps were performed in a pre-cleaned UV sterilization cabinet (Cleave Scientific, Rugby, UK). Clean gloves were worn during each sampling step and all materials used, e.g. water sample containers or membrane tweezers, were sterilized by washing with 3% bleach and/or UV sterilization where possible.

2.3. Environmental DNA extraction

eDNA was extracted from water according to Renshaw et al. (2015). In brief, filtered and frozen PES membranes were placed in a 2 ml tube and then supplied with 700 μL of CTAB buffer (2% CTAB (w/v), 1.4 M NaCl, 100 mM Tris, 20 mM EDTA) and incubated at 65°C for 10 min with agitation at 650 rpm. Subsequently, 900 μL of PCI Phenol/chloroform/isoamyl alcohol (25:24:1) was added and vortexed for 5 s,

Table 1

The geographical coordinates of water and fish sampling locations used in the present study. Trawling was performed directly after water sampling.

Station	Latitude	Longitude
1	54° 35.03	007° 37.11
2	54° 41.52	007° 20.16
3	54° 47.94	007° 32.71
4	54° 47.20	006° 44.91
5	54° 49.65	006° 39.16
6	54° 56.98	007° 02.23
7	55° 01.05	007° 00.21
8	55° 06.11	007° 20.08
9	55° 01.01	007° 47.92
10	55° 06.04	007° 38.89
11	55° 01.02	007° 24.04
12	55° 50.07	007° 40.43

before centrifugation at $15,000\times g$ for 5 min 700 μL of supernatant was transferred to a new 2 ml tube, prior to adding 700 μL of chloroform, followed by centrifugation at $15,000\times g$ for 5 min. After transferring 500 μL of the supernatant to a new 2 ml tube, the Monarch® cleaning kit (New England Biolabs, Frankfurt, Germany). The elution was transferred into 40 μL of elution buffer. DNA extracts were stored at -20°C until analysis. All DNA extraction steps were performed under a chemical hood in a dedicated area for eDNA analysis at the Thünen Institute in Bremerhaven.

2.4. Metabarcoding

2.4.1. Library preparation

All libraries were prepared with MiFish primers targeting the 12S rRNA gene according to the protocol of [Miya et al. \(2015\)](#). Briefly, the library preparation consists of two PCR steps. The first PCR cycle (1st PCR) aimed at specifically amplifying the fish 12S rRNA gene with adaptor sequences according to Nanopore recommendations. This PCR was performed in 8 replicates with a total reaction volume of 12 μL , including 2 μL of 1:5 diluted eDNA, 6.0 μL of $2\times$ KAPA HiFi HotStart ReadyMix (Roche, Germany), 2.8 μL of primer mix (Primer Mix: MiFish-E-F/R-v2: MiFish-U-F/R: MiFish-U2-F/R = 0.125:0.250:0.125 μM), and 1.2 μL sterile distilled water. These primer pairs co-amplify a hypervariable region of the fish mitochondrial 12S rRNA gene (approximately 180 bp).

The 1st PCR product was purified using the GeneRead size selection kit (Qiagen, Germany) to remove all sequences shorter than 150 bp and primer dimers. The purified product was then analyzed using the TapeStation 4150 to quantify and check for the presence of the fish band (approximately 380 bp), then diluted to a concentration of 0.1 ng/ μL . A second cycle PCR (2nd PCR) using the diluted 1st PCR product were performed to add Nanopore unique index to each sample as well as the Nanopore sequencer adapters. A total volume of 20 μL was used for the 2nd PCR, containing 7.5 μL of KAPA Master Mix, 5 μL of DNA, 0.5 μL of barcode primers and 7 μL of water. The thermal cycling profile after an initial denaturation at 95°C for 3 min was as follows (12 cycles): denaturation at 98°C for 20 s; annealing at 62°C for 15 s (as recommended by Nanopore) and extension at 72°C for 15 s with a final extension at 72°C for 5 min. The second PCR pooled equal volumes from all samples, and therefore, the product was isolated by size at approximately 370 bp. The 'fish band' was purified using the MinElute gel extraction kit (Qiagen, Germany), then quantified using the Qubit fluorometer.

Pool preparation for sequencing was done using the Ligation Sequencing Amplicons - Barcode PCR protocol with EXP-PBC096 and SQK-LSK112 kits (Oxford Nanopore Technologies) (Version: NBE_9065_v109_revY_9Mar2021). Briefly, approximately 200 fmol of purified amplification products were subjected to DNA repair and final preparation using NEBNext DNA Repair Mix and NEBNext Ultra II End Repair/dA-Tailing Module (New England Biolabs). The purpose of which is to repair the DNA and pre-prepare the DNA ends for adapter attachment, followed by attachment of the sequencing adapters to the DNA ends using T4 Ligase (New England Biolabs). Each step was followed by DNA purification with AMPure XP magnetic beads (provided in the sequencing kit).

Sequencing was performed on a MinION 10.4 cell according to Oxford Nanopore Technologies (ONT) instructions. Libraries were loaded into the Nanopore MinION Spot-on Flow Cell (FLO-MIN112, version R10.4) and sequenced for 10 h. The baseline call was performed automatically by the MinKnow program. Raw reads were obtained in FAST5 and FASTQ formats from which 'pass' quality reads were subjected to further analysis.

2.4.2. Environmental DNA data analysis

2.4.2.1. Raw data pre-processing. Reads were demultiplexed and adapters trimmed using MinKNOW Nanopore software. Primers and adapters were removed using Cutadapt with default settings and a 10% error tolerance. Quality was checked using Epi2m and FASTQC. Therefore, sequences were read quality filtered to a minimum of 6, while read length was limited to 145 bp using MinKNOW. The FASTQs of each sample were assembled into a single file using a "cat" command in a Linux environment. All analyses were performed in a unix-ubuntu 21.04 environment. The FASTQs were converted to FASTA using a cut adapter.

2.4.2.2. Taxonomic assignment of consensus sequences. To taxonomically assign the sequences in the fasta files, they were BLAST-ed against the fish specific MARE-MAGE database (12S North and Baltic Sea sub-database) ([Kasmi et al., 2023](#)). The unassigned ASVs were aligned to the NCBI database ([Benson et al., 2013](#)). A BLASTN (Basic Local Alignment Search Tool) search ([Chen et al., 2015](#)) was performed using the GenBank nucleotide database and MARE-MAGE locally installed via the BLAST package version 2.10.0. The complete nucleotide database was downloaded (Feb 1, 2023), version December 2022 was used for the MARE-MARE database. An R script was applied to select the OTUs assigned to species, genus and family levels according to the percentage of identity, based on Fishbase taxonomy ([Froese, 1990](#); [Froese and Pauly, 2019](#)). At similarities higher than 98% sequences were assigned to a species level, between 90 and 98% to a genus and at less than 90% to a family level.

2.4.2.3. Alpha and beta diversity. The assessment of fish biodiversity from metabarcoding eDNA was carried out using measures of alpha and beta diversity. Alpha diversity, which refers to the intrinsic diversity of a specific habitat, was measured using Shannon and Chao1 indices, which take into account the abundance, evenness and potential richness of species. These measures were calculated using the 'vegan' package in R. Beta diversity, which captures differences between fish communities in different environments, was analyzed using non-metric multidimensional scaling (NMDS) and Bray-Curtis dissimilarity. NMDS is a rank-based method that represents pairwise dissimilarity between samples in a low-dimensional space, while Bray-Curtis dissimilarity calculates compositional dissimilarity between different sites. The beta diversity analysis was performed using the 'vegan' package in R, and the results were visualised using 'ggplot2'.

2.4.2.4. Data visualization. Visualization of the results was performed in Krona and Rstudio using the ggplot and phylum packages.

3. Results

3.1. Bottom trawl results

Fish species diversity and abundance were assessed through bottom trawl sampling at 12 designated stations in the Sylt Outer Reef (SOR). A total of 17 fish species were identified and quantified during the monitoring. The most abundant species observed were common dab (*Limanda limanda*), with 2427 individuals, followed by plaice (*Pleuronectes platessa*) with 1063 individuals. Scaldfish (*Arnoglossus laterna*) and solenette (*Buglossidium luteum*) were also relatively abundant with 798 and 620 individuals, respectively. Common dragonet (*Callionymus lyra*), hooknose (*Agonus cataphractus*), and sand goby (*Pomatoschistus minutus*) were present in moderate numbers, while the remaining species were observed in lower numbers, ranging from 19 to 1 individual ([Fig. 2](#)).

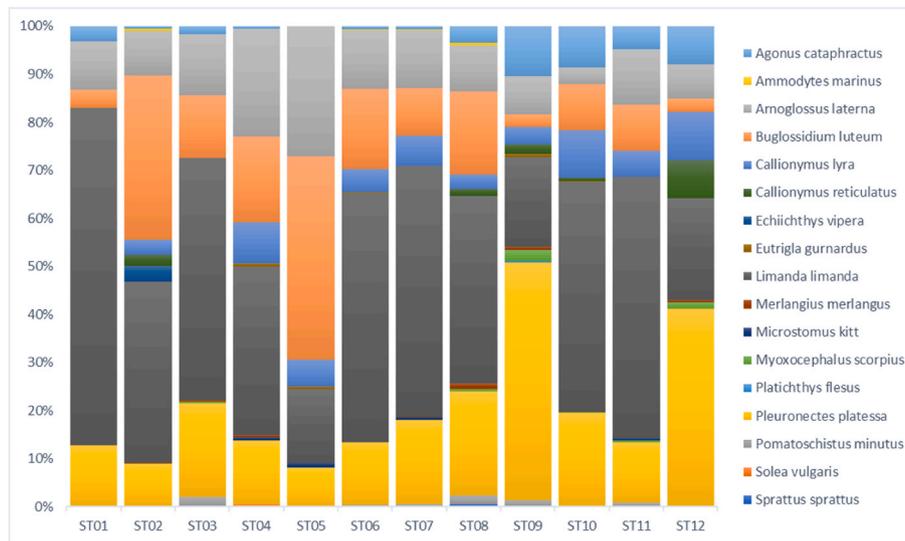


Fig. 2. Percentage of fish species by station sampled by a 3m beam trawl.

3.2. eDNA using metabarcoding

To reveal the ichthyodiversity in the Natura 2000 site Sylt Outer Reef, a metabarcoding method targeting the 12S rRNA gene was applied to 24 eDNA water samples from 12 stations. In total, two million reads were generated by the Nanopore Minion sequencer. 0.5 million reads (28.54%) were discarded due to the presence of chimeras, quality score and copy number, while 1.5 million reads (72.55%) were retained for ASV (Amplicon Sequence Variants) analysis. Of these, 42% could be assigned to distinct fish species, while 58% were assigned as unknown fish sequences. All sample files were of good quality for reads up to 260 bp. The percentage of fish reads ranged from 50% to 83% per sample, with a median of 72%. Negative controls were all without fish reads. The BLAST algorithm was used to taxonomically assign the ASVs, using the GenBank database.

The BLAST results identified diverse fish species with differences in species composition among the 12 sites sampling sites. A total of 36 fish species were detected (Table S1 and Fig. S1 A and B), with the higher abundant reads: The Atlantic mackerel (*Scomber scombrus*), cod (*Gadus morhua*) common dab (*Limanda limanda*), common dragonet (*Callionymus lyra*), European pilchard (*Sardina pilchardus*), grey gurnard (*Eutrigla gurnardus*) and Raitt's sand eel (*Ammodytes marinus*) accounting for 70% of all assigned ASVs (Fig. 2 A). Common mola (*Mola mola*), common sole (*Solea solea*), European anchovy (*Engraulis encrasicolus*), European eel (*Anguilla anguilla*), Greenland halibut (*Reinhardtius hippoglossoides*), haddock (*Melanogrammus aeglefinus*), lemon sole (*Microstomus kitt*), lesser weever (*Echiichthys vipera*), short-snouted seahorse (*Hippocampus hippocampus*) and turbot (*Scophthalmus maximus*) has much less reads, each representing less than 1% of the total fish ASV. About 98% of the ASVs were classified as bony fishes (Actinopterygii). Nevertheless, some sequences of mammals, namely of the harbor porpoise (*Phocoena phocoena*), were also detected, representing on average 1% of the relative abundance. The BLAST-results also revealed two non-native, North Pacific species as false-positives: The Pacific herring (*Clupea pallasii pallasii*) (132 reads) and Alaska pollock (*Gadus chalcogrammus*) (93 reads). A phylogenetic analysis revealed that Mifish primer-based 12S-sequences are unable to distinguish between these two species and their North Atlantic congeners (*Clupea harengus* and *Gadus morhua*) because the similarities among the two species-pairs is less than 2% (1–3 mutations in the 180 pb fragments). Considering the known error rate of the Nanopore technology, Pacific herring and Alaska pollock reads were therefore assigned to Atlantic herring and Atlantic cod, respectively.

The alpha and beta diversity indices were calculated in order to

characterize the fish diversity at the different stations. Alpha-diversity analysis revealed a mean richness of 35 and a Shannon diversity of 2.9., whereas the beta-diversity analysis showed significant differences between samples ($p < 0.05$), indicating relative spatial variation in the fish community composition (Fig. 3a).

3.3. Method comparison

The eDNA-analysis of water samples included almost all species caught by bottom trawling (Fig. 3), except for three species: the scaldfish (*Arnoglossus laterna*), reticulated dragonet (*Callionymus reticulatus*) and solenette (*Buglossidium luteum*) which at the time of the analysis were not available in any public genetics databases with regard to their mitochondrial 12S gene. However, considerably more species were detected through eDNA analyses compared to bottom trawling. Fig. 3b A total of 22 species were exclusively found by eDNA analysis, among them various pelagic species known to be underrepresented in beam trawls (Fig. 4). With the Harbour porpoise (*Phocoena phocoena*), even the presence of one marine mammal was confirmed.

4. Discussion

In the quest to understand and preserve marine biodiversity, the utilization of advanced scientific methods has proven pivotal. Environmental DNA analysis could be a game-changer in this context. However, case studies showing the general efficiency of the use of eDNA as compared to standardized reference methods in the marine realm are still scarce. The present study employed metabarcoding technology to assess fish diversity at 12 sampling stations in a Natura 2000 site in the North Sea.

The results show a significant difference in the number of detected fish species using eDNA metabarcoding compared with traditional bottom trawling. The metabarcoding approach detected a total of 36 species of fish as well as one marine mammal in the Sylt Outer Reef (SOR), whereas the beam trawl caught only 17 species. Many of the additional species detected with eDNA metabarcoding were pelagic and are - by nature - not caught with a beam trawl, which underpins the high efficiency of the method. The high concordance rate of 87% between trawling and eDNA metabarcoding further emphasizes the reliability and accuracy of the genetic approach. This suggests that eDNA metabarcoding can serve as a robust and reliable complement to traditional survey methods, depending on the study objectives. Using short reads technology (Illumina NovaSeq 6000) on the same area, Barco et al.

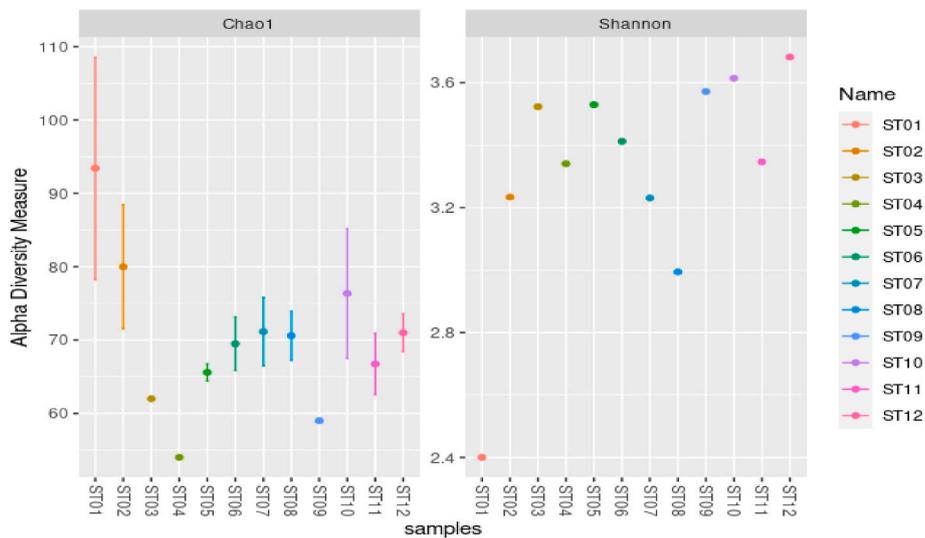


Fig. 3a. Chao1 and Shannon indices as measures for Alpha diversity. The error bars in the left panel represent the variability or uncertainty of the Chao1 index. On the right represents the alpha diversity measured using the Shannon index for different samples.

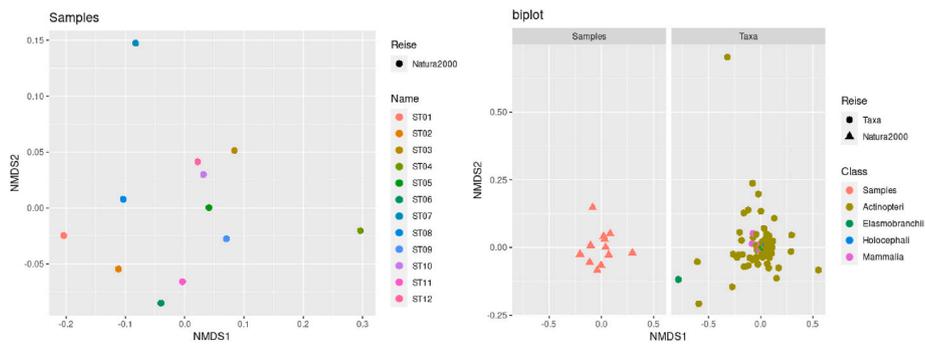


Fig. 3b. Beta-diversity analysis using non-metric multidimensional scaling (NMDS) and the Bray-Curtis dissimilarity.

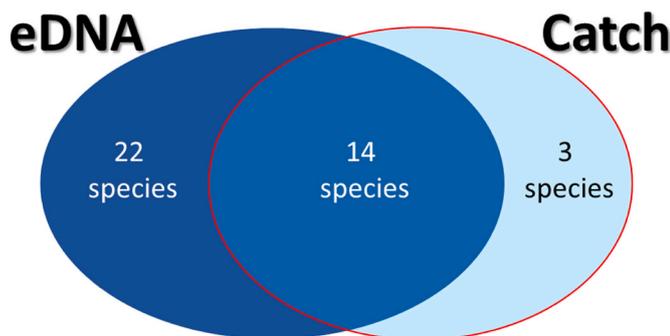


Fig. 4. Venn diagram showing the intersection between eDNA and catch results (per fish). In global, 22 species were detected only by eDNA, 14 species were detected by eDNA and Trawl, while 3 species were detected only by the trawl.

(2022) detected 24 fish species at 16 stations in the SOR using eDNA metabarcoding, which may be due to a higher threshold (99%) for alignment with reference data. The eDNA outputs of that study were compared to the online Database for Trawl Surveys (DATRAS) of the International Council for the Exploration of the Sea (ICES), in which it was found that 37% of the species were detected with both methods, which is in good agreement with our results (Barco et al., 2022).

4.1. Method comparison

A major advantage of eDNA analyses is undoubtedly its non-invasive nature. The use of classical fisheries methods potentially poses a risk especially for rare or even protected species, like marine mammals, and can further have a negative impact on habitats. Zhang et al. (2023) demonstrated that eDNA metabarcoding based on Mifish primers originally targeting fish diversity is also capable to detect whales, verified by the parallel use of species-specific primers and the visual observation. Therefore, the detection of the Harbour porpoise in the present study must be considered a valid observation. Valsecchi et al. (2021) already proposed primers for marine mammals designed on the basis of Mifish. In the case of cartilaginous fishes (sharks, skates and rays), Miya (2022) and Miya et al. (2015) proposed a slightly adapted primer set (Mifish-E). Despite using Mifish-E in this study, no cartilaginous species were detected.

One of the main obstacles for a standardized use of eDNA metabarcoding in biodiversity monitoring is the incomplete availability of quality-assured reference sequences in DNA databases. This can result in false statements about species absences and therefore negative findings. However, the available databases do often also not reflect genotypic plasticity of single species and, as a consequence, possible overlaps between closed related species. This often results in false positive results, so the erroneous detection of non-native species to a certain region, in our case of Pacific herring (*Clupea pallasii*) and Alaska pollock (*Gadus chalcogrammus*), highlighting the limitations of the Mifish primers in distinguishing between some North Sea and Pacific Ocean species due to

high similarity across the target region.

An eDNA advantage is its long half-life compared to reference methods: several studies on the kinetics of eDNA degradation in fresh and salt water show that eDNA has a half-life of 24 h, whereas trawling only covers a few minutes, with a maximum of 1 h (Collins et al., 2018; Harper et al., 2019). However, the eDNA decay can vary from taxon to another or depending on the biotic and abiotic factors (Scriver et al., 2023). Therefore, more research should be addressed to two fundamental questions: i) the persistence of eDNA in the water column dependent on physical and chemical parameters; and ii) the influence of hydrodynamic features, such as currents, on eDNA dispersal. Understanding these factors is important for an accurate interpretation of eDNA data and for inferring about the targeted area of interest.

However, it is crucial to acknowledge some limitations of eDNA. While eDNA metabarcoding offers a presumably rather comprehensive overview of fish diversity, it provides limited information on species abundance and individual sizes. Additionally, accurately identifying certain fish species based solely on their DNA sequences, especially for closely related taxa, remains a challenge. To address these limitations, future research efforts should focus on refining and expanding reference databases to enhance the accuracy of species identification. It is also important to note that eDNA analyses have a detection limit for each species, although it is rarely mentioned in eDNA research. This detection limit refers to the minimum amount of DNA from a particular species that must be present in a sample for it to be reliably detected. When the amount of DNA falls below this limit, the species may not be detected in the sample, leading to potential false negatives in the analysis. Therefore, understanding and accounting for the detection limit is crucial for accurately interpreting eDNA results and determining the presence or absence of species in a given environment. This may also explain the absence of three species in eDNA output, despite being present in the trawl catches.

Despite these limitations, the present results demonstrate the effectiveness of eDNA metabarcoding as a powerful tool for assessing fish diversity in the North Sea. The advantages of this approach over traditional survey methods are evident, providing a more complete understanding of species composition and enabling the detection of rare species. Integrating eDNA analysis into conservation and management strategies holds immense promise for the preservation and sustainable use of marine resources in the Natura 2000 network and beyond.

4.2. eDNA: comparison between long and short read sequencing technologies

The essential advantage of analysing eDNA with long-read technology, such as Nanopore, is their increased sensitivity for discriminating between closely related species (Davidov et al., 2020; De Vivo et al., 2022; Fonseca, 2018; Franco-Sierra and Díaz-Nieto, 2020; Gaonkar and Campbell, 2024; Stevens et al., 2023; Toxqui Rodríguez et al., 2023) and for reducing false positives as well as taxonomic artefacts as compared to short reads (Ramírez-Amaro et al., 2022). Thanks to continued progress in the development of long-read sequencers and chemistry, the reduction in error associated with these techniques promises to address more complex issues in biodiversity monitoring (Bogaerts et al., 2024; Stevens et al., 2023; T. Zhang et al., 2023), including quantitative assessments based on random short repeat sequences or micro-satellites (Adams et al., 2019; Andres et al., 2021, 2023; Couton et al., 2023; Hellström et al., 2023). By applying a ligation approach, long-reads also open up the possibility of PCR-free library preparations to prevent any PCR bias (Fonseca, 2018; Manu and Umapathy, 2023).

5. Conclusion

The application of metabarcoding in eDNA analysis has provided valuable insights into species distribution and relative abundance. However, to further enhance our understanding, it is crucial to combine

eDNA approaches with other non-invasive methods. This integrated approach holds great potential in supporting sustainable fisheries management and conservation strategies in the future.

The findings from the metabarcoding approach have revealed a significantly higher number of fish species compared to bottom trawling with a beam trawl, highlighting the limitations of conventional sampling techniques in capturing the full spectrum of species present. However, limitations have been observed in accurately quantifying species abundance using eDNA metabarcoding alone. These results contribute to the expanding body of knowledge on the application of eDNA in monitoring fish populations, forming a foundation for informed decision-making and the development of conservation strategies.

Further research is necessary to refine species identification, address limitations in quantification methods, and validate the models developed across different regions and fish species. The integration of eDNA analysis into conservation and management frameworks, such as the Natura 2000, holds great promise for the sustainable use of marine resources and the preservation of biodiversity.

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CRedit authorship contribution statement

Yassine Kasmi: Writing – original draft, Resources, Methodology, Formal analysis, Data curation. **Hermann Neumann:** Writing – review & editing, Conceptualization. **Holger Haslob:** Writing – review & editing, Validation, Resources, Methodology, Data curation, Conceptualization, Funding acquisition. **Tina Blancke:** Writing – review & editing, Formal analysis, Data curation. **Benita Möckel:** Resources, Investigation, Data curation. **Ute Postel:** Visualization, Validation, Supervision. **Reinhold Hanel:** Writing – review & editing, Supervision, Conceptualization.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.marenvres.2024.106602>.

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Supplementary Data

Title	Comparative analysis of bottom trawl and nanopore sequencing in fish biodiversity assessment: the Sylt outer reef example
Authors	Yassine Kasmi(1), Tina Blancke(1), Hermann Neumann(2), Holger Haslob(2), Benita Möckel(1), Ute Poste(1), Reinhold Hanel(1)
Date	01.05.2024
Resume	<p>This document presents the outcomes of metabarcoding analyses conducted using the MiFish primer, with sequencing performed by the Nanopore MinION technology. The primary aim of this analysis is to evaluate the impact of various threshold levels on the accuracy of species identification, particularly focusing on minimizing the occurrence of false positives during the assignment and subsequent BLAST analysis.</p> <p>To determine the impact of similarity threshold levels on the accuracy of species identification and minimize false positives in the assignment and subsequent BLAST analysis, we evaluated thresholds of 97%, 98%, 99%, and 100% against eDNA reads from a reference database. Our study reveals that a 98% similarity threshold delivers the most reliable results by effectively balancing the accuracy of species identification and the exclusion of artifact species. This stands as a marked improvement compared to the 96% and 97% thresholds. The chosen threshold also provides a broader view of the read contents than the more restrictive 99% and 100% thresholds. Our findings indicate that a threshold of 98% yields the most reliable results, striking an optimal balance between accuracy and the exclusion of artifact species—a notable improvement over the outcomes at the 96% and 97% thresholds. Additionally, this threshold level provides a more comprehensive overview of read contents in comparison to higher thresholds of 99% and 100%, which tend to be overly restrictive.</p> <p>In summary, the 98% threshold emerges as the most effective for aligning with expected results and reducing the occurrence of mistakenly identified species.</p>
100%	Results of BLAST with NCBI for only reads has hit 100%

99%	Results of BLAST with NCBI for only reads has over than hit 99%
98%	Results of BLAST with NCBI for only reads has over than hit 98%
97%	Results of BLAST with NCBI for only reads has over than hit 97%
Fig S1	Figure S1A: eDNA derived list of fish species and their Amplicon Sequence Variant (ASV) frequencies per sampling station.
Fig S2	Figure S1B: eDNA derived list of vertebrates (fish and mammal) families and their Amplicon Sequence Variant (ASV) frequencies per sampling station.
Table01	Sampling data localisation and time of sampling
Table02	Number of reads obtained in the taxon assignment.
	mifish primer

Results of BLAST with NCBI for only reads has hit 100%

Species	ST_01	ST_02	ST_03	ST_04	ST_05	ST_06	ST_07	ST_08	ST_09	ST_10	ST_11	ST_12
<i>Ammodytes personatus</i>	1	0	0	0	0	0	0	0	0	0	0	0
<i>Callionymus lyra</i>	11	1	0	0	0	0	0	0	0	0	1	0
<i>Clupea harengus</i>	0	0	0	0	0	0	1	0	0	0	0	1
<i>Clupea pallasii</i>	0	0	0	0	0	1	0	0	0	0	0	0
<i>Gadus morhua</i>	1	0	0	0	0	0	0	0	0	0	0	1
<i>Limanda limanda</i>	0	0	0	0	0	1	0	2	0	0	0	0
<i>Sardina pilchardus</i>	4	0	1	0	0	0	0	0	0	0	0	0
<i>Scomber scombrus</i>	0	0	0	0	0	0	1	0	0	0	0	0

Results of BLAST with NCBI for only reads has over than hit 99%

Species	ST_01	ST_02	ST_03	ST_04	ST_05	ST_06	ST_07	ST_08	ST_09	ST_10	ST_11	ST_12
<i>Ammodytes marinus</i>	2	1	0	0	0	0	0	1	0	1	0	1
<i>Callionymus lyra</i>	267	5	1	2	5	4	2	2	0	1	1	2
<i>Clupea harengus</i>	0	0	0	0	0	0	2	0	1	0	0	1
<i>Clupea pallasii</i>	0	0	0	0	0	1	6	1	0	1	0	0
<i>Cyclopterus lumpus</i>	1	0	0	0	0	0	0	0	0	0	0	0
<i>Eutrigla gurnardus</i>	1	2	0	1	0	0	1	1	0	0	3	0
<i>Gadus chalcogrammus</i>	0	0	0	0	0	1	0	0	0	0	0	0
<i>Gadus morhua</i>	1	1	0	0	0	2	2	1	0	0	4	2
<i>Hippoglossoides platessoides</i>	0	0	1	0	0	1	0	0	0	0	0	0
<i>Hippoglossus stenolepis</i>	0	0	0	0	0	0	0	0	0	0	0	2
<i>Limanda limanda</i>	6	2	1	0	0	3	1	4	0	2	2	0
<i>Myoxocephalus scorpius</i>	1	0	0	0	0	0	0	0	0	0	0	0
<i>Platichthys stellatus</i>	0	0	0	0	0	1	0	0	1	2	0	0
<i>Pleuronectes platessa</i>	2	0	0	0	2	0	0	2	1	2	0	1
<i>Pomatoschistus minutus</i>	0	3	0	0	0	0	0	0	0	0	0	0
<i>Sardina pilchardus</i>	18	1	1	0	2	0	2	1	0	0	2	1
<i>Scomber scombrus</i>	2	1	0	0	0	0	1	0	0	0	0	0
<i>Scophthalmus maximus</i>	2	0	0	0	0	0	0	0	0	0	0	0
<i>Sprattus sprattus</i>	0	0	0	0	0	1	5	1	0	0	0	0
<i>Trachurus trachurus</i>	0	0	0	0	0	0	0	0	0	1	0	0

Results of BLAST with NCBI for only reads has over than hit 98%

Name	ST01	ST02	ST03	ST04	ST05	ST06	ST07	ST08	ST09	ST10	ST11	ST12
<i>Agonus cataphractus</i>	10	6	3	1	4	1	2	0	1	20	99	3
<i>Ammodytes marinus</i>	498	229	17	0	23	67	23	602	14	44	33	24
<i>Ammodytes tobianus</i>	284	130	12	0	13	37	12	365	8	42	16	28
<i>Anguilla anguilla</i>	1	0	1	0	0	8	2	2	0	0	10	3
<i>Callionymus lyra</i>	6614	127	100	8	164	58	56	58	23	57	59	55
<i>Clupea harengus</i>	8	1	0	1	25	6	207	7	3	0	2	25
<i>Cyclopterus lumpus</i>	94	0	0	0	0	0	0	0	0	0	0	2
<i>Echiichthys vipera</i>	0	0	0	0	0	0	6	0	0	0	0	0
<i>Engraulis encrasicolus</i>	21	0	0	0	0	1	0	0	0	0	0	0
<i>Eutrigla gurnardus</i>	219	335	16	9	123	46	65	30	7	29	110	32
<i>Gadus morhua</i>	325	184	29	8	40	158	65	63	28	74	156	71
<i>Gymnammodytes semisquamatus</i>	57	8	18	5	13	2	39	27	1	14	26	18
<i>Hippocampus hippocampus</i>	8	6	1	2	3	1	0	2	4	5	3	2
<i>Hippoglossoides platessoides</i>	63	27	12	4	28	24	13	29	5	14	25	10
<i>Hippoglossus hippoglossus</i>	47	28	13	11	17	32	20	24	3	14	31	18
<i>Hyperoplus immaculatus</i>	42	9	2	0	0	6	1	41	0	3	2	5
<i>Hyperoplus lanceolatus</i>	232	117	5	0	11	38	17	268	7	22	21	16
<i>Limanda limanda</i>	446	177	107	38	221	175	97	193	42	129	211	121
<i>Melanogrammus aeglefinus</i>	5	3	1	0	6	0	1	3	0	0	1	0
<i>Merlangius merlangus</i>	115	39	37	8	60	28	30	38	9	36	32	66
<i>Microstomus kitt</i>	5	1	0	2	1	2	0	7	2	0	1	1
<i>Mola mola</i>	15	2	1	1	4	5	0	3	0	6	4	8
<i>Myoxocephalus scorpius</i>	141	0	5	1	1	1	0	0	0	8	0	1
<i>Platichthys flesus</i>	47	13	15	1	21	21	13	14	9	16	17	26
<i>Pleuronectes platessa</i>	236	90	33	2	101	47	39	35	15	107	52	127
<i>Pomatoschistus minutus</i>	206	213	24	2	9	5	14	37	6	15	37	13
<i>Reinhardtius hippoglossoides</i>	11	4	5	1	5	6	3	8	3	2	6	4
<i>Sardina pilchardus</i>	1368	241	92	49	89	120	84	77	46	110	257	136
<i>Scomber scombrus</i>	562	252	28	17	102	55	90	124	31	71	47	61

<i>Scophthalmus maximus</i>	6	1	0	8	0	0	0	1	0	1	0	0
<i>Solea solea</i>	1	2	0	0	2	2	12	1	0	1	3	1
<i>Sprattus sprattus</i>	69	35	24	13	57	29	385	52	11	16	10	18
<i>Trachurus trachurus</i>	47	32	90	4	40	20	40	13	17	38	35	72
<i>Phocoena phocoena</i>	48	11	10	0	15	12	9	15	6	18	8	19
<i>Clupea pallasii</i>	5	3	1	2	24	6	84	5	0	1	0	1
<i>Gadus chalcogrammus</i>	22	18	3	1	2	14	5	7	3	4	13	1

Results of BLAST with NCBI for only reads has over than hit 97%

Species	ST01	ST02	ST03	ST04	ST05	ST06	ST07	ST08	ST09	ST10	ST11	ST12
<i>Callionymus lyra</i>	6614	127	100	8	164	58	56	58	23	57	59	55
<i>Sardina pilchardus</i>	1368	241	92	49	89	120	84	77	46	110	257	136
<i>Limanda limanda</i>	446	177	107	38	221	175	97	193	42	129	211	121
<i>Ammodytes marinus</i>	498	229	17	0	23	67	23	602	14	44	33	24
<i>Scomber scombrus</i>	562	252	28	17	102	55	90	124	31	71	47	61
<i>Gadus morhua</i>	325	184	29	8	40	158	65	63	28	74	156	71
<i>Buglossidium luteum</i>	273	183	13	12	94	129	103	63	10	34	153	44
<i>Eutrigla gurnardus</i>	219	335	16	9	123	46	65	30	7	29	110	32
<i>Ammodytes tobianus</i>	284	130	12	0	13	37	12	365	8	42	16	28
<i>Pleuronectes platessa</i>	236	90	33	2	101	47	39	35	15	107	52	127
<i>Hyperoplus lanceolatus</i>	232	117	5	0	11	38	17	268	7	22	21	16
<i>Chimaera monstrosa</i>	216	43	49	12	83	38	50	42	21	66	39	92
<i>Sprattus sprattus</i>	69	35	24	13	57	29	385	52	11	16	10	18
<i>Pomatoschistus minutus</i>	206	213	24	2	9	5	14	37	6	15	37	13
<i>Syngnathus typhle</i>	105	37	36	13	73	28	29	34	17	42	28	62
<i>Merlangius merlangus</i>	115	39	37	8	60	28	30	38	9	36	32	66
<i>Chirolephis ascanii</i>	119	47	26	12	50	15	19	24	12	49	19	63
<i>Trachurus trachurus</i>	42	29	82	2	29	14	37	6	14	37	30	54

<i>Naucrates ductor</i>	68	19	22	4	40	9	19	20	10	28	24	63
<i>Zeus faber</i>	75	25	28	5	39	18	14	20	11	32	21	35
<i>Zoarces viviparus</i>	74	18	24	2	26	19	20	14	8	29	17	45
<i>Clupea harengus</i>	8	1	0	1	25	6	207	7	3	0	2	25
<i>Hippoglossus hippoglossus</i>	47	28	13	11	17	32	20	24	3	14	31	18
<i>Hippoglossoides platessoides</i>	63	27	12	4	28	24	13	29	5	14	25	10
<i>Myzopsetta ferruginea</i>	57	16	23	3	26	17	15	18	4	16	24	28
<i>Gymnammodytes semisquamatus</i>	57	8	18	5	13	2	39	27	1	14	26	18
<i>Platichthys flesus</i>	47	13	15	1	21	21	13	14	9	16	17	26
<i>Myoxocephalus scorpius</i>	141	0	5	1	1	1	0	0	0	8	0	1
<i>Agonus cataphractus</i>	10	6	3	1	4	1	2	0	1	20	99	3
<i>Muraena helena</i>	3	8	1	16	53	4	4	5	2	8	7	22
<i>Triglops murrayi</i>	25	3	7	4	12	4	6	9	5	15	5	24
<i>Hyperoplus immaculatus</i>	42	9	2	0	0	6	1	41	0	3	2	5
<i>Chelon ramada</i>	30	4	6	0	11	3	9	9	4	12	9	12
<i>Pagellus acarne</i>	35	7	7	1	7	6	7	7	4	10	10	6
<i>Ranzania laevis</i>	21	7	9	3	9	11	3	3	3	10	10	17
<i>Cyclopterus lumpus</i>	94	0	0	0	0	0	0	0	0	0	0	2
<i>Regalecus glesne</i>	19	7	5	1	10	6	2	7	3	4	6	11
<i>Nemichthys scolopaceus</i>	14	5	10	1	11	6	1	6	1	8	6	9
<i>Trachyrincus murrayi</i>	5	3	8	2	11	6	3	7	3	1	5	18
<i>Mullus surmuletus</i>	11	4	4	1	8	5	1	3	3	4	7	7
<i>Reinhardtius hippoglossoides</i>	11	4	5	1	5	6	3	8	3	2	6	4
<i>Macroramphosus scolopax</i>	15	5	6	0	3	7	1	3	2	5	7	2
<i>Leuciscus idus</i>	13	1	6	1	4	1	4	3	2	5	4	10
<i>Maurolicus muelleri</i>	7	1	4	1	8	3	4	2	5	3	6	8
<i>Mola mola</i>	15	2	1	1	4	5	0	3	0	6	4	8
<i>Coris julis</i>	9	4	1	0	7	0	2	3	3	5	1	11
<i>Gobius niger</i>	7	4	1	1	11	2	1	4	2	3	1	6
<i>Gadiculus thori</i>	9	4	2	1	4	0	2	3	2	1	2	9
<i>Hippocampus hippocampus</i>	8	6	1	2	3	1	0	2	4	5	3	2
<i>Argentina silus</i>	13	1	0	0	0	3	5	3	1	2	0	5

<i>Xiphias gladius</i>	6	2	5	0	0	1	3	4	0	3	0	9
<i>Argentina sphyraena</i>	12	5	0	0	2	3	1	1	1	1	3	3
<i>Spinachia spinachia</i>	9	4	1	1	2	2	1	2	2	3	0	5
<i>Nerophis ophidion</i>	5	2	2	6	6	0	3	1	0	3	1	2
<i>Anguilla anguilla</i>	1	0	1	0	0	8	2	2	0	0	10	3
<i>Solea solea</i>	1	2	0	0	2	2	12	1	0	1	3	1
<i>Engraulis encrasicolus</i>	21	0	0	0	0	1	0	0	0	0	0	0
<i>Microstomus kitt</i>	5	1	0	2	1	2	0	7	2	0	1	1
<i>Melanogrammus aeglefinus</i>	5	3	1	0	6	0	1	3	0	0	1	0
<i>Enchelyopus cimbrius</i>	3	1	0	0	0	7	1	0	0	1	1	4
<i>Scophthalmus maximus</i>	6	1	0	8	0	0	0	1	0	1	0	0
<i>Osmerus eperlanus</i>	3	0	0	0	1	0	0	0	0	8	0	0
<i>Echiichthys vipera</i>	0	0	0	0	0	0	6	0	0	0	0	0
<i>Pholis gunnellus</i>	0	6	0	0	0	0	0	0	0	1	0	0
<i>Clupea pallasii pallasii</i>	5	3	1	2	24	6	84	5	0	1	0	1
<i>Gadus chalcogrammus</i>	22	18	3	1	2	14	5	7	3	4	13	1

Figure S1A: eDNA derived list of fish species and their Amplicon Sequence Variant (ASV) frequencies per sampling station.

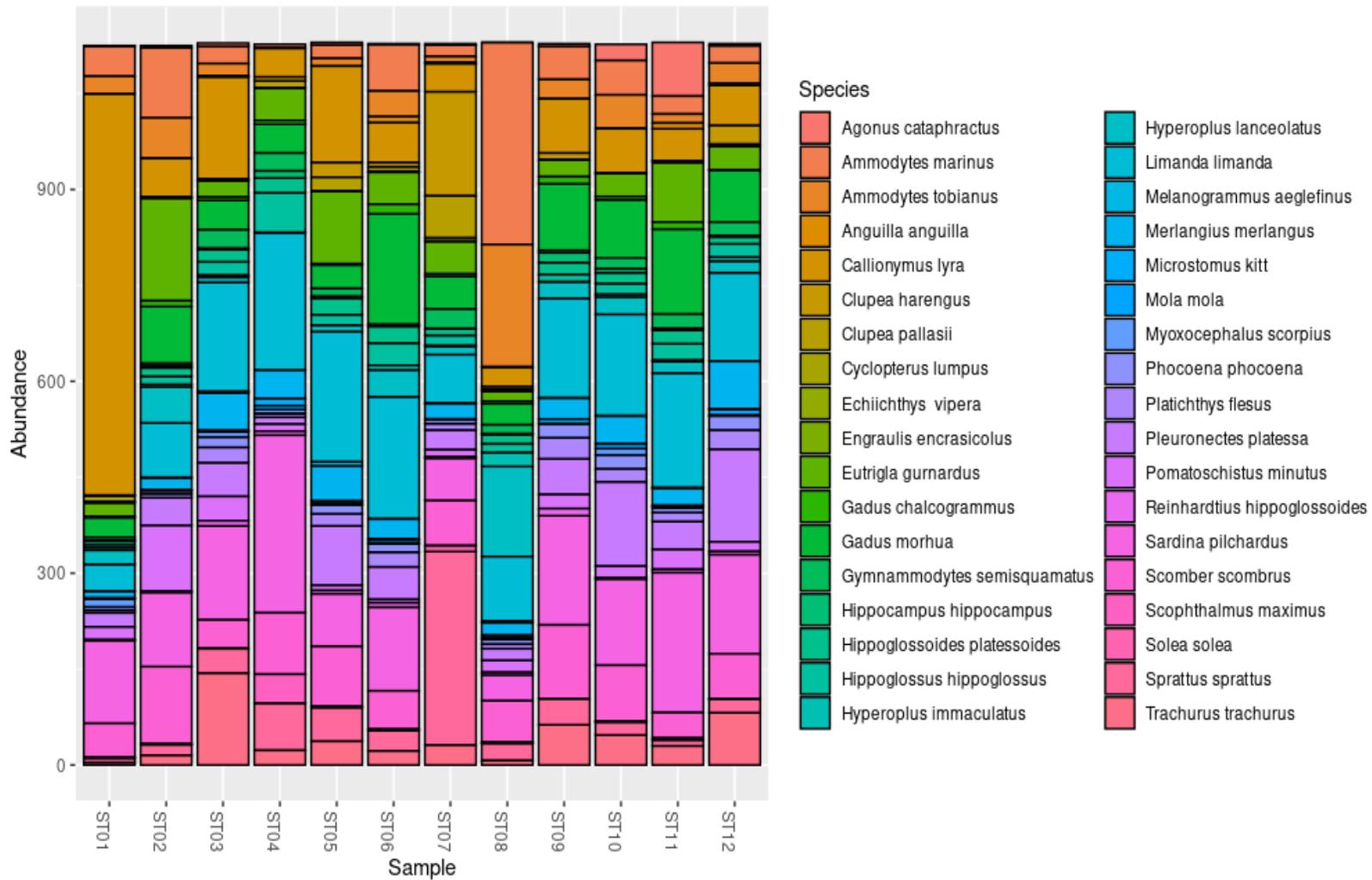
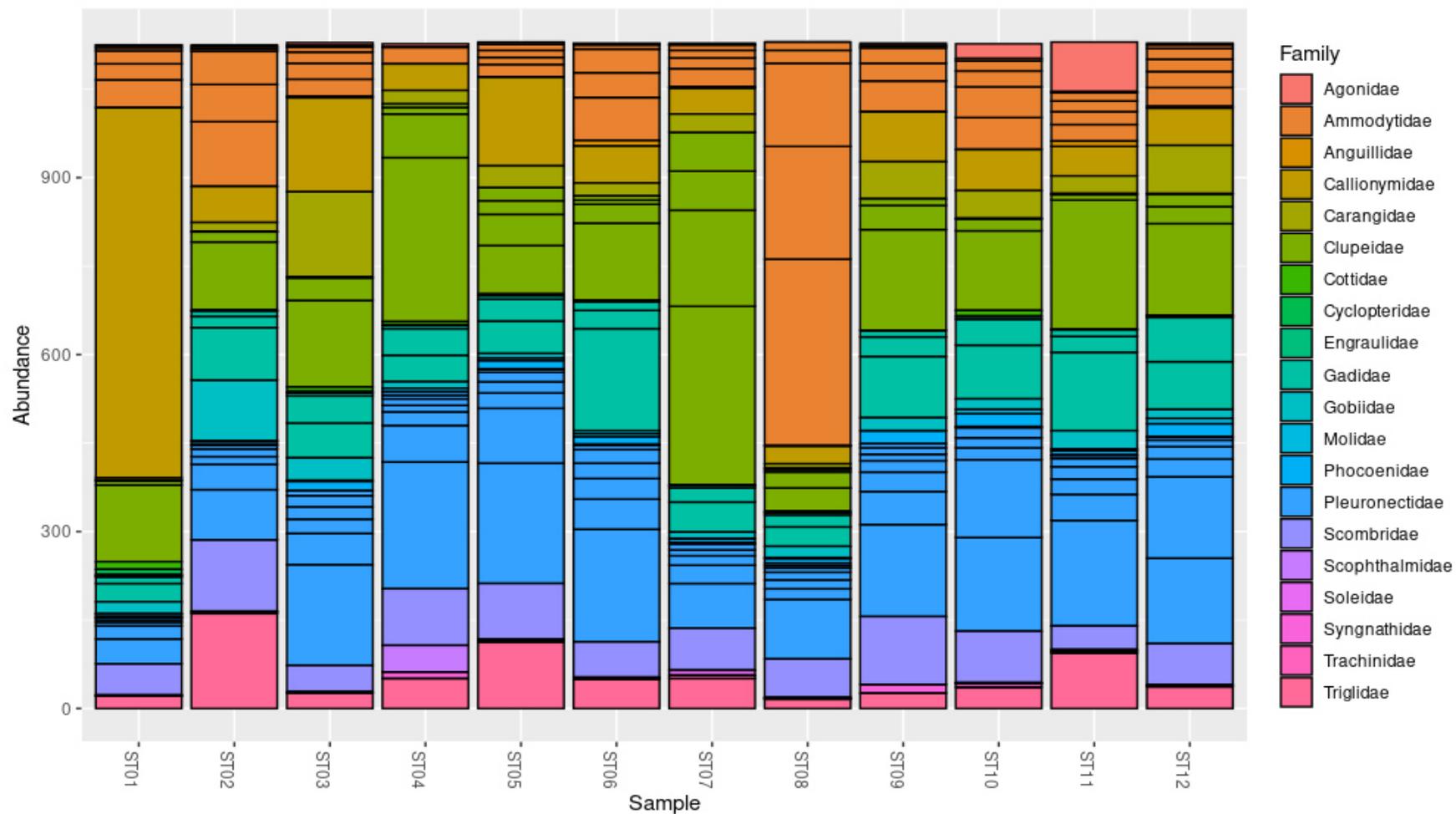


Figure S1B: eDNA derived list of vertebrates (fish and mammal) families and their Amplicon Sequence Variant (ASV) frequencies per sampling station.



Sampling data localisation and time of sampling

Station	Latitude E	Longitude N	Date of water sampling and trawling
1	54° 35.03	007°37.11	01.07.2022
2	54°41.52	007°20.16	01.07.2022
3	54°47.94	007°32.71	01.07.2022
4	54°47.20	006°44.91	01.07.2022
5	54°49.65	006°39.16	02.07.2022
6	54°56.98	007°02.23	02.07.2022
7	55°01.05	007°00.21	02.07.2022
8	55°06.11	007°20.08	02.07.2022
9	55°01.01	007°47.92	04.07.2022
10	55°06.04	007°38.89	04.07.2022
11	55°01.02	007°24.04	04.07.2022
12	55°50.07	007°40.43	04.07.2022

Table 2: Number of reads obtained in the taxon assignment. No-color is assigned to samples with no read, red color highlights the species which has less than 6 reads per sample and the green highlights the species detected with at least 6 reads.

Name	ST01	ST02	ST03	ST04	ST05	ST06	ST07	ST08	ST09	ST10	ST11	ST12	Sum	
<i>Callionymus lyra</i>	6614	127	100	8	164	58	56	58	23	57	59	55	7379	Not pelagic
<i>Sardina pilchardus</i>	1368	241	92	49	89	120	84	77	46	110	257	136	2669	Pelagic
<i>Limanda limanda</i>	446	177	107	38	221	175	97	193	42	129	211	121	1957	Not pelagic
<i>Ammodytes marinus</i>	498	229	17	0	23	67	23	602	14	44	33	24	1574	Not pelagic
<i>Scomber scombrus</i>	562	252	28	17	102	55	90	124	31	71	47	61	1440	Pelagic

<i>Gadus morhua</i>	325	184	29	8	40	158	65	63	28	74	156	71	1201	Not pelagic
<i>Eutrigla gurnardus</i>	219	335	16	9	123	46	65	30	7	29	110	32	1021	Not pelagic
<i>Ammodytes tobianus</i>	284	130	12	0	13	37	12	365	8	42	16	28	947	Not pelagic
<i>Pleuronectes platessa</i>	236	90	33	2	101	47	39	35	15	107	52	127	884	Not pelagic
<i>Hyperoplus lanceolatus</i>	232	117	5	0	11	38	17	268	7	22	21	16	754	Not pelagic
<i>Sprattus sprattus</i>	69	35	24	13	57	29	385	52	11	16	10	18	719	Pelagic
<i>Pomatoschistus minutus</i>	206	213	24	2	9	5	14	37	6	15	37	13	581	Not pelagic
<i>Merlangius merlangus</i>	115	39	37	8	60	28	30	38	9	36	32	66	498	Not pelagic
<i>Trachurus trachurus</i>	47	32	90	4	40	20	40	13	17	38	35	72	448	Pelagic
<i>Clupea harengus</i>	8	1	0	1	25	6	207	7	3	0	2	25	285	Pelagic
<i>Hippoglossus hippoglossus</i>	47	28	13	11	17	32	20	24	3	14	31	18	258	Not pelagic
<i>Hippoglossoides platessoides</i>	63	27	12	4	28	24	13	29	5	14	25	10	254	Not pelagic
<i>Gymnammodytes semisquamatus</i>	57	8	18	5	13	2	39	27	1	14	26	18	228	Not pelagic
<i>Platichthys flesus</i>	47	13	15	1	21	21	13	14	9	16	17	26	213	Not pelagic
<i>Myoxocephalus scorpius</i>	141	0	5	1	1	1	0	0	0	8	0	1	158	Not pelagic
<i>Agonus cataphractus</i>	10	6	3	1	4	1	2	0	1	20	99	3	150	Not pelagic
<i>Hyperoplus immaculatus</i>	42	9	2	0	0	6	1	41	0	3	2	5	111	Not pelagic
<i>Cyclopterus lumpus</i>	94	0	0	0	0	0	0	0	0	0	0	2	96	Not pelagic

<i>Reinhardtius hippoglossoides</i>	11	4	5	1	5	6	3	8	3	2	6	4	58	Not pelagic
<i>Mola mola</i>	15	2	1	1	4	5	0	3	0	6	4	8	49	Pelagic
<i>Hippocampus hippocampus</i>	8	6	1	2	3	1	0	2	4	5	3	2	37	Not pelagic
<i>Anguilla anguilla</i>	1	0	1	0	0	8	2	2	0	0	10	3	27	Pelagic
<i>Melanogrammus aeglefinus</i>	5	3	1	0	12	0	1	3	0	0	1	0	26	Not pelagic
<i>Microstomus kitt</i>	5	1	0	2	1	2	0	11	2	0	1	1	26	Not pelagic
<i>Solea solea</i>	1	2	0	0	2	2	12	1	0	1	3	1	25	Not pelagic
<i>Engraulis encrasicolus</i>	21	0	0	0	0	1	0	0	0	0	0	0	22	Pelagic
<i>Scophthalmus maximus</i>	6	1	0	8	0	0	0	1	0	1	0	0	17	Not pelagic
<i>Echiichthys vipera</i>	0	0	0	0	0	0	10	0	0	0	0	0	10	Not pelagic
<i>Clupea pallasii</i>	5	3	1	2	24	6	84	5	0	1	0	1	132	
<i>Gadus chalcogrammus</i>	22	18	3	1	2	14	5	7	3	4	13	1	93	
<i>Phocoena phocoena</i>	48	11	10	0	15	12	9	15	6	18	8	19	171	

5. Chapter V: Fish diversity and semi-quantitative stock assessment through metabarcoding of environmental DNA

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5.1. Abstract

A sustainable management of marine fisheries resources requires the continual development and adaptation of monitoring and tracking techniques, both in terms of species diversity and abundance. In order to find alternatives to common invasive catch-based biodiversity assessments, we tested a fish-specific environmental DNA (eDNA) metabarcoding approach, targeting the mitochondrial 12S rRNA gene. Analysis of 64 seawater and sediment samples revealed a high detectability of fish DNA in the North Sea, with more reliable results in water compared to sediment samples. Species diversity assessments overlapped by 81% between eDNA and bottom trawl catch analyses, with more than 32% of the species detected only by eDNA compared to bottom trawling as reference value (100%).

With regard to quantitative biomass estimates for common dab (*Limanda limanda*), the eDNA-based stock assessment revealed similar results as the bottom-trawl catch. Modelling of trawl outputs as a function of read counts and sampling depth yielded up to 90% correlation between the model and the observed data for common dab.

The outcomes of this study again highlight the potential of eDNA for marine biodiversity monitoring, not only for presence/absence assessments of species, but also for biomass estimates, with a high degree of reliability as compared to reference methods. The model reveals the importance to include environmental data to correct the error linked to the persistence of the eDNA in seawater.

Keywords: Marine biodiversity, environmental DNA, metabarcoding, 12S Mifish primers, semi-quantitative, Machine learning, whiting, European sprat, common dab.

1. Introduction

Many marine ecosystems are outstanding in terms of species richness (Alidoost Salimi et al., 2021; McQuatters-Gollop et al., 2022). However, biodiversity assessments in the sea are often biased due to water column and sediment structure related habitat complexity as well as rapid temporal changes. Classical monitoring schemes therefore mostly cover only a fraction of the diversity in a given area. For a more comprehensive overview of the present state of marine resources, the development and adaptation of new monitoring and tracking methods is inevitable, especially when they are the basis for management decisions.

The application of environmental DNA (eDNA)-based methods in fisheries research started in the beginning of the last decade (Foote et al., 2012). The increasing attention to the implementation of eDNA-based assessments is mainly due to their non-invasive nature and facilitated by the relatively long half-life of eDNA of up to 48 h in seawater (Collins et al., 2018). Numerous studies have demonstrated the potential of eDNA to reveal species richness in freshwater and the seas. Mathon et al. (2022) detected 16% more species by eDNA in 1224 stations than in underwater visual surveys in the Central and Southwestern Pacific and the Western Indian Ocean. Similarly, Doorenspleet et al. (2021) found eDNA results well suited to assess marine biodiversity as compared to trawl catches. In addition, Skelton et al. (2022) demonstrated a strong correlation between the number of eDNA reads and species abundance or biomass, although the detective power was weak for non-dominant species. Since a reliable detection of non-dominant and rare species still remains a major challenge in marine monitoring, new eDNA metabarcoding approaches could be a suitable tool (Evans et al., 2017). Various studies demonstrated the ability of Mifish primers targeting the mitochondrial 12S gene (Miya et al., 2015) to reveal fish diversity in marine and freshwater habitats. Other studies demonstrated the capacity of Mifish primers to detect also marine mammals (Valsecchi et al., 2021; Zhang et al., 2023).

Metabarcoding via high-throughput sequencing (HTS) generates millions to billions of sequence reads covering several giga to tera-bases in a single run. However, most metabarcoding studies only focus on qualitative (presence/absence) assignments of species, largely ignoring the quantitative information in the generated data, mostly due to a lack of adequate bioinformatic tools. Ladetto et al. (2014) and Xuan et al. (2013) already demonstrated that next-generation sequencing (NGS) has a sensitivity comparable to that of quantitative PCR (qPCR) in the assessment of minimal/measurable residual disease in B cell disorders. In marine

science, however, only few studies on quantitative aspects of NGS data exist (Kimmerling et al., 2018, p. 1; Lamb et al., 2019; Thomas et al., 2016).

However, one aspect often largely ignored is the potential impact of abiotic factors on the persistence and degradation of eDNA in the aquatic ecosystems. Qian et al., (2022) found that higher water temperature increases the degradation rate of eDNA. Other studies highlighted the influence of sampling season, temperature, pH, salinity and UV on eDNA-based biomass and abundance estimates (Doi et al., 2019; Doi, Inui, et al., 2017; Doi, Katano, et al., 2017).

The present study aimed at determining the effectivity of eDNA to assess marine fish diversity using high-throughput DNA sequencing in the North Sea environment. Furthermore, the possibility of extracting quantitative information from NGS outputs, as opposed to solely qualitative information, was tested in combination with modelling approaches comparing the number of reads obtained from HTS with bottom trawl fisheries data.

2. Method

2.1. Sampling

2.1.1. Water and Sediments

Sampling was conducted from 8 July to 6 August 2019 during the scientific research survey WH428 aboard the German fisheries research vessel Walther Herwig III. A total of 16 International Bottom Trawl Survey (IBTS) monitoring stations covering a wide gradient of environmental conditions were sampled in the North Sea (Figure 1).

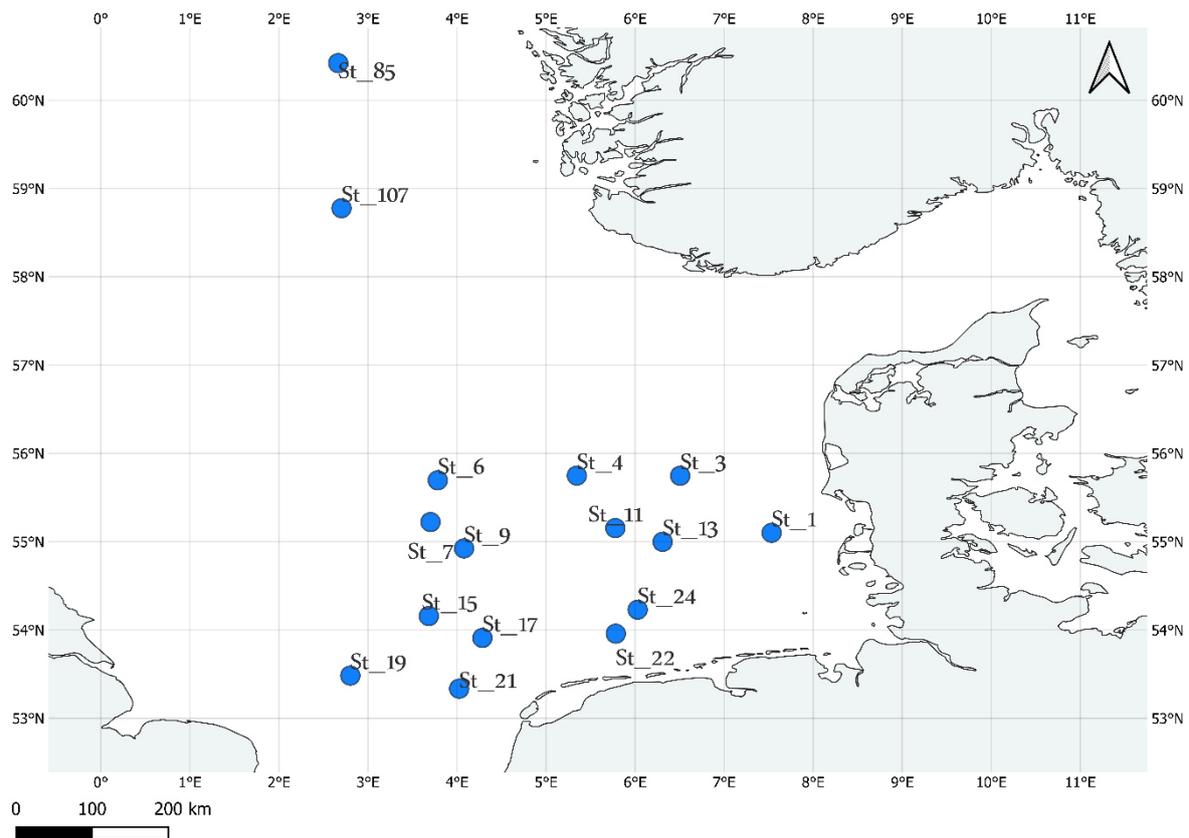


Figure 1: Sampling map. Blue circles indicate sampling stations.

Water: Water samples were collected in two 5 L Niskin bottles of a CTD (conductivity-temperature-depth) rosette at approximately four meters above the seafloor at each of the 16 sampling stations to avoid sediment contamination while still matching the average bottom trawl height of around five meters. The 10 L of water at each sampling station were divided in two 5 L replicates and immediately filtered on board through Sartorius™ PES membranes (pore size: 0.45 μm , diameter: 47 mm) with a vacuum pump. After filtration, the filters were stored at -20°C until DNA extraction. In addition, 32 negative samples of ultrapure water used to rinse the Niskin bottles prior to sampling were taken. Contamination avoidance included the thorough rinsing of the CTD rosette and the Niskin bottles with fresh water on deck immediately after recovery, followed by rinsing with ultrapure water. The on-board workbench area was covered with aluminium foil and rinsed with a 20% commercial bleach solution followed by ultrapure water. Each lab bottle was rinsed three times with sample water and then filled to a 2.5 L mark. All further processing of the samples took place in a sterile environment in a molecular biology lab on land.

Sediments: In total, 32 superficial sediment samples were collected at the 16 sampling positions in duplicates (two per station) using a common Van Veen grab. The grab was rinsed with sterile water in between sampling. Approximately 50 g (\approx 40 ml) of the superficial strata of the sediment were taken with a plastic spoon (one per sample) and placed into a 100 ml sterile container filled with 60 ml of pure ethanol and stored at -20°C until extraction.

Depth, salinity and temperature data were obtained in parallel with a CTD probe (CTD 48 M, Sea and Sun, Germany).

2.1.2. Trawl assessment

Demersal trawling was carried out according to the International Bottom Trawl Survey (IBTS) standards (ICES, 2020). Trawling was conducted for up to 30 min at a trawl speed of approximately 4 knots using the standard bottom trawl GOV (chalut à Grande Ouverture Verticale), immediately after water sampling at each station. Each catch was sorted by species. Weights of the total catch and of each sorted species were recorded together with length measurements of all specimens. For selected species, such as common dab (*Limanda limanda*), European sprat (*Sprattus sprattus*) and whiting (*Merlangius merlangus*) (ICES, 2020), single fish weight measurements, sex and maturity stage discrimination were performed according to IBTS standard species for subsequent laboratory age determination. In this study, catch per unit effort (CPUE) is defined as the number of fishes of a certain species that are caught per hour of trawling.

2.2. DNA Extraction

2.2.1. eDNA extraction from Water samples

eDNA was extracted from water according to Renshaw et al. (2015). In brief, filtered and frozen PES membranes were placed in a 2 ml tube and then supplied with 700 μL of CTAB buffer (2% CTAB (w/v), 1.4 M NaCl, 100 mM Tris, 20 mM EDTA) and incubated at 65°C for 10 min with agitation at 650 rpm. Subsequently, 900 μL of PCI phenol/chloroform/isoamyl alcohol (25:24:1) were added and vortexed for 5 s, prior to centrifugation at $15,000 \times g$ for 5 min. 700 μL of supernatant was transferred to a new 2 ml tube, prior to adding 700 μL of chloroform, followed by centrifugation at $15,000 \times g$ for 5 min. After transferring 500 μL of the supernatant to a new 2 mL tube, the Monarch® cleaning kit (New England Biolabs, Frankfurt, Germany) was used by adding 1000 μL of binding buffer. The elution was performed into 20 μL of elution buffer. DNA extracts were stored at -20°C until analysis. All DNA extraction steps were performed under a chemical hood in a dedicated eDNA area of a genetics laboratory.

2.2.2. eDNA extraction from Sediments

The extraction of eDNA from the sediments was performed by using the DNeasy PowerSoil Pro Kit (Qiagen, Germany) according to the manufacturer's instructions. Ultrapure water was used as negative extraction control and treated the same way as regular samples throughout the experiment. DNA extracts were stored at -20°C until analysis. All DNA extraction steps were performed under a chemical hood in a dedicated eDNA area of the genetics laboratory.

2.3. Sequencing and NGS

All libraries were prepared with MiFish primers (Table 1) targeting the 12S rRNA gene, according to the protocol of Miya et al. (2015). The library preparation followed two PCR steps. The first PCR cycle (1st PCR) aimed at specifically amplifying the 12S rRNA gene of fishes with adapter sequences. The second PCR cycle (2nd PCR) was performed using the diluted 1st PCR product and Illumina adapters for binding to the Illumina sequencer flow cell (MiSeq). NGS was performed with an Illumina MiSeq sequencer.

Table 1: 12S rRNA primers

Name	Sequence (5' – 3')
<i>MiFish-U-F</i>	ACACTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNGTCGGTAAAACTCGTGCCAGC
<i>MiFish-U-R</i>	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NNNNNNCATAGTGGGGTATCTAATCCCAGTTG
<i>MiFish-E-F-v2</i>	ACACTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNRGTGGTAAATCTCGTGCCAGC
<i>MiFish-E-R-v2</i>	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NNNNNNGCATAGTGGGGTATCTAATCCTAGTTG
<i>MiFish-U2-F</i>	ACACTCTTTCCCTACACGACGCTCTTCCGATCT NNNNNNGCCGGTAAAACTCGTGCCAGC
<i>MiFish-U2-R</i>	GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT NNNNNNCATAGGAGGGTGTCTAATCCCCGTTG

The 1st PCR was performed in a reaction volume of 12 µl containing 6.0 µl of 2 × KAPA HiFi HotStart ReadyMix (Roche, Germany), 2.8 µl of each primer (Primer Mix: MiFish-E-F/R-v2: MiFish-U-F/R : MiFish-U2-F/R = 0.125 : 0.250 : 0.125 µM), 1.2 µl of H₂O sterile distilled water, and 2.0 µl of eDNA. The primer pairs co-amplify a hypervariable region of the fish mitochondrial 12S rRNA gene (approximately 180 bp) and add primer binding sites (5' ends of the five N forward sequences) for sequencing at both ends of the amplicon. The five random bases were used to improve cluster separation on flow cells during initial base-calling calibrations on the MiSeq platform. The thermal cycling profile after an initial 3 min denaturation at 95 °C was as follows (35 cycles): denaturation at 98°C for 20 s; annealing at

65°C for 15 s; and extension at 72°C for 15 s, with a final extension at the at 72°C for 5 min. Four replicates were performed for the 1st PCR, and replicates were pooled to minimise PCR dropouts into a single 1.5 ml tube. Then, the products were purified with a GeneRead Size Selection Kit (Qiagen, Germany) to remove all sequences less than 150 bp in length. The purified products were diluted to a concentration of 0.1 ng/μL after quantitative and qualitative verification by TapeStation4200. The 2nd PCR was performed in a reaction volume of 15 μl containing 7.5 μl of 2 × KAPA HiFi HotStart ReadyMix, 0.88 μl of each primer (5 μM), 3.88 μl of sterile distilled H₂O and 1.86 μl of template. MiSeq primers included the dual index sequences (8 nucleotides indicated by X's) and flow cell binding sites for the MiSeq platform (5' ends of sequences before eight X's) were used. The thermal cycling profile after an initial 3 min denaturation at 95°C was as follows (12 cycles): denaturation at 98°C for 20 s; combined annealing and extension at 72°C for 15s, with a final extension at 72°C for 5 min. The concentration of each 2nd PCR product was measured by Qubit. Subsequently, the pooled 2nd PCR product was size-isolated to approximately 370 bp and the "fish band" was purified by the MinElute Gel Extraction Kit (Qiagen, Germany). The product was sequenced on the MiSeq platform using a MiSeq v2 Reagent Kit (2 × 150 bp) (Illumina, Inc.).

2.4. Bioinformatics analysis for metabarcoding data

The quality of the reads was checked by using the FASTQC program (Babraham Bioinformatics). Subsequently, forward and reverse reads were assembled by vsearch via the *-fastq_mergepairs* option. Cutadapt was used to trim the reads and remove primers. Subsequently, Operational Taxonomic Unit (OTU) analysis was performed on sequences with a similarity of 98% or more, while Amplicon Sequence Variant (ASV) analysis took place on those with a confidence percentage $\geq 75\%$. For both methods, only sequences with a fragment length between 160 and 260 bp were selected. The processed reads were subjected to a BLASTN search of the complete NCBI (Macher et al., 2017) and MAREMAGE (Kasmi et al., 2023) databases. All sequences originating from organisms other than bony fish, sharks and rays or with lengths less than ≤ 150 bp were eliminated. All sequences with similarities of more than 97% between the queries and the BLASTN were assigned to a distinct species. Sequences with a similarity below 97% but higher than 92% were assigned to a distinct genus, while all sequences with similarities less than 92% but higher than 75% were assigned to a family level (Xiong et al., 2022).

2.5. Computational modelling of metabarcoding data

Assuming that there is a possible quantitative relationship between the number of fish caught in the bottom trawl and the number of DNA reads from metabarcoding, we investigated the possibility of modelling the number of fish caught as a function of the number of reads. Data from the four most abundant fish species, Grey gurnard (*Eutrigla gurnardus*), Common dab (*Limanda limanda*), whiting (*Merlangius merlangus*), and European sprat (*Sprattus sprattus*), were used for this purpose. These four species were chosen because they were observed at high abundance (according to Tukey's divergence index results) at most stations and they represent distant taxonomic bony fish groups. The number of reads was modelled against the trawl results by univariate analysis using linear regression modelling methods, namely generalized least squares (GLS). In addition, we considered applying multivariate analysis using depth, salinity and water temperature as well as number of reads per litre as predictive descriptors and catch data as response value/model output. Pressure, salinity and temperature were chosen as parameters with potential effects on the persistence of eDNA in seawater.

To assess the relationship between biomass and eDNA counts from the metabarcoding data, two generalised linear models (GLM) were selected out of 150 models based on their correlation with biomass predictor, lower AIC values and a significant number of model terms. The GLM response in both models was catch per unit effort (CPUE) in km²/h combined with log link functions. Predictors included the observed number of eDNA copies per litre of sampled water (referred to as $eDNA_{observed}$ in this paper) and the expected number of eDNA copies at the time of sampling ($eDNA_{expected(t_0)}$ or eDNA0) (eq 01). Utilizing $eDNA_{expected(t_0)}$ instead of $eDNA_{observed}$ allows us to standardize the initial amount of eDNA present at the time of sampling. This standardization helps to address and mitigate potential biases that arise due to variations in the timing of filtration and the different conservation methods used, ensuring a more accurate and consistent comparison across samples.

$$eDNA_{expected(t_0)} = \frac{eDNA_{observed}}{e^{-(t_1+r_1+t_2+r_2)}}, \quad \text{Eq01}$$

with $eDNA_{observed}$ is the number of eDNA reads observed in this data. While r_1 and r_2 are the decay rate of eDNA at 24°C equivalent to filtration temperature and 4°C (conservation temperature), respectively according to Scriver et al., (2023), multiplied by the filtration and conservation time represented by t_1 and t_2 in this equation, respectively.

We also included environmental factors derived from the CTD data, mainly sampling depth as a proxy for correlated environmental factors and water temperature. To reduce multicollinearity, variables with more than 70% correlation were removed, leaving only two key variables for the final analysis: 1. Sampling depth, expressed as a positive value starting from 0 at sea surface and 2. Temperature expressed in degrees Celsius. Given the high correlation between temperature and depth (approximately 70%), only one environmental factor was included in each model to reduce the risk of false positive correlations. To assess the relationship between biomass and each predictor, we calculated partial model effect by varying the one predictor while holding all other variables fixed to their sample average.

To further explore the relation between the response variable and the model predictors, we constructed non-linear models based on the two chosen GLMs, by inserting a parameter into a logarithmic function (Eq. 2 and 3). The model parameters were estimated using the Nelder-Mead method (Nelder & Mead, 1965). Initial values of the non-linear model coefficients were estimated from the GLM model, with the coefficients of the environmental factors within the logarithm initially set to 1. The Nelder-Mead method is a robust numerical method widely used to find the minimum of an objective function in a multidimensional space. To estimate the significance of the model coefficients, 1000 Monte Carlo simulations were performed using random data within the standard deviation limits of the original data.

$$\log(\text{CPUE}(eDNA_0, \text{Depth})) = (\alpha + \beta \cdot (\text{Depth})) + \gamma \cdot \log(eDNA_{\text{expected}(t_0)} - \delta \cdot \text{Depth})$$

Eq :02

$$\log(\text{CPUE}(eDNA_0, T_p)) = (\alpha \cdot T_p + \beta \cdot (T_p^2)) + \gamma \cdot \log(eDNA_{\text{expected}(t_0)} - \delta \cdot T_p)$$

Eq :03

The Nelder-Mead model equation serves as a tool for estimating biomass levels based on a combination of eDNA and various environmental factors. This model uses two interdependent complementary functions to elucidate different aspects of the relationship between eDNA, environmental conditions and biomass $\log(\text{CPUE}(eDNA_{\text{expected}(t_0)}, \text{environmental factor})) = g(\text{environmental factor}) + f(eDNA_{\text{expected}(t_0)}, \text{environmental factor})$. The first function describes the influence of environmental factors on biomass, denoted $g(\text{environmental factor})$, and provides insight into how factors such as depth affect the overall biomass composition within a given ecosystem. The second function $f(eDNA_{\text{expected}(t_0)}, \text{environmental factor})$ explores the interplay between eDNA concentration ($eDNA_{\text{expected}(t_0)}$),

environment (e.g., eDNA degradation) and catch per unit effort (CPUE), revealing nuanced relationships between these variables. In this function, CPUE is modelled as a logarithmic function of both eDNA concentration and depth, recognizing the non-linear nature of these relationships. Depth is used as a proxy for other environmental factors that may not be directly measurable but are nonetheless influential. The coefficients within the equation, including α , β , γ and δ , represent the strength and direction of these relationships and provide insight into how changes in eDNA concentration and depth affect CPUE. Importantly, the inclusion of an interaction term (δ) highlights the nuanced relationship between eDNA concentration and depth, suggesting that the influence of eDNA on CPUE may vary with depth in the water column. This comprehensive model provides a valuable framework for understanding the complex dynamics of biomass estimation in aquatic ecosystems, offering insights that can inform ecological studies and resource management strategies.

All analyses and data visualizations were performed in R. The GLM was implemented using the GLM package and the `glm.r` function, while the Nelder-Mead optimization was performed using the 'nelder mead' package.

Replicates from the same station were treated as independent samples. CPUE in a specific area was used as a basis for prediction. CPUE was used in kg/h and fish/h.

eDNA copy numbers in the original seawater samples (X , copies/L) were calculated based on the volume of filtered seawater (a : 5 L), the re-suspended volume of purified DNA (b : 20 μ L), the amount of purified DNA added to each NGS-reaction volume (c : 2 μ L/ reaction), and the number of reads in your NGS data (Y : reads/NGS reaction).

3. Results:

3.1. Fish Biodiversity

3.1.1. Results of eDNA

3.1.1.1. Sequencing performance and pre-processing

Metabarcoding of 34 water samples generated a total of 15,982,574 12S rRNA paired-end reads, in the MiSeq platform (Illumina), compared to 7,208,776 reads in the sediment samples (3,604,388 each for forward and reverse). All sample files were of high quality for reads of up to 260 bp. The reads were filtered for primers, chimeras, quality score and copy number. From

all water and sediment libraries, 85% of all reads were merged. The percentage of fish reads was between 40% and 98% per sample, with an average of 84%. All negative control water samples were tested negative. For the sediments' negative controls 2 samples included less than 10 reads of European pilchard, therefore considered as negative as well.

3.1.1.2. eDNA sequencing from water samples

The species composition revealed from the water samples was dominated by bony fishes (Actinopterygii) with an average relative abundance of 92% (48 species) (Figure 2). Cartilaginous fishes (Elasmobranchii) were only represented by the Thorny skate (*Amblyraja radiata*) and the Porbeagle shark (*Lamna nasus*). In addition, with the harbour porpoise (*Phocoena phocoena*) a marine mammal was recorded using MiFish primers at stations 6 and 7, in the central North Sea.

The most abundant bony fish orders in our results were the Clupeiformes, Pleuronectiformes, Scombriformes, Perciformes and Gadiformes, respectively, covering more than 75% of the total number of species. At the genus level, an average of 27.5% and 13.7% of the reads were attributed to *Clupea* and *Limanda*, respectively. The fish species present in almost all stations within the study area were Atlantic herring (*Clupea harengus*), Grey gurnard (*Eutrigla gurnardus*), Common dab (*Limanda limanda*), whiting (*Merlangius merlangus*), starry flounder (*Platichthys stellatus*), European plaice (*Pleuronectes platessa*), Atlantic mackerel (*Scomber scombrus*), Common sole (*Solea solea*), and European sprat (*Sprattus sprattus*) (p-value <0.001) (Figure 2). 38% of the reads couldn't be assigned to any fish genus or species, 3% were assigned to human or mouse.

Amplicon sequence variant (ASV) analysis (based on confidence percentages $\geq 75\%$) increased the number of assigned species to 53 compared to 45 from Operational Taxonomic Unit (OTU) analysis. Only sequences with a fragment length between 160 and 260 bp were assigned. ASV also reduced the number of unassigned reads from 41% to 38%.

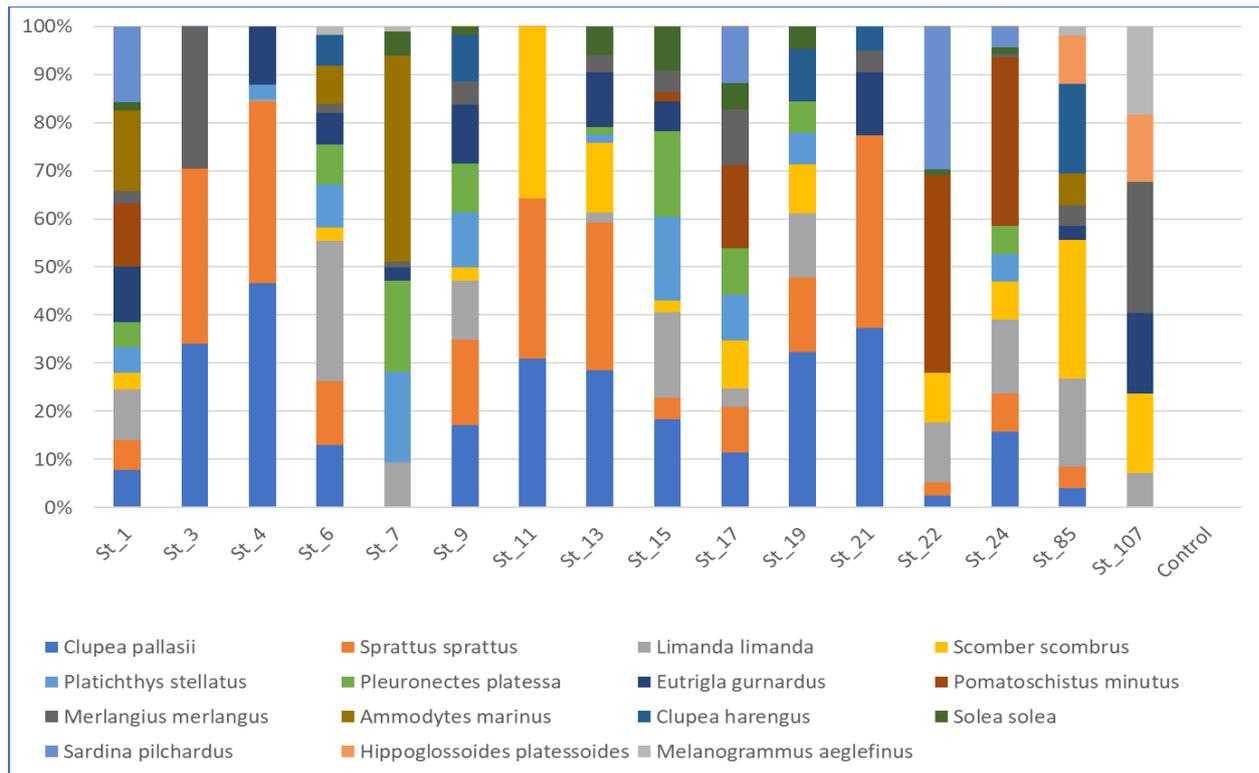


Figure 2: The 15 most common fish species in water samples. The Y-axis represents the percent of reads for each species into each sample (relative abundance), while the X-axis presents the stations.

Fish composition clearly differed between sampling sites, with a general increase in abundance and diversity with increasing latitude, as revealed from both, water and sediment eDNA analyses. Results of sediment eDNA were almost all negative in the southern stations and the closer to the German coasts, with a low presence of Common dab and American sole, an obvious misidentification. Diversity increased significantly at the northernmost stations and along the northern Danish coasts.

Overall misidentification rate amounted to 19%, with 6 of the falsely identified species (11%) originating from the Pacific Ocean and 4 species (8%) from the North Atlantic and Arctic. However, many of the obviously false records have closely related, often congeneric relatives in the North Sea, especially in the family Gadidae, with differences of only one to three (0.5-1.5%) nucleotides in the target region of the 12S rRNA gene. The most common water-derived false positive hit was Starry flounder (*Platichthys stellatus*), for sediment-derived eDNA White perch (*Morone americana*).

3.1.1.3. eDNA sequencing from Sediments samples

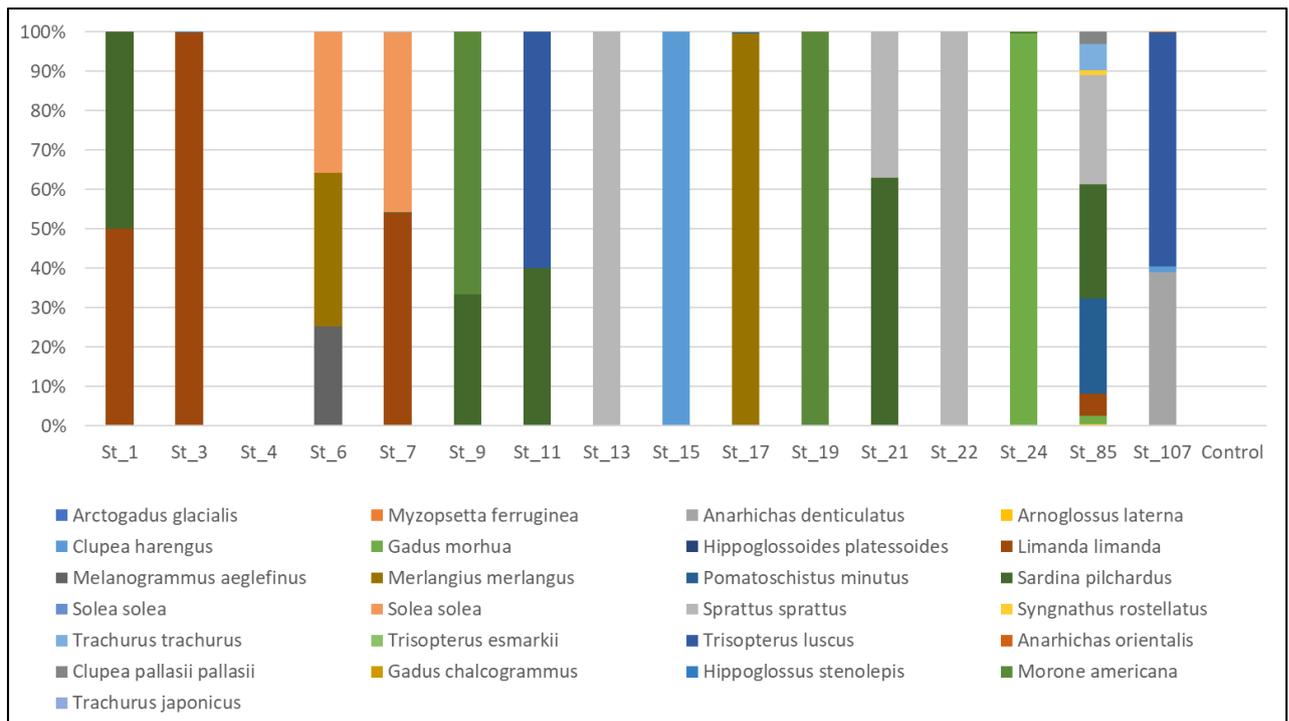


Figure 3: Fish biodiversity in sediment samples. The Y-axis represents the percent of reads for each species into each sample (relative abundance), while the X-axis presents the stations.

The metabarcoding results of the sediments showed a heterogeneous diversity together with an uneven distribution of species between stations. Many southern stations showed low richness, in which 10 from 14 stations showing less than 5 species.

In total, 25 fish species were recorded in sediment samples, of which 18 (72%) were detected only in station no. 85. The most common species detected in sediment eDNAs were European pilchard, European sprat, sand goby and Common dab. (Figure 3)

The fish composition in sediment samples was dominated by bony fish with an average relative abundance of 99.3% (24 species) (Figure 3), dominated by the orders Clupeiformes, Gadiformes and Pleuronectiformes, (Table S3). Furthermore, 69% of the reads remained unassigned and 19% were of human or mouse origin.

3.1.2. Trawl catches

3.1.2.1. Geographical distribution of fish species

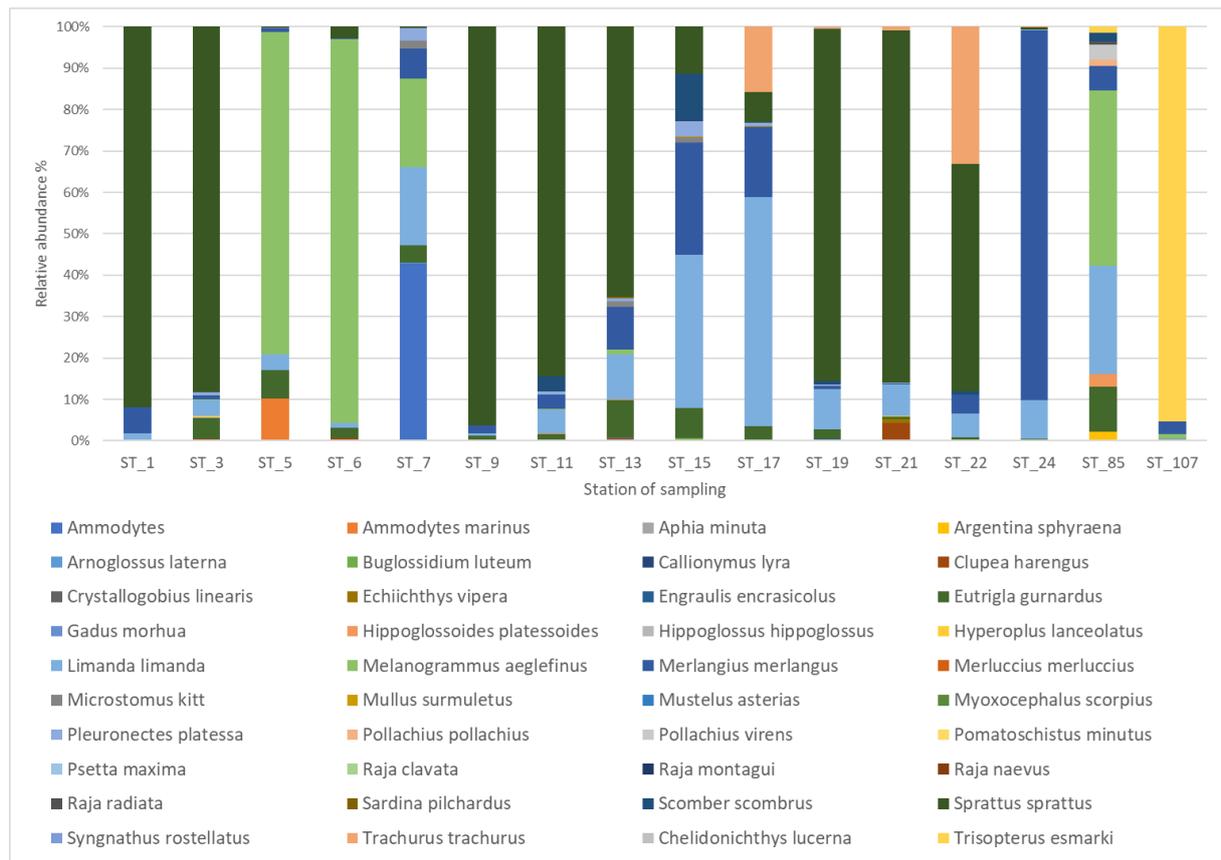


Figure 4: Fish biodiversity as relative abundance (%) in the trawl samples (by station).

Trawl data analysis confirmed the observation trend of an increase in fish diversity and abundance with increasing latitude (Figure 4), with a maximum catch of 48 950 kg/h in western Norway and a minimum of 12.4 kg/h at station 13 in the southern North Sea. The most abundant species across all stations were European sprat, Norway pout, whiting and haddock.

3.2. Comparison between trawl and DNA

81% of the fish species detected by the bottom trawling were also detected by eDNA. Moreover, eDNA was able to reveal the presence of more species than bottom trawling. Seven of the bottom trawls caught species were not detected in the respective eDNA analyses due to the unavailability of their 12S rRNA sequences in public genetic databases at the time of the study: solenette (*Buglossidium luteum*), lesser weever (*Echiichthys vipera*), Common dragonet (*Callionymus lyra*), Great sand eel (*Hyperoplus lanceolatus*), starry smooth-hound (*Mustelus asterias*), Spotted ray (*Raja montagui*), and Tub gurnard (*Chelidonichthys lucerna*). Another 8 species were not detected by eDNA most likely because their DNA was below the detection limit (1 to 3 individuals per catch): European anchovy (*Engraulis encrasicolus*), Atlantic halibut (*Hippoglossus hippoglossus*), Striped red mullet (*Mullus surmuletus*), shorthorn sculpin

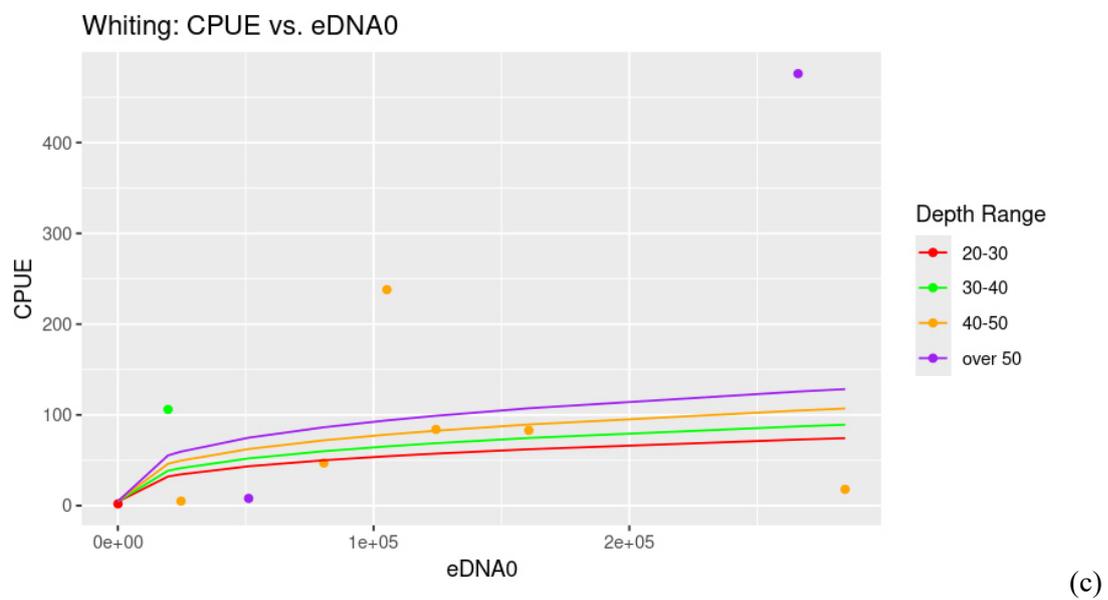
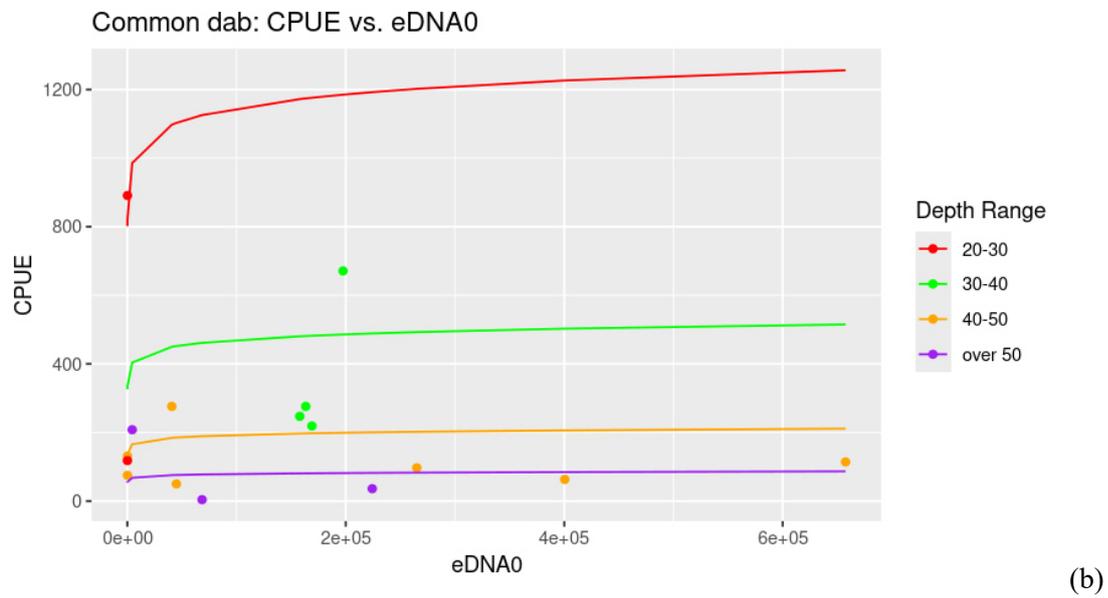
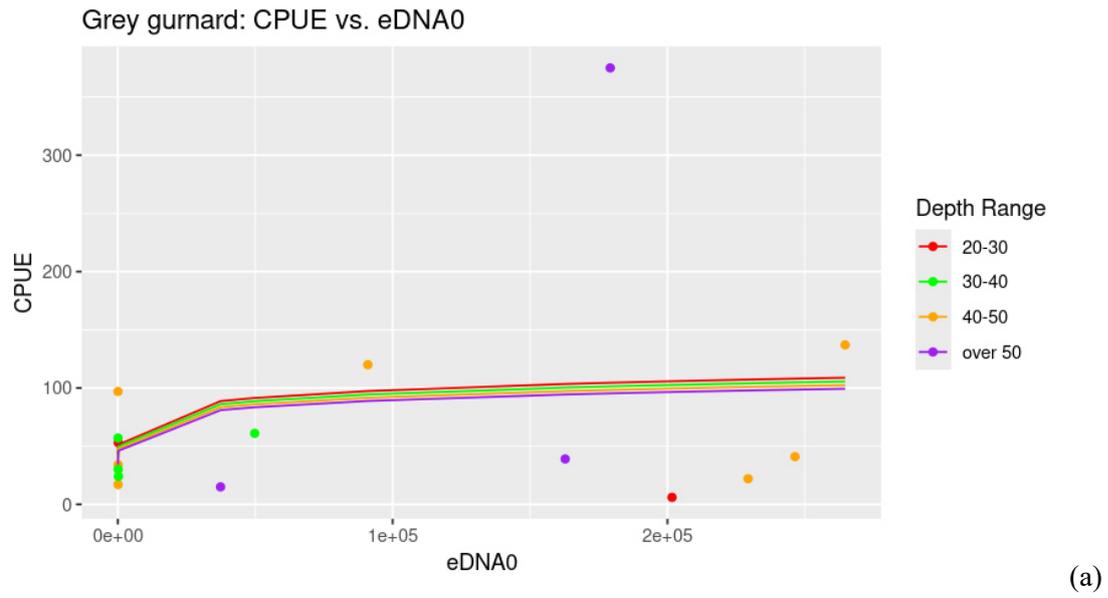
(*Myoxocephalus scorpius*), turbot (*Scophthalmus maximus*), thornback ray (*Raja clavata*) and Cuckoo ray (*Leucoraja naevus*).

3.3. Modelling reads vs trawling

The univariate correlation between log read counts and fish quantity in CPUE/h was low (between 14 and 35%) for the four targeted species. The correlation rate increased significantly by adding sampling depth as a predictive descriptor in a multivariate approach. The non-linear model led to a correlation of up to 90% for European sprat (Table 2). The latter was revealed as best method for predicting fish biomass as a function of read count per litre including sampling depth (eq. 02). For a second model (eq. 03) that included temperature instead of depth, the correlation between eDNA and CPUE achieved 70%. Applying the same model to whiting, and common dab, the correlation between model outputs and observed values was between 70% and 90% for the model with depth, except for Grey gurnard that has showed low correlation, around 13% (Table 2 and Figure 5), but the predictors significance was lower than in model of depth.

Table 2: Comparison of the regression between the model outputs and the observed catch data.

Model	Link	Grey gurnard		Common dab		Whiting		European sprat	
		AIC	R2	AIC	R2	AIC	R2	AIC	R2
Eq02	log	117	0.13	161	0,70	151	0.90	312	0.91
Eq03	log	131	0.91	196	0,31	151	0.90	380	0.69



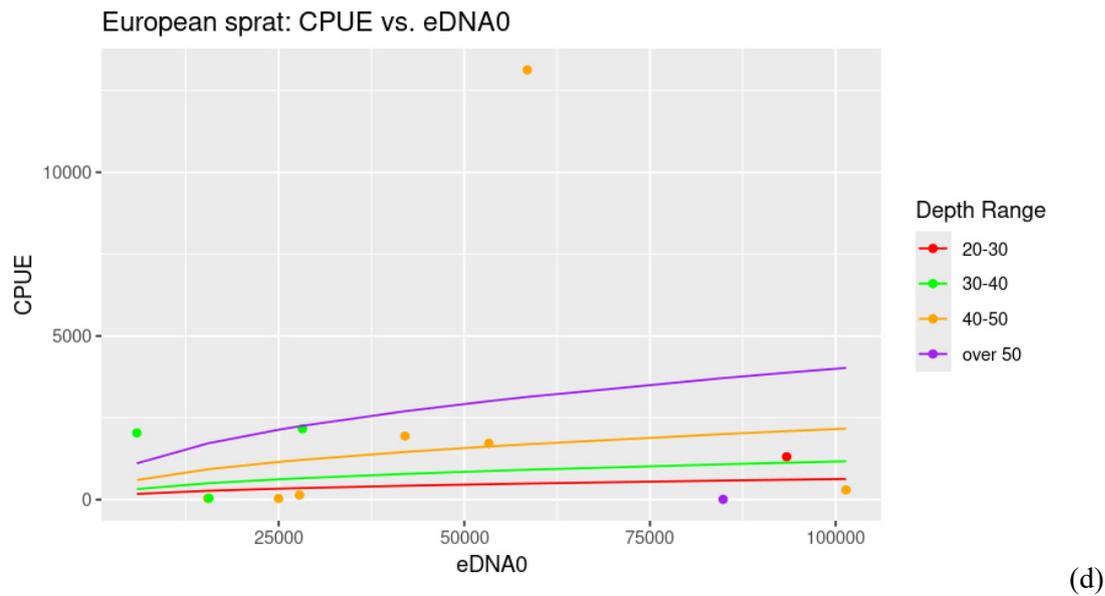


Figure 5: Model simulation of Biomass (CPUE) in function of eDNA at time of sampling ($eDNA_{expected}(t_0)$).

4. Discussion

A direct comparison of the results of an eDNA metabarcoding approach and standardized bottom trawl catches revealed the general suitability of non-invasive genetic methods for fish biodiversity assessments in the North Sea. In addition, a quantitative analysis based on the number of eDNA reads showed a positive correlation with fish biomass and density for common dab, European sprat, gurnard and whiting. Modelling approaches for fish biomass and abundance in relation to the number of eDNA reads and sampling depths based on ensemble learning techniques revealed strong non-linear correlations for the selected target species, except for the Grey gurnard, which showed a relatively weak correlation between eDNA and biomass using the depth model.

4.1. Biodiversity

Water-derived eDNA analyses much better reflected fish biodiversity compared to eDNA from sediment sources. By analyzing sediments, fish were only detected in 50% of the stations, whereas water-derived eDNA yielded a 100% detection rate. In contrast, the proportion of non-fish reads (including high numbers of phytoplankton and microbial) was significantly higher in sediments compared to water.

The primary reason for this disparity can be explained by a higher homogeneity of water compared to sediment. Sediments, being less homogeneous, provide a favourable niche for

bacteria to form biofilms, resulting in 2-1000 times richer bacterial contents compared to water (Sander & Kalff, 1993). This disparity poses challenges in obtaining reliable information, and it needs to be tested, if an increase of the number of sediment samples per station would result in a more comprehensive understanding of fish composition. Sediments are reported as valuable archives that potentially enable to trace back the ecological history of marine regions and the determination of the impacts of biotic and abiotic factors on biodiversity over a period spanning more than two million years (Kjær et al., 2022; Ogata et al., 2021; Sakata et al., 2020; Turner et al., 2015). However, targeting ancient DNA requires alternative sequencing techniques, preferably the application of shotgun methods, such as whole mitochondrial metagenomics (Doane et al., 2018).

In contrast, water-derived eDNA results, though rather providing a snapshot picture, matched very well with the concomitant bottom trawl catches. Exceptions include species yet unrepresented for 12S rRNA sequences in genetic public databases and rather rare, mostly benthic species. However, eDNA results also revealed species missing in the bottom trawl, including various mostly pelagic bony fish but also sharks (porbeagle) and cetaceans (harbor porpoise). This is not surprising, considering net avoidance capabilities of fast swimming large pelagic species. The standard IBTS trawl (GOV) differs in its catchability for different fish species (Fraser et al., 2007). In addition, the trawl has a scare effect by e.g. the action of the otter doors and trawl sweeps on the seabed stirring up a sediment cloud, allowing some fish species to avoid the net (Kaartvedt et al., 2012), while other species are herded into the path of the net (Engås & Godø, 1989). Considering a half-life of eDNA in seawater of approximately 24 hours (Barnes et al., 2014), it is plausible for fish passing the area minutes or hours before the net was deployed but still contributing to the eDNA data. Barnes et al. (2014) and Maruyama et al. (2014) demonstrated that eDNA from fish in ambient water has a relatively slow degradation rate of 10% per hour and 90% per day, allowing for a longer-term view of fish diversity compared to short-duration bottom trawl surveys. Govindarajan et al. (2021) also found that fish composition from eDNA remains stable for up to 16 hours, making it a sensitive and relatively long-term monitoring tool. This highlights the advantage of eDNA in providing meaningful information and a comprehensive overview of species richness over a longer timeframe compared to fisheries-based reference methods.

Another advantage of eDNA, particularly from water samples, is its ability to identify all species regardless of fish size, unlike traditional methods that may be limited by mesh-size selectivity. This is in line with previous studies demonstrating eDNA to be compatible with

trawl or other reference methods both in marine and freshwater environments (Andruszkiewicz et al., 2017; Baker et al., 2018; Ficetola et al., 2015; Hinlo et al., 2017; Sakata et al., 2020) and an explanation for eDNA detecting more fish species than trawling at most stations in this study. Additionally, sampling water at different depths can provide a more comprehensive understanding of fish species richness, with qualitative results comparable to reference methods (Govindarajan et al., 2021). However, it is important to note that this aptitude to detect all sizes of species can also be perceived as a disadvantage. Indeed, knowledge of the length and/or life stage of fish can be crucial for fish stock assessments. Furthermore, eDNA cannot provide additional information on population structure, such as age, sex or size distribution, which is often key information for species management and conservation. It is also crucial to mention that the persistence time of eDNA in water can distort the results. The long persistence of eDNA in the water, after the organism has left the area, can lead to an overestimation of species abundance. Consequently, it is essential to take eDNA persistence into account when interpreting eDNA results.

The integration of different target genes can be beneficial to enhance the sensitivity of eDNA methods, potentially even allowing stock differentiation (Kumar et al., 2022). Furthermore, combining eDNA with other non-invasive monitoring methods like hydroacoustic and visual surveys into a unified can offer a more comprehensive overview of fish biodiversity, distribution and biomass estimation.

Despite a generally high coherence between eDNA results and the known distribution of the respective species, some misidentifications occur, mostly based on high similarities of closely related species in the 12S rRNA region covered by the Mifish primers. While the total number of misidentified species is not significant (7 from 53 species), the total number of reads for some of them is high, suggesting the potential presence of undiscovered 12S rRNA haplotypes in the target species. In the same line Miya et al. (2020), Nester et al. (2020) and Takeuchi et al. (2019) mentioned the possibility to detect false positive species by Mifish primers in marine and freshwater eDNA due to various reasons and conclude on the inability of the primers to distinguish between closely related species. To solve this problem, primer modifications and target sequencing fragments of up to 1000 bp might be necessary to enhance the discriminative power as compared to current methods limited to 180-200 bp. However, new methods need to include solutions to also mitigate the high error rates associated with longer fragment sequencing. Another problem is the misannotation of available sequences in public databases, emphasizing the need for curated reference genomic databases. It is also recommended to

increase the number of PCR replications and pooling them in order to reduce the risk of false negatives in eDNA metabarcoding studies (Miya et al., 2015, 2020).

One of the main limitations of the application of eDNA approaches remains the lack of genetic information for a multitude of fish species, reducing the percentage of overlapping between eDNA and trawling to 75% (Duhamet et al., 2023; Hestetun et al., 2020; Kumar et al., 2022; Xiong et al., 2022). In the North Sea, for only 123 (56%) out of 220 recorded fish species mitochondrial genetic information is publicly available. For others, only a single sequence is published, limiting insights into genetic variability and potential North Sea-specific haplotypes. This calls for greater efforts to integrate more species-specific DNA sequences in open access databases (Duhamet et al., 2023; Hestetun et al., 2020; Kasmi et al., 2023; Kumar et al., 2022; Xiong et al., 2022).

Nothing is yet known about species-specific detection limits, even though they may play a crucial role in presence/absence findings of metabarcoding outputs. The absence of some species in the eDNA results that were present in low abundance in single trawl catches may give a hint towards potential detection thresholds.

Data analysis clearly revealed the ASV method to be better suited than the OTU method for marine species, especially with 12S as target gene, as it is characterized by its high similarity between species, as previously shown by Antognazza et al. (2021) and Forster et al. (2019). OTU methods better fit to microbial studies, which often focus on genera or families, and thus less frequently touch the species or subspecies level. Since the ASV approach aims at assigning each read as a unique output and not as a cluster output, this increases its specificity.

From a biogeographic point of view, a clear pattern of an increase of fish diversity from the German and Danish coastal areas in the southeast to off the Norwegian coast in the northwest was observed, as previously reported by Bom et al. (2022).

4.2. Estimation of biomass from number of reads

Despite of the fact that NGS metabarcoding has long been used in marine science, its application remains primarily a qualitative rather than quantitative approach. Its quantitative value is still controversially discussed, because of claims that PCR amplifications during library preparation can negatively influence the final read counts or result in a lack of correlation between biomass and number of reads (Bik et al., 2012; Bucklin et al., 2016). Consequently, the millions of reads generated by NGS at each run are often not adequately used.

In order to overcome this issue, some studies have used internal standards for biomass estimations (Bell et al., 2019; Zemb et al., 2020). However, this method has limitations as it requires the development of a specific standard for each species, and applying the same standard curve to all species may introduce biases. Other studies reported weak correlations between organism abundance and sequence reads using simple correlation methods (Bucklin et al., 2016; Ershova et al., 2021; Loos & Nijland, 2020; Mariac et al., 2022; Pont et al., 2018). In contrast, studies in the field of medical science have shown good correlations between the outputs of quantitative PCR (qPCR) and the number of reads (Flügge et al., 2023; Forster et al., 2019; Min et al., 2020; Robin et al., 2016; Song et al., 2021). Robin et al., (2016) were able to demonstrate that a quantitative analysis for the hTERT gene was similar in qPCR and NGS. In lymphoid neoplasms, NGS showed a high reproducibility, accuracy and precision in detecting and quantifying immunoglobulin (IG) and T cell receptor (TR) gene rearrangements compared to standard methods (Knecht et al., 2019). The number of reads achieved by amplicon NGS was also able to provide an absolute quantitative estimation of bacterial load in food comparable to qPCR as reference method (Kallastu et al., 2023). In virology, NGS showed a high sensitivity and also quantitative aptitude to detect Human papillomavirus (HPV) in early stage of infection compared to droplet digital PCR, and quantitative real-time PCR (Mattox et al., 2022).

The results of this study show that biomass positively correlates with read numbers, under consideration of sampling depth and other ecological parameters (Table 2 and Figure 5).

Incorporating environmental variables such as temperature and sampling depth is crucial for enhancing the robustness and predictive accuracy of the generated model aiming at estimating fish biomass from eDNA copies. Previous research has indicated that environmental factors can significantly affect eDNA degradation rates and, consequently, its detectability (Qian et al., 2022; Doi et al., 2019). For instance, water temperature is known to accelerate eDNA degradation, which could lead to underestimations of biomass if not accounted for. Moreover, depth and therefore pressure are factors that can impact eDNA solubility and diffusion, thus affecting the measured eDNA copies per litre (Duhamet et al., 2023).

While this study introduces a model for estimating fish biomass from eDNA, it is recognized that there are some limitations that need to be addressed in future research studies to improve its application. A key question that needs to be addressed in future studies is the standardization of the sequencing data for ensuring accurate and comparable results across different studies and conditions. The current model does not account for sequencing depth and competitive amplification between different barcodes during the metabarcoding procedure. Moreover, the

model does not take into account the variations introduced by the different sampling methods and types of net used. Future research should focus on integrating these variables to develop more comprehensive models. This includes not only the standardisation of sequencing data, but also taking into account environmental and methodological variations. By addressing these limitations, it could be possible to improve the accuracy, reliability and applicability of eDNA metabarcoding as a quantitative ecological assessment tool.

5. Conclusion

The results obtained show that metabarcoding is an effective method for qualitatively determining marine fish diversity in the North Sea, with a relatively long detection time compared to bottom trawling as the reference method, which can be up to 24 hours before sampling. However, it is necessary to choose the read assignment method very carefully, hence we recommend adopting ASV read assignment instead of the OTU method, because it allows to assign reads more precisely, eliminating the false assignment error due to the close similarity between species, especially in the 12S gene.

On the other hand, the results obtained in this study present evidence that NGS outputs can be exploited quantitatively, compatible with qPCR output or quantitative reference methods. This also reveals the presence of a strong non-linear relationship between the number of reads and biomass in CPUE, calling for more work on the modelling part and the development of quantitative and semi-quantitative models for estimating stock sizes.

Supplementary data S02

Table S3 Water samples taxonomy assignment with the number of reads obtained for each taxon at each station. The colour green is used to distinguish stations where more than 10 copies were obtained in the Miseq outputs, and therefore more accurate. The reading is assigned to stations with reading numbers between 1 and 9 (available as an Excel table).

Niche	Species	St						St_1	St_1	St_1	St_1	St_1	St_2	St_2	St_2	St_	St_1	Cont
		_1	St_3	St_4	St_6	St_7	St_9	1	3	5	7	9	1	2	4	85	07	rol
North																		
Sea	<i>Amblyraja radiata</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0	0
North																		
Sea	<i>Ammodytes dubius</i>	0	0	0	0	16	0	0	0	0	0	0	0	0	0	4	0	0
Arctic	<i>Ammodytes hexapterus</i>	0	0	0	3	26	0	0	0	0	0	0	0	0	0	3	0	0
North	<i>Ammodytes marinus</i>	19	5	1	1	8	9	2	2	21	14	1	3	9	7	5	2	0
Pacific	<i>Ammodytes personatus</i>	0	0	0	1	21	0	0	0	0	0	0	0	0	0	2	0	0
North																		
Sea	<i>Anarhichas minor</i>	0	0	0	1	0	0	0	1	0	0	0	1	0	0	0	8234	0

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North Sea	<i>Aphia minuta</i>	0	1	0	0	0	1	0	0	0	8602	0	0	0	1	0	0	0
Arctic	<i>Arctogadus glacialis</i>	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	1	0
North Sea	<i>Argentina silus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	771	2974	0
North Sea	<i>Argentina sphyraena</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	719	0
North Sea	<i>Arnoglossus laterna</i>	0	0	0	514	0	0	0	0	598	1730	0	0	8	21	0	0	0
North Sea	<i>Clupea harengus</i>	0	18	80	4	0	3	22	34	134	18	0	9381	2	58	81	1	0
Pacific	<i>Clupea pallasii</i>	9	4411	8274	2073	12	0	3	2	2	0	7	6	3940	0	5	13	0
North Sea	<i>Crystallogobius linearis</i>	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0
North Sea	<i>Cyclopterus lumpus</i>	0	0	1	0	0	0	2	0	0	0	7	0	0	0	116	64	0

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North	<i>Enchelyopus</i>							1078										
Sea	<i>cimbrius</i>	0	2	0	0	0	0	2	4	0	4	0	1	0	5	0	1	0
North				2135	1050			3336	2821	2238			2403			445	1864	
Sea	<i>Eutrigla gurnardus</i>	13	8	6	0	6276	0	12	7	0	12	9	4	26	17	5	0	0
Pacific	<i>Gadus</i>																	
	<i>chalcogrammus</i>	0	0	0	0	0	0	0	142	0	0	0	0	0	0	0	5	0
North																		
Sea	<i>Gadus morhua</i>	0	1	0	0	0	0	1	2473	0	0	1	1	0	0	2	2265	0
North	<i>Gaidropsarus</i>																	
Sea	<i>vulgaris</i>	0	2	0	0	0	0	0	1	0	4	0	1	0	4	0	1	0
North	<i>Gobiusculus</i>																	
Sea	<i>flavescens</i>	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0
North	<i>Hippoglossoides</i>																148	1566
Sea	<i>platessoides</i>	0	0	3	13	12	13	1	7	32	2	5	2	8	8	08	7	0
Pacific	<i>Kareius</i>																	
	<i>bicoloratus</i>	0	0	0	2	5	2	0	1	9	0	2	0	0	1	1	0	0
North																		
Sea	<i>Lamna nasus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	20	0

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North				4622	2059	3340			6416		2071		2014	2762	267			
Sea	<i>Limanda limanda</i>	12	14	533	8	2	6	10	5145	6	5257	1	10	0	4	24	7836	0
North	<i>Lophius</i>																283	
Sea	<i>piscatorius</i>	0	0	1	0	0	0	2	0	0	0	0	0	0	0	4	0	0
North	<i>Melanogrammus</i>																277	2046
Sea	<i>aeglefinus</i>	0	0	1	2897	2498	0	0	5	1	0	3	4	0	0	7	7	0
North	<i>Merlangius</i>		3821							1568	1606						609	3045
Sea	<i>merlangus</i>	3	5	3	2850	2473	0	0	9221	0	0	5	7937	1	1336	4	8	0
North	<i>Merluccius</i>																116	
Sea	<i>merluccius</i>	0	0	1	0	0	1	2	3	2570	1	2	0	0	0	12	2745	0
North																		
Sea	<i>Microstomus kitt</i>	0	0	5778	0	0	4579	1	0	2	2	2	0	0	2	2	0	0
Pacific	<i>Microstomus</i>																	
	<i>pacificus</i>	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0
North																	229	1424
Sea	<i>Molva molva</i>	0	0	0	8	0	0	0	2	0	0	0	5	0	0	4	3	0
Norway	<i>Myzopsetta</i>																	
sea	<i>ferruginea</i>	0	0	0	10	38	29	0	3	90	8	7	0	2	17	9	4	0

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North Sea	<i>Nesiarchus nasutus</i>	0	0	0	0	1	0	0	0	0	1	1	0	1	0	0	2	0
North Sea	<i>Phocoena phocoena</i>	0	0	0	11	20	0	0	0	0	0	0	0	0	0	0	0	0
Pacific	<i>Platichthys stellatus</i>	6	8	5683	3	5	4	2	4064	8	3	8	6	8	9	7	5	0
North Sea	<i>Pleuronectes platessa</i>	6	8	3	6	6	3	2	4110	5	9	2	7	7	7	1	4	0
North Sea	<i>Pollachius pollachius</i>	0	0	1	0	0	0	0	0	0	0	1	0	0	0	3	47	0
North Sea	<i>Pollachius virens</i>	0	0	0	2	0	0	0	9	0	0	1	6	0	0	1	4	0
North Sea	<i>Pomatoschistus minutus</i>	15	14	0	0	17	13	0	0	7413	2	0	0	1	1	4	3	0
Pacific	<i>Psettichthys melanostictus</i>	0	0	0	0	1	0	0	0	0	1	1	0	0	0	0	0	0
Atlantic	<i>Pseudopleuronectes americanus</i>	0	0	1	6	14	7	0	3	17	3	4	0	0	2	0	0	0

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North	<i>Reinhardtius</i>																			
Sea	<i>hippoglossoides</i>	0	0	2	5	3	5	0	2	14	2	2	0	1	4	5	1	0		
North													1630				4833			
Sea	<i>Sardina pilchardus</i>	18	5	0	0	36	4	0	0	59	7	0	0	2	7873	0	0	0		
North								3543	3606				1406	1608			1682	1425	424	1855
Sea	<i>Scomber scombrus</i>	4	8	10	4535	3	7622	7	9	8862	1	3	15	0	1	03	1	0		
North	<i>Scyliorhinus</i>																			
Sea	<i>canicula</i>	0	0	0	0	0	0	0	0	0	0	6009	0	0	0	0	0	0	0	0
North						1078			1454	3308										
Sea	<i>Solea solea</i>	2	0	0	0	0	4900	0	7	2	7699	7291	2	2115	2393	0	0	0		
North			4728	6674	2119		4864	3305	7663	1609	1318	2443	7347		1442	652				
Sea	<i>Sprattus sprattus</i>	7	4	7	0	15	8	7	2	9	6	1	5	4651	1	5	20	0		
North	<i>Trisopterus</i>																			
Sea	<i>esmarkii</i>	0	2	4	4	0	4	5	6	0	4	9	5	0	2	5	9	0		
North																	175	1821		
Sea	<i>Trisopterus luscus</i>	0	2	4	3	0	4	5	6	0	4	11	5	0	3	2	6	0		
North	<i>Trisopterus</i>																			
Sea	<i>minutus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0		

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North Sea	<i>Xiphias gladius</i>	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0
North Sea	<i>Chelidonichthys lucerna</i>	9	7	6	2	6177	0	10	0	0	0	12	7	6	20	15	9	7	0
				2099	1030			3283		2773	2203			2364			438	1830	

Table S4: Sediments samples taxonomy assignment with the number of reads obtained for each taxon at each station. The colour green is used to distinguish stations where more than 10 copies were obtained in the Miseq outputs, and therefore more accurate. The reading is assigned to stations with reading numbers between 1 and 9 (available as an Excel table)

Niche	Species	St_1	St_3	St_4	St_6	St_7	St_9	St_1_1	St_1_3	St_1_5	St_1_7	St_1_9	St_2_1	St_2_2	St_24	St_8_5	St_1_07	Contr ol
Arctic	<i>Arctogadus glacialis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0
Atlantic north	<i>Myzopsetta ferruginea</i>	0	2	0	0	1	0	0	0	0	0	0	0	0	10	0	0	0
North Sea species	<i>Anarhichas denticulatus</i>	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	6	0
North Sea species	<i>Arnoglossus laterna</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	2853	0	0	0

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North species	Sea	<i>Clupea harengus</i>	0	0	0	0	0	0	0	0	5068	5	0	0	3	0	270	0	824	0
North species	Sea	<i>Gadus morhua</i>	0	0	0	0	0	0	0	0	0	0	0	1	0	0	1326	731	0	0
North species	Sea	<i>Hippoglossoides platessoides</i>	0	4	0	0	2	0	0	0	0	0	0	0	0	0	20	0	0	0
North species	Sea	<i>Limanda limanda</i>	1	1760	0	0	755	4	0	0	0	1	0	0	0	0	3556	1	0	0
North species	Sea	<i>Melanogrammus aeglefinus</i>	0	0	0	138	0	0	0	0	0	0	0	0	0	0	0	0	0	0
North species	Sea	<i>Merlangius merlangus</i>	0	0	0	213	3	1	0	0	0	1451	1	0	6	0	2	0	0	0
North species	Sea	<i>Pomatoschistus minutus</i>	0	0	0	0	5	0	0	0	0	9	0	0	0	0	1556	01	0	0
North species	Sea	<i>Sardina pilchardus</i>	1	1	0	0	9	1	2	0	1	1842	20	5	9	0	1859	68	4	3
North species	Sea	<i>Solea solea</i>	0	0	0	195	638	2	7	0	0	0	0	0	0	0	4	0	0	0

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North species	Sea	<i>Sprattus sprattus</i>	0	0	0	0	6	0	0	428	1	0	2	0	1086	3	2	1779	31	0	4	0
North species	Sea	<i>Syngnathus rostellatus</i>	0	0	0	0	1	0	0	0	0	1	0	0	0	0	0	7336	0	0	0	0
North species	Sea	<i>Trachurus trachurus</i>	0	0	0	0	1	0	0	0	0	0	4	0	0	0	0	4287	5	0	0	0
North species	Sea	<i>Trisopterus esmarkii</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	0
North species	Sea	<i>Trisopterus luscus</i>	0	0	0	0	0	0	3	0	0	0	0	0	2	0	0	0	0	0	2	3452
Pacific		<i>Anarhichas orientalis</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	18	0
North species	Sea	<i>Chupea pallasii</i>	0	0	0	0	0	0	0	0	5	1	0	0	3	0	2005	7	0	1	0	0
Pacific		<i>Gadus chalcogrammus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	42	11	0	0	0	0
Pacific		<i>Hippoglossus stenolepis</i>	0	7	0	0	1	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0

Pacific	<i>Morone americana</i>	0	0	0	0	0	2	0	0	0	0	0	1030	4	0	0	3	0	0
Pacific	<i>Trachurus japonicus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	0	0	0

Table S5: Bottom trawl results by CPUE per station

Species	ST_1	ST_3	ST_5	ST_6	ST_7	ST_9	ST_1 1	ST_1 3	ST_1 5	ST_1 7	ST_1 9	ST_2 1	ST_2 2	ST_2 4	ST_8 5	ST_1 07
<i>Ammodytes</i>	0	0	0	0	626	0	0	0	0	0	0	0	0	0	0	0
<i>Ammodytes marinus</i>	0	0	553	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Aphia minuta</i>	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
<i>Argentina sphyraena</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	3	1
<i>Arnoglossus laterna</i>	0	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0
<i>Buglossidium luteum</i>	0	0	0	0	0	0	0	0	2	0	2	0	0	1	0	0
<i>Callionymus lyra</i>	0	1	1	0	1	0	0	0	0	0	0	0	1	1	0	0
<i>Chelidonichthys lucerna</i>	0	0	0	0	0	0	0	0	0	0	1	0	0	1	0	0

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<i>Clupea harengus</i>	2	9	3	34	0	2	3	2	0	0	1	67	2	0	0	9
<i>Crystallogobius linearis</i>	0	0	0	0	0	18	0	2	0	0	0	0	0	0	0	0
<i>Echiichthys vipera</i>	3	0	0	0	0	0	0	0	0	0	4	15	1	0	0	0
<i>Engraulis encrasicolus</i>	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0
<i>Eutrigla gurnardus</i>	53	97	375	120	61	137	34	41	22	17	57	6	24	30	15	39
<i>Gadus morhua</i>	0	2	0	0	1	0	0	0	0	0	0	0	0	0	0	11
<i>Hippoglossoides platessoides</i>	0	2	0	0	0	1	3	1	0	0	0	0	0	0	4	15
<i>Hippoglossus hippoglossus</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
<i>Hyperoplus lanceolatus</i>	17	6	3	2	0	0	0	0	0	0	1	4	0	1	0	0
<i>Limanda limanda</i>	891	75	208	63	276	97	131	50	114	276	247	118	219	671	36	4
<i>Melanogrammus aeglefinus</i>	1	2	4256	4646	311	1	6	5	0	0	0	0	0	0	58	183
<i>Merlangius merlangus</i>	3232	18	53	5	106	238	80	47	83	84	17	2	164	6452	8	476
<i>Merluccius merluccius</i>	0	2	0	0	0	0	0	0	0	0	0	0	0	0	0	5
<i>Microstomus kitt</i>	0	1	4	0	28	9	0	6	4	0	0	0	0	0	0	3

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<i>Mullus surmuletus</i>	0	0	0	0	0	0	0	0	0	1	1	2	0	1	0	0	0
<i>Mustelus asterias</i>	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0
<i>Myoxocephalus scorpius</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Pleuronectes platessa</i>	0	14	1	1	43	3	15	4	11	3	9	4	1	4	0	1	
<i>Pollachius pollachius</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
<i>Pollachius virens</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5	2
<i>Pomatoschistus minutus</i>	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0
<i>Psetta maxima</i>	0	0	0	0	0	0	0	0	0	1	0	0	0	0	1	0	0
<i>Raja clavata</i>	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
<i>Raja montagui</i>	0	0	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0
<i>Raja naevus</i>	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
<i>Raja radiata</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0
<i>Sardina pilchardus</i>	0	0	0	1	0	0	0	1	0	0	0	0	0	0	0	0	0
<i>Scomber scombrus</i>	38	0	0	0	0	0	86	0	35	1	11	0	32	4	3	0	
<i>Sprattus sprattus</i>	47544	1721	8	142	4	13127	1942	299	35	36	2163	1310	2037	44	0	0	
<i>Syngnathus rostellatus</i>	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0

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<i>Trachurus trachurus</i>	4	0	0	0	0	0	0	0	0	0	79	11	14	1232	4	0	0
<i>Trisopterus esmarki</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2	15378

Conflict of Interest

The information and views set out in this manuscript are based on scientific data and information collected under Service Contract “Improving cost-efficiency of fisheries research surveys and fish stocks assessments using next-generation genetic sequencing methods [EMFF/2018/015]” signed with the European Climate, Infrastructure and Environment Executive Agency (CINEA) and funded by the European Union. The information and views set out in this publication are those of the author(s) and do not necessarily reflect the official opinion of CINEA or of the European Commission. Neither CINEA nor the European Commission can guarantee the accuracy of the scientific data/information collected under the above Specific Contract or the data/information included in this publication. Neither CINEA nor the European Commission or any person acting on their behalf may be held responsible for the use which may be made of the information contained therein.

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Supplementary Material

File S1: List of models screened.

File S2: Metabarcoding results.

Data Accessibility

The fastq file, assignment data and script will be accessible after publication in the repository Zenodo at the DOI: [10.5281/zenodo.11242794](https://doi.org/10.5281/zenodo.11242794).

Including:

- Water samples fastq, output of MiSeq with Mifish primers;
- Sediments samples fastq, output of MiSeq with Mifish primers;
- List of barcodes of each sample
- Sampling information and data

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Ethical

No ethical permission is required.

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6. Chapter VI: Atlantic cod (*Gadus morhua*) assessment approaches in the North and Baltic Sea: A comparison of environmental DNA analysis versus bottom trawl sampling.

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6.1. Abstract

The assessment of fish stocks is often dependent on scientific trawl fisheries surveys, which are both invasive and costly. The analysis of environmental DNA (eDNA) from water samples is regarded as a non-invasive and cost-effective alternative, but meaningful performance evaluations are required for a wider application. The goal of this study was to comparatively analyze a newly developed, more sensitive real-time PCR based eDNA approach with bottom trawl fisheries catches to locally detect and quantify Atlantic cod (*Gadus morhua*) in the North and Baltic Seas.

With a species-specificity of the qPCR assay of 100%, a minimal limit of 15 Cytochrome b eDNA copies was determined for the detection of cod. In addition, a Gaussian processing regression proved a significant correlation (95%) between eDNA (copies per L of water) and cod biomass (CPUE/Ha) found by bottom trawling. The results presented here prove the potential of eDNA analyses for quantitative assessments of commercial fish stocks in the open ocean, although additional comparative analyses are needed to demonstrate its performance under different oceanographic conditions.

Keywords : quantitative environmental DNA (eDNA) analysis, bottom trawl sampling, Atlantic cod, *Gadus morhua*



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Atlantic cod (*Gadus morhua*) assessment approaches in the North and Baltic Sea: A comparison of environmental DNA analysis versus bottom trawl sampling

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The assessment of fish stocks is often dependent on scientific trawl fisheries surveys, which are both invasive and costly. The analysis of environmental DNA (eDNA) from water samples is regarded as a non-invasive and cost-effective alternative, but meaningful performance evaluations are required for a wider application. The goal of this study was to comparatively analyze a newly developed, more sensitive real-time PCR based eDNA approach with bottom trawl fisheries catches to locally detect and quantify Atlantic cod (*Gadus morhua*) in the North and Baltic Seas. With a species-specificity of the qPCR assay of 100%, a minimal limit of 15 Cytochrome b eDNA copies was determined for the detection of cod. In addition, a Gaussian processing regression proved a significant correlation (95%) between eDNA (copies per L of water) and cod biomass (CPUE/Ha) found by bottom trawling. The results presented here prove the potential of eDNA analyses for quantitative assessments of commercial fish stocks in the open ocean, although additional comparative analyses are needed to demonstrate its performance under different oceanographic conditions.

KEYWORDS

Environmental DNA (eDNA), quantitative eDNA analysis, bottom trawl sampling, *Gadus morhua*, North Sea, Baltic Sea, eDNA modeling

1 Introduction

A sustainable fishery requires accurate and up-to-date information on the status of the fished stocks. Over the last decade, several studies have demonstrated the potential of exploiting eDNA for marine biodiversity assessment and monitoring (Tillotson et al., 2018; Andruszkiewicz et al., 2019; Jerde, 2019; Jo et al., 2021; Stoeckle et al., 2021). The increased interest in applying eDNA tools is mainly due to their non-invasive properties with a half-life of the eDNA in sea water of up to 48 h (Tsuiji et al., 2017; Collins et al., 2018).

The monitoring and surveillance of fish diversity and the estimation of fish abundance are usually carried out with methods based on visual census, remotely operated vehicles (ROVs), as well as fishing techniques, such as bottom trawls (Thompson et al., 1982; Groeneveld, 2000; Sward et al., 2019; Trenkel et al., 2019). Despite all the standardization and optimization protocols, these traditional sampling methods provide information only on short sampling/monitoring time within the stations that span over a few minutes to an hour (Baudrier et al., 2018; Sward et al., 2019; Jourdain et al., 2020) and can be altered by behavioral responses, like observer or gear avoidance. In addition, fishing methods are invasive and cause disturbances and disequilibrium in the marine ecosystems, and can therefore threaten the conservation efforts for marine species.

Advances in molecular biology have allowed the extraction of eDNA from water samples avoiding any invasive effects (Lacoursière-Roussel et al., 2016; Dickie et al., 2018; Jerde, 2019). Water-extracted eDNA is therefore increasingly used for biodiversity assessments as well as for semi-quantitative biomass surveys based on eDNA copy numbers of specific species.

Knudsen et al. (2019) reported on the development of a new PCR assay for quantifying eDNA copy numbers with a Limit of Quantification (LoQ) of 665 copies per reaction, equivalent to a minimum detection limit of 200 kg/h of trawling for Atlantic cod (*Gadus morhua*). However, a significant correlation between eDNA concentration and bottom trawl catches could not be found. A similar study by Salter et al. (2019), based on a commercial qPCR kit (Techne) to target Atlantic cod mitochondrial control region, showed significantly positive correlations between regional integrals of cod biomass (kg) and eDNA copy number ($R^2 = 0.79$, $P = 0.003$) as well as between catch per unit effort, normalized by sampling effort (kg/h), and eDNA concentrations (copies L^{-1}) ($R^2 = 0.71$, $P = 0.008$). Nonetheless, despite first promising results in the interpretation of eDNA copy number analysis, the technology is still in its infancy and needs a much more robust calibration to assist or even replace invasive routine methods for quantitative fish stock assessments. Furthermore, eDNA based methods can also not yet provide important stock structure information such as length or age class distributions, even though a few studies tried to tackle this issue with methylome approaches (Sigsgaard et al., 2020; Minamoto, 2022; Zhao et al., 2023). The goal of this study was to refine the existing quantitative eDNA based assessment approaches for Atlantic cod, by focusing on the development of a more sensitive qPCR assay with an improved LoQ value compared to existing methods. In addition, the suitability of various computational models to establish a

correlation between eDNA copy number and bottom trawl sampling are evaluated.

2 Methods

2.1 Primer development

2.1.1 Sequence selection

Reference sequences of whole mitochondrial genomes of northeastern Atlantic fishes were downloaded from the public databases **Aquagene** (Thünen Institute of Fisheries Ecology) (Hanel, 2019) and **NCBI-GenBank** (Benson et al., 2013) together with single mitochondrial sequences of cytochrome oxidase (COI) subunit 1, small unit ribosomal RNA (12S rRNA), NADH dehydrogenase (NADH) subunits, cytochrome b (CYTB), ATPase6 genes and control region (D-loop) of Atlantic cod (*Gadus morhua*) and untargeted gadid species (Table S1: Sequences Accession).

2.1.2 Primer development

The collected sequences were aligned using Bioedit (Hall et al., 2011) and MAFFT (Katoh et al., 2018). Plotcon plots were produced for every alignment to check for conserved regions between species and the regions that distinguish Atlantic cod from other gadid species. Subsequently, primers and probes were designed for the COI, 12S rRNA, NADH5, NADH3 and CYTB regions manually or by using PrimerMiner (Elbrecht and Leese, 2017) and the R script DECIPHER (Wright et al., 2012). All primers and probes targeted regions with low intraspecific divergence while maximizing mismatches between related species at the 3' end as described by (Wilcox et al., 2013). Primers were designed to amplify fragments in the size range of 80 to 250 bp. Annealing temperatures and cross-amplifications of untargeted species were verified using Primer Blast (Ye et al., 2012) and were adjusted to be adequate to the PCR/qPCR kit used.

All designed primers and probes underwent a screening according to their ability to amplify the target species (*G. morhua*) versus cross-amplification of various untargeted species, abundant in the region (*Brosme brosme*, *Chelidonichthys lucerna*, *Clupea harengus*, *Enchelyopus cimbrius*, *Engraulis encrasiolus*, *Helicolenus dactylopterus*, *Lota lota*, *Melanogrammus aeglefinus*, *Merlangius merlangus*, *Merluccius merluccius*, *Micromesistius poutassou*, *Molva molva*, *Pollachius virens*, *Scophthalmus maximus*, *Trachurus trachurus*, *Trisopterus luscus*, *Trisopterus minutus*) (Table S2).

A preliminary primer screening was performed in a conventional PCR using Phusion[®] High-Fidelity PCR Master Mix with HF Buffer, 2X (New England Biolabs, Germany) on a BioRad T100[™] PCR system in a final volume of 20 μ l: 10 μ l of Phusion Master Mix (2X), 0.5 μ l of each primer (10 μ M), 3 μ l of DNA (5–10 ng) and 6 μ l of ultrapure distilled water, following these conditions: 98°C for 30 s, 34 cycles \times [98°C for 10 s, 54–70°C for 30 s], 72°C for 20s, in which (*) refers to a gradient PCR. Then, the PCR products were visualized on a 1% electrophoresis agarose gel.

Those primer pairs that showed strong amplification of the target and weak or even no amplification of the non-targeted species were selected and passed on for a second screening. This was performed on SYBR Green qPCR, using Luna[®] Universal qPCR Mastermix (New England Biolabs, Germany) in a qTOWER³ real-time PCR thermal cycler (Analytik Jena, Germany), in a final volume of 20 μ l: 10 μ l of Luna[®] Universal qPCR Master Mix (2X), 0.5 μ l of each primer (10 μ M), 2 μ l of DNA (5–10 ng) and 7 μ l of ultrapure distilled water, following these conditions: 95°C for 60 s, 44 cycles \times [95°C for 15 s, 54–70°C for 30 s]. Data collection was enabled at each combined annealing/extension step. The amplification cycle was followed by a melting curve protocol: 60–95°C each for 15 s with an increment of 1°C.

At this stage, the selection of the potential primers was done using two criteria: the specificity to the target species (i. e. Atlantic cod) and in which cycle the first amplification signal started. The two primers/probe pairs that fulfilled these criteria were then tested on different polymerases: TaqMan assay with TaqMan[™] Environmental Master Mix 2.0 (ThermoFisher, Germany), KAPA PROBE FORCE qPCR kit (Roche, Germany) and Luna Universal probe qPCR mastermix (New England Biolabs, Germany), according to the manufacturer's protocols. All probes carried 5'FAM fluorescence modifications and BHQ1 as a 3' quencher. Some probes were additionally tested with alternative fluorescence technologies (Locked Nucleic Acids (LNA) and Minor Groove Binder (MGB)) to increase specificity.

All PCR preparations were performed in a designated DNA-free hood in a pre-PCR room.

2.2 Standard curve and assay sensitivity

A standard curve experiment was performed using purified and diluted target amplicons as templates. The PCR products were first purified by Monarch[®] PCR & DNA Cleanup Kit (New England Biolabs, Frankfurt, Germany) and after 24 h at 4°C quantified by Qubit using Qubit[™] dsDNA BR-Assay-Kits (ThermoFisher).

PCR-products of Atlantic cod amplified with universal primers for the complete CytB gene (Sevilla et al., 2007): FishCytbF (5' ACC ACC GTT GTT ATT CAA CTA CAA GAA C-3'), TruccytbR (5' CGA CTT CCG GAT TAC AAG ACC G-3') served as a target amplicon for CytB standard curves. For the NADH5 gene, the corresponding qPCR primers were used to create the template including the target amplicon sequence.

From the amplicon stock solutions, diluted to $1.00E^{+10}$ copies/ μ l, a series of tenfold dilutions (1,000,000, 1,00,000, 10,000, 1,000, 100, 10, 1, as well as 5000, 500, 50, and 25 copies per reaction) were prepared. Nine replicates of each dilution were run to determine the amplification efficiency and limit of detection defined as the lowest copies per reaction with >95% amplification success for each primer/probe set (Bustin et al., 2009).

In real-time PCR (qPCR), false positives can be caused by various sources of molecular biological noise. Establishing a false-positive threshold for the quantification of nucleic acids is essential for the performance of a robust and reliable qPCR assay. Establishing the analytical performance indicators of an assay,

including the limit of blank (LoB) that can also be referred to as the Negative Control, and limit of detection (LoD), is fundamental and must be done according to precise procedures. The LoB is the upper limit of the target concentration that is considered acceptable in a blank sample. The LoB is then used to calculate the LoD, the target concentration limit above which the presence of the target can be asserted and quantified with a given statistical confidence. The method to calculate the LoB, LoD and LoQ is described in the [Supplementary Data](#).

2.3 Sea sampling

2.3.1 Baltic Sea

In the Baltic Sea, 21 water samples (5L per sample) were collected from seven sampling stations during research survey No. 441 of FRV Walther Herwig III from 30.11. to 20.12.2020, conducted by the Thünen Institute of Fisheries Ecology in the frame of the North and Baltic Sea monitoring of environmental radioactivity. Water samples were collected from 3 different positions along the drag line of each bottom trawl station (start, middle and end of each drag line). The sampling was performed as described in the North Sea section.

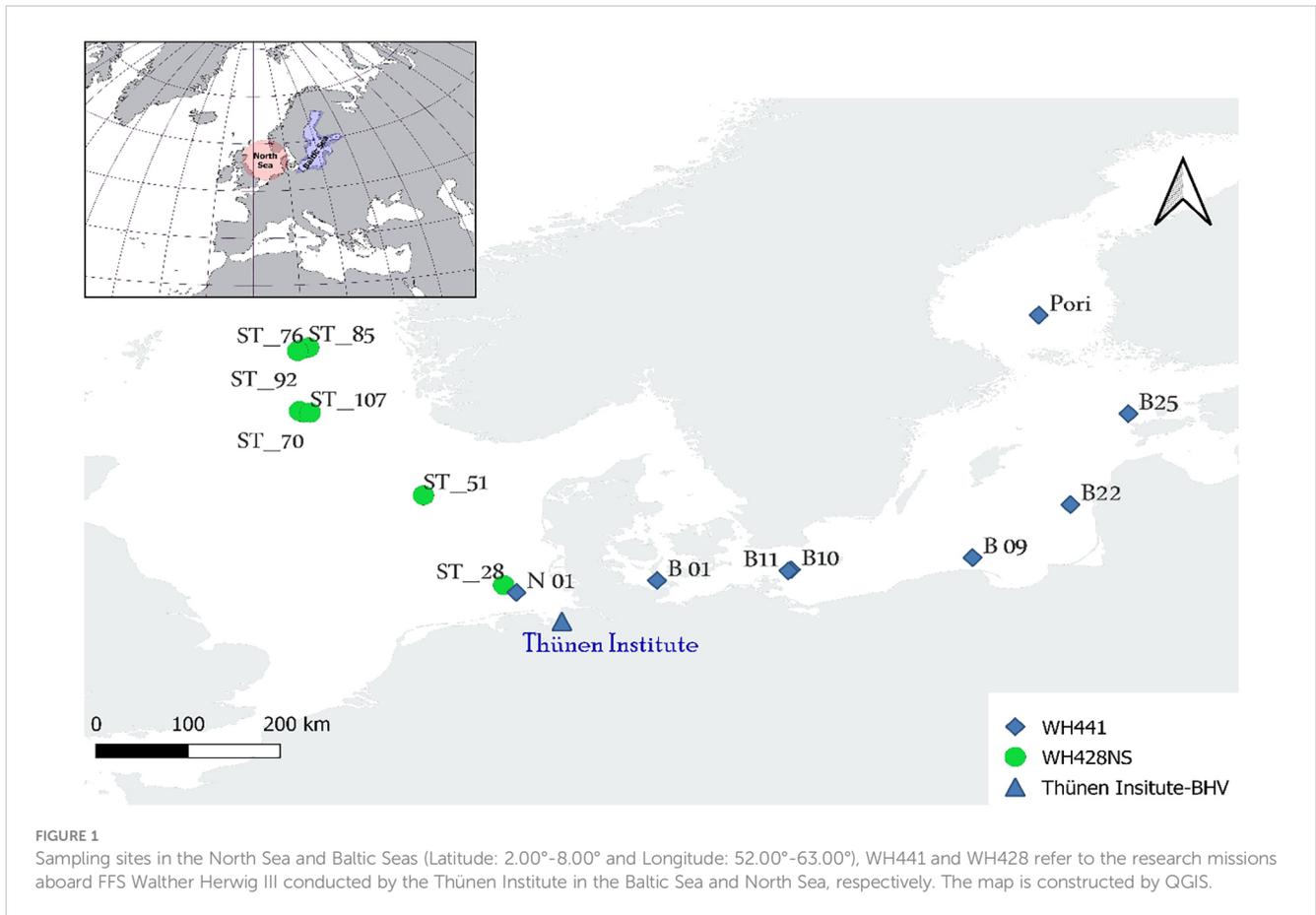
In the Baltic Sea a 140 ft. bottom trawl gear with rock hoppers and a mesh size of 20 mm in the cod-end was used immediately after water sampling to collect Baltic cod. Catch time was 60 min each with a towing speed of 3.9–4.1 kn during daytime. Atlantic cod catches varied between 0.00 and 9.25 kg/Ha.

2.3.2 North Sea

A total of 32 water samples consisting on 5L each were collected during research survey No. 428 of FRV Walther Herwig III in the North Sea in summer 2019, conducted by the Thünen Institute of Sea Fisheries (Figure 1). The procedure was performed before trawling to minimize contamination from trawl-derived DNA sources and possible disturbance due to the resuspension of sediment caused by the bottom trawler gear. Six water samples were collected along the drag line of each bottom trawl station (two replicates each at the start, the middle and at the end).

Demersal trawling was carried out immediately after water sampling to collect North Sea cod, according to International Bottom Trawl Survey (IBTS) standards (ICES, 2020). Catch time was up to 30 min each at a trawling speed of approximately 4 knots, Total weight of each trawl catch was recorded, before sorting by species. On predefined species, such as Atlantic cod (ICES, 2020), individual length and weight were measured, sex and maturity stage discrimination were performed and otoliths were removed for subsequent age determination in the laboratory.

A total of 32 5 L seawater samples were collected at 16 trawl positions (Figure 1) by Niskin bottles mounted on a stainless steel CTD frame at a depth of 4 m above the seafloor to match the trawl height of 5 m and to minimize the possibility of sampling eDNA from non-recent sediment sources. Immediately after sampling, the water was filtered through Sartorius[™] PES membranes (pore size: 0.45 μ m, diameter: 47 mm) with a vacuum pump. Filters were subsequently stored at -20°C until DNA extraction at the laboratory.



2.3.3 Negative controls and contamination avoidance

Contamination avoidance was key throughout the analysis. Two negative control samples were taken from the ultrapure water which was used to rinse the Niskin bottles before each sampling. Field blanks consisted of 2.5 L of ultrapure water rinsed through the corresponding Niskin Bottles before water sample collection onboard the research vessel. After recovery of the CTD rosette, all Niskin sample bottles were thoroughly rinsed with fresh water on deck, removed from the sampling frame and transported to a CTD control laboratory isolated from the deck area where they were mounted on wall mounts for further processing. Prior to sub-sampling, the exterior of the Niskin bottles and the sampling nozzle were rinsed with a solution of sodium hypochlorite (10% commercial bleach), followed by ultrapure water. The on-board workbench area was covered with aluminium foil and rinsed with a 20% commercial bleach solution followed by ultrapure water. Each subsample bottle was rinsed three times with sample water and then filled to a 2.5 L mark. Negative samples were treated as real samples and were filtered immediately. All further processing of the samples took place in a sterile environment in a molecular biology lab on land.

2.4 eDNA extraction

eDNA was extracted from the water according to the protocol of (Renshaw et al., 2015). In brief, the frozen PES membranes from

each samples were placed in 2 ml tubes filled with 700 μ L of CTAB-buffer (2% CTAB (w/v), 1.4 M NaCl, 100 mM Tris, 20 mM EDTA) and incubated at 65°C for 10 min while shaking at 650 rpm. Subsequently, 900 μ L PCI phenol/chloroform/isoamyl alcohol (25:24:1) was added and vortexed for 5 s, prior to centrifugation at 15,000 \times g for 5 min. 700 μ L of supernatant were transferred to a new 2 ml tube, and 700 μ L of chloroform was added, followed by centrifugation at 15,000 \times g for 5 min. 500 μ L of supernatant was transferred to a new 2 mL tube, and from here we proceeded with Monarch[®] Cleanup Kit (New England Biolabs, Frankfurt, Germany) from the step of adding 1000 μ L of binding buffer. The final elution step was accomplished with 20 μ L of elution buffer. The eDNA extracts were either stored at -20°C until qPCR or archived at -80°C. All steps of eDNA extractions were performed in a dedicated lab area under a chemical hood.

2.5 Atlantic cod qPCR from North Sea water samples

Quantitative Real-time PCR of Atlantic cod eDNA was performed using the best primer/probe couple developed in the first and second phase of this study. The primers Gm_Cytb_For2a (5'-TACACTATACCTCAGACATCGAGAC-3') and Gm_Cytb_Rev2b (5'-GGCAATGTGCATATAAAGACAAATG-3'), coupled with the LNA-based TaqMan probe GmCytb-LNA-P (5'-[FAM] A[+C]TA[+C]GGCTGA[+C]TAATTCG[+G]A[BHQ1]-3')

were used for the amplification of the CytB gene. A qTOWER³ Real-time PCR thermal cycler (Analytik Jena, Germany) was used for amplification. The 20 μ L qPCR reaction mix contained 5.5 μ L eDNA template, 10 μ L 2 \times TaqManTM Environmental Master Mix 2.0 (ThermoFisher, Germany), 0.4 μ M of each primer, 0.2 μ M of Atlantic cod probe (GmCytb-LNA-P probe), 0.1 μ L of AmpEraseTM Uracil N-Glycosylase (UNG) (ThermoFisher, Germany) and 2.4 μ L of ultrapure water. qPCR reactions were performed under thermal cycler conditions of 10 min at 95°C followed by 45 cycles of 15 s at 95°C and 59 s at 62°C. Data collection was enabled at each combined annealing/extension step. eDNA samples were measured in triplicate reactions without dilution. Numerous qPCR assays were carried out to determine the optimal PCR condition, including primers/probe concentrations as well as the DNA amount to be added in each reaction.

During the DNA extraction and qPCR assays, negative controls for extractions and no template controls (NTCs) were included in all manipulations to ensure that no cross-contamination occurred. All controls and NTCs contained only nuclease-free water. A positive control consisting of Atlantic cod genomic DNA was used to prove the qPCR reaction performance. Extractions were performed identically. Field blanks consisted of 2.5 L of ultrapure water rinsed through the corresponding Niskin Bottles before water sample collection onboard the research vessel. Neither the extraction blanks nor the field sample blanks showed amplification with Atlantic cod qPCR primers.

2.6 Computational modeling of trawling and eDNA data

The relationship between Atlantic cod eDNA concentrations and trawl catches in the standard survey area was assessed by

various combinations of covariates where eDNA levels were above LoQ. The response variable, eDNA level per fishing position, and all explanatory variables were log₁₀ transformed with the exception noted in the comment column in section 3.3. The Shapiro-Wilk test was used to validate the dependent and independent variables before the regression model. All variables and residuals used in the regional regression models were characterized by Shapiro-Wilk p-values > 0.05 confirming normal distributions.

The correlation between the number of Atlantic cod gene copies obtained by qPCR and the catch per unit effort (CPUE) was tested by simple correlation regression, generalized least squares (GLS), forest-tree, nonlinear regression, and neural network. A model was developed based on the data obtained by Knudsen et al., 2019 and then readjusted according to the output of this study. The model with the best correlation degree between real and predictive data with a minimal RMSD and MRE was selected as the best model. The model was developed using R and MATLAB scripts.

3 Results

3.1 Atlantic cod primer-probes development

3.1.1 Primer screening

Four primer pairs targeting the mitochondrial genes ATPase6, ND5, COI, and CytB, were designed in silico to distinguish Atlantic cod from other species of the family *Gadidae*. By comparing the four designed primers with the ones published by Knudsen et al. (2019), ATPase6, ND5, and CytB showed more than 6 additional mutations (Table 1). A first screening for cross-amplification of other gadids *via* conventional PCR for ATPase6, ND5, COI, and CytB was negative in each of the three technical replicates (Tables 2, 3).

TABLE 1 The number of mutations observed *In-silico* between the primers designed or published compared to the reference *Gadidae* mitochondrial genomes.

Species	GenBank Acc. Nr.	ATPase6_Cod	ND5_Cod	COI_Cod	GmCytb-LNAP	(Knudsen et al., 2019)
<i>Gadus morhua</i>		0	0	0	0	0
<i>Gadus macrocephalus</i>	MK990531.1	6	9	2	4	1
<i>Boreogadus saida</i>	MG100545.1	7	9	2	7	2
<i>Gadus ogac</i>	LN908945.1	7	9	2	5	2
<i>Gadus chalcogramma</i>	DQ356946.1	9	8	3	4	2
<i>Eleginus gracilis</i>	MH061057.1	9	N	-	N	6
<i>Theragra finnmarchica</i>	AM489719.1	10	8	-	4	2
<i>Pollachius virens</i>	KP644330.1	10	15	4	N	6
<i>Micromesistius australis</i>	AB550326.1	11	N	4	N	6
<i>Trisopterus minutus</i>	KP644339.1	N	N	4	N	6
<i>Microgadus proximus</i>	DQ174066.1	10	N	4	N	6
<i>Melanogrammus aeglefinus</i>	KP644328.1	8	13	4	8	5

The presence of more than 5 mutations between the primer and the non-target species sequence and therefore a high distinction of Atlantic cod is highlighted in red. 4 to 5 primer mutations with the non-target species and therefore a medium risk of detection is highlighted in yellow, less than 3 mutations in green. The letter N means that no combination/similarity was observed between the primer and the untarget species.

TABLE 2 List of primers/probes pairs developed and tested, with the optimal concentrations and lengths of target fragments.

species	Primer/Probe name	Sequence	Optimal concentration [nM]	Fragment length (bp)	
<i>Gadus morhua</i>	Gm_Cytb_For	TACTACTATACCTCAGACATCGAGAC	400 nM	140	
	Gm_Cytb_Rev	GGCAATGTGCATATAAAGACAAATG	400 nM		
	GmCytb-LNA-P	[FAM]A[+C]TA[+C]GGCTGA[+C]TAATTCG[+G]A[BHQ1]	200 nM		
	Target fragments for each assay				
	Sequence Cytb	TACTACTATACCTCAGACATCGAGACAGCCTTCTCATCCGTAGTCCACATCTGTCGTG ATGTAAACTACGGCTGACTAATTCGGAATATACATGCTAATGGTGCCTCTTCTTTT TCATTTGTCTTTATATGCACATTGCC			
	ND5_Cod_For	GCAAGAATTTGGACATAACTCTCCCTCTA	400 nM	250	
	ND5_Cod_Rev	AATATAGTGGTTAAGGCTCCTAGACAGA	400 nM		
	ND5_Cod-P	[FAM] CCT AAT TCG GAT GAG CCC [MGBEQ]	200 nM		
	Target fragments for each assay				
	Sequence ND5	GCAAGAATTTGGACATAACTCTCCCTCTACTCGGTTTAATCTTGGCTGCCACTGGTAA ATCCGCCAGTTTGGACTTCACCCATGACTACCAGCCGCAATAGAAGGTCCAACGCC AGTGTCTGCCCTACTTCATTCTAGCACAATAGTTGTAGCAGGAATTTTCTCCTAATT CGGATGAGCCCTCTTATAGAAAATAATCAGACTGCACTAACTCTGTCTCTGTCTA GGAGCCTTAACCACTATATT			
	COI_Cod_For	TATTAATATGAAACCTCCGGCA	400 nM	98	
	COI_Cod_Rev	CGGGGAGAGATAATAGTAGAA	400 nM		
	COI_Cod-P	[FAM]CCTATTTGTTTGGAGCAGTACTAATTACAGCTGTG [BHQ1]	200 nM		
	Target fragments for each assay				
	Sequence COI	GCAAGAATTTGGACATAACTCTCCCTCTACTCGGTTTAATCTTGGCTGCCACTGGTA AATCCGCCAGTTTGGACTTCACCCATGACTACCAGCCGCAATAGAAGGTCCAACG			
	ATPase6Cod-F	ACCCTGACTTTTAATTCCTACACCTAC	400 nM	209	
	ATPase6Cod-R	TAGGAGTGAAGATATATGGTATTAAGC	400 nM		
	Target fragments for each assay				
	Sequence 12S	ACCCTGACTTTTAATTCCTACACCTACTTCCCGATGACTAAGCAATCGAGTTGTATCTCTA CAAGGATGGTTTATCGCCCGCTTACTAATCAACTCTTTTACCTCTAAATGTGGGAGGAC ACAAATGAGCTCCTCTTCTTGCCTCACTAATAATGTTTTTACTCACTCTAAATATGTTAGG CTTAATACCATATATCTTCACTCCTA			

-For, forward primer; -Rev, reverse primer; -P, Probe.

TABLE 3 Comparison of polymerase performance based on LoD and LoQ values.

Primer	Polymerase	LoD	LoQ	Slope
<i>GmCytb-LNA</i>	Environmental TF	12	16	-3.57
<i>GmCytb-LNA</i>	KAPA	16	22	-3.74
<i>GmCytb-LNA</i>	probe Luna	34	70	-3.37
<i>ND5_Cod</i>	probe Luna	26	70	-4.03
<i>ND5_Cod</i>	SybrGreen Luna	7.88	16	-3.85
<i>Techne</i>	Techne	770	781	-4.35
Knudsen et al. (2019)		660	660	-

However, when using SybrGreen PCR, some replicates showed unintended cross-amplification of untargeted gadid species, generally in the late cycles of the qPCR. The same bias was observed when using the probe, resulting in a strategy of defining specificity and sensitivity individually for each primer/probe pair with LoQ being a minimum accepted specificity value, and LoD the minimum sensitivity of a qPCR assay.

For ATPase, even both primers (forward and reverse) are located in hypervariable sites and should allow high specificity, it was problematic to determine probe regions for an unambiguous discrimination of Atlantic cod. Therefore, the ATPase-targeted primer pair is proposed for a qPCR assay coupled to SyberGreen. For this primer pair, the initial Ct value for DNA control was in the range of 12.02, while all investigated untargeted species would start from 32, with an equivalent LoQ Ct value of 31.

For the COI primers/probes, we obtained an initial amplification at Ct 18.3 for genomic DNA control, however, all untargeted species started to show some Ct values equal to 33.43. The same genomic DNA amount were used in all assays, the quantification of the genomic DNA were made by Qubit 4.0. The in-silico evaluation revealed a borderline number of mutations which allowed a distinction between Atlantic cod (*Gadus morhua*), the congeneric Pacific cod (*Gadus macrocephalus*), Greenland cod (*Gadus ogac*) and Alaska pollock (*Gadus chalcogramma*) as well as the Polar cod (*Boreogadus saida*).

The GmCytb-LNAP and ND5_Cod primers/probes were found to amplify only Cytb, and ND5 DNA of Atlantic cod as the target species. Cross-amplification tests on untargeted species collected during different missions to the North Sea were performed to test the specificity of the designed primers in qPCR. For the GmCytb-LNA primer pair, DNA extracted from cod tissue samples amplified at an initial Ct value of 18.8 ± 0.25 , whereas for non-target species amplification was only proven after Ct 36.5, knowing that the LoQ

value equals Ct=35.5, equivalent to 15 copies per reaction. The ND5_Cod primer/probe pair was able to reliably detect cod DNA at an initial Ct value of 11.56 corresponding to 8×10^7 copies, its LoQ value was 16.27 copies per reaction, equivalent to a Ct value of 29, while non-target species appeared after a Ct value of 31. To prevent the inclusion of false-positive test results, LoQ Ct values were established as the positive threshold for the test. The standard curve based on the genomic control had a slope of -3.57 and -3.85 for CytB and ND5, respectively.

3.1.2 Comparison of polymerase screening

The use of TaqMan™ Environmental Master Mix 2.0 (ThermoFisher, Germany) increased the specificity and sensitivity of the assay compared to KAPA qPCR kit and Luna Universal probe qPCR mastermix kit, observed at the initial Ct level for the control, which was in the order of 16.80 and a final Ct of 36.1 for 10 copies per reaction, whereas non-target species only began to appear after a Ct of 36.01, with an LoQ value that went up to 16 copies per reaction when the Environmental Master Mix 2.0 kit was used (Figure 2). Similarly, the sensitivity also increased with the KAPA qPCR kit, but less significantly than with the Environmental Master mix. Using KAPA qPCR kit, the initial control Ct value was 16.45, and final Ct was 35.04 for 10 copies per reaction, while the non-target species started only after a Ct of 34.9, making the LoQ value equal to 22 copies per reaction (equivalent to a Ct of 33.8). Whereas for the Luna Universal probe qPCR mastermix kit, **untarget** species started to appear at a Ct of 34.5 which is equivalent to 57 copies per reaction, and therefore the LoQ value was 70 copies per reaction. Similarly, the ND5_Cod results had almost similar results concerning the sensitivity of the Environmental Taq polymerase compared to Luna Probe (Table 3). Technne has an LoQ value equivalent to 781 copies per reaction.

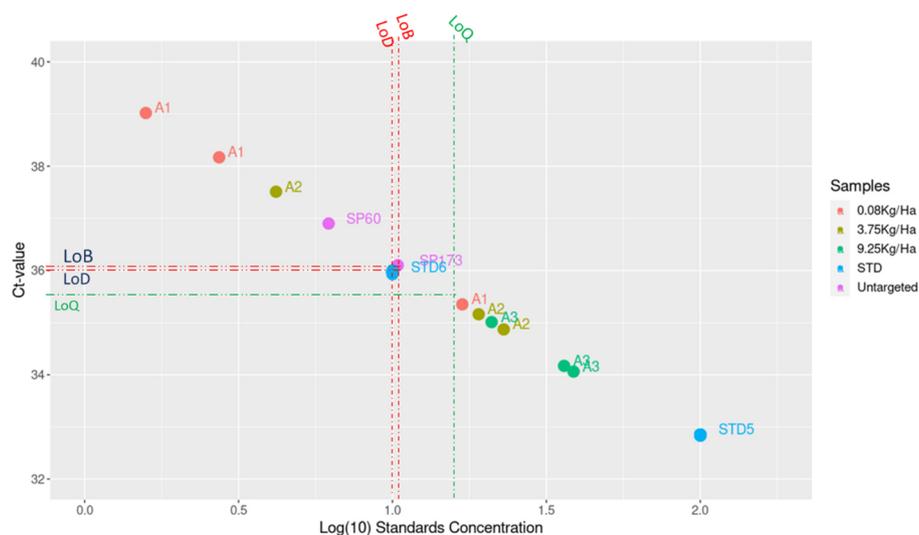


FIGURE 2

The results from different qPCR assays of *in situ* validation as well as the standard curve of threshold cycle number (Ct values) plotted against the log concentration (copy number). Dark green dots represent 9 replicates for each dilution. STD: the qPCR Standards. Positive control sample A1, A2, A3 with 0.08, 3.75, 9.25 kg/Ha of Atlantic cod in catch, respectively.

3.2 Artificial and *in situ* validation

Considering that the GmCytb-LNA assay performed best in the evaluation part (with a slope of about -3.5), we opted to proceed with *in situ* validation steps only with the primer/probe pair, setting the threshold of positive signal to a value of 1.7 for the GmCytb-LNA primer/probe pair.

For the Baltic sea samples, all technical replicates from stations with trawl catches of 9.25 and 3.75 kg/Ha of Atlantic cod were positive with eDNA signals between Ct 34.06 and 35.48, equivalent to copy numbers between 38.75 and 19.06 copies per reaction. For the stations with trawl catches of 0.08 kg/ha, only one of the three replicates was positive.

In the analysis of eDNA North Sea samples, the obtained Atlantic cod eDNA copy numbers were above the LOQ in 12 of the 17 samples, with a maximum copy number equivalent to 153 copies L⁻¹, clearly higher than those found in the Baltic Sea. Within single stations, water samples taken in the middle of each drag line were generally richer in Atlantic cod eDNA than those at the start and the end (Figure 3). The highest Atlantic cod biomass recorded by bottom trawling in this study was 9.5 kg/Ha in the Baltic Sea (Station B10:54°49,377N; 013°55,848E), which corresponded to a concentration of 134 eDNA copies per L. Conversely, with 153 copies per L, the highest eDNA concentration was found in the North Sea, corresponding to a trawl biomass of 1.25 kg/Ha.

Overall, the GmCytb-LNA qPCR analysis of 15 eDNA samples resulted in the detection of Atlantic cod at all sites where the species was found in the concomitant trawl catches. Additionally, at station ST51-2, where cod was absent in the trawl catch, eDNA analysis was positive with 20 copies per reaction while all other negative trawl stations tested also negative using qPCR. This means that our qPCR assay was able to detect *G. morhua* in 112% of eDNA samples. All the expected positive samples produced an amplification signal in all three

qPCR replicates for each sample (Table 4). The qPCR assays had an efficiency of 94.04% +/- 1.0%, with a slope equal to -3.53 +/-0.27. The R2 values ranged from 0.99 +/-0.2%. All negative extraction and PCR controls tested were negative with no sign of contamination.

Overall, Atlantic cod was detected in 60% (9/16) of the analyzed trawl hauls at the North Sea.

3.3 Computational modeling of trawling and eDNA data

The correlation between eDNA copy number and the amount of Cod found by the simple models was insignificant and weak. Despite attempts to standardize the data by logarithmic, exceptional, (max-min)/max, (max-min)/(average-x) functions, the best regression obtained was 28% between eDNA copy number per L and biomass, for the abundance in CPUE/Ha the best correlation obtained with the Log of eDNA copies/L was 40%. The correlation between eDNA copy number and CPUE in ordinary least squares (OLS) models was less than 25%, while for biomass it was around 0. By adopting Gaussian process regression (GPR), Neural network and non-linear regression modelling, we observed an increase in the regression rate. However, the use of only one variable (eDNA/L copy number) achieved only 55%. The use of two variables (eDNA/L copy number and sampling depth) significantly increased the regression rates. This allowed us to achieve 96% regression between the trawl results and the model outputs by GPR and Neural Network method. At the same time, the error rate was significantly reduced to 0.002 (MAE) (Table 5).

The Gaussian process regression (GPR) model (GPR_4) with two explanatory variables was the best at explaining eDNA levels for the fishing position (GPR_4, R2 98%, MAE=0,002), compared to

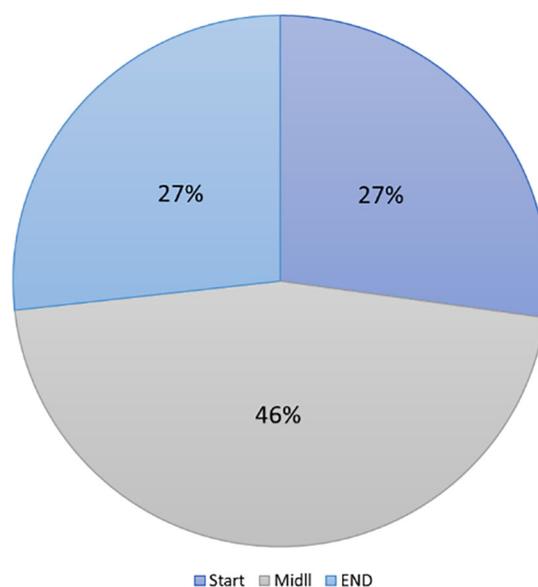


FIGURE 3

Percent of the number of copies per station, 46% of DNA copies obtained by qPCR were in middle stations of trawling. ($p=0.004168^*$).

TABLE 4 The average measured eDNA (copies/L of seawater) in seawater samples from 17 stations is presented together with catches (kg), eDNA levels (copies/L of filtered water), and catches per unit effort (unit/ha).

Sampling Mission Number	Station Sampling Nr.	rep. pos	Kg/ha	eDNA (copies eDNA/L)
WH441	N01	1	0.1	16,5
	B09	3	3.8	76
	B10	3	9.3	134
	B 01	0	0,3	–
	B25	0	0,3	–
	B11	3	0,4	19.56
	B22	1	0,8	21.54
	Pori	0	0	0
WH428	ST28-1	0	0	–
	ST28-2	0	0	–
	ST28-3	0	0	–
	ST51-1	0	0	–
	ST51-2	1	0	20.36
	ST51-3	0	0	–
	ST70-1	3	1.25	110.32
	ST70-2	3	1.25	153.69
	ST70-3	3	1.25	109.82
	ST76-1	3	1.0	0
	ST76-2	3	1.0	64.24
	ST76-3	3	1.0	27.86
	ST98-1	3	1.0	67.46
	ST98-2	3	1.0	121.36
	ST98-3	3	1.0	49.69
	ST85	0	0	0

All water samples were analyzed by quantitative PCR in three replicates. The number of replicates with positive eDNA detection (eDNA level above the LOQ) is indicated for each sample (columns designated “pos.”). If one or more replicates were above the level of quantification, LOQ = 16 copies of eDNA/L, an average was calculated using the positive replicates. Rep.pos, number of replicates positive.

other models with only one (eDNA/L copy number) or two (eDNA/L copy number and sampling depth) explanatory variables. In this model, we did not consider the fishing position and the latitude of the fishing position. The positive correlation between eDNA concentrations and catch per unit effort (CPUE) per Hectare on a station-by-station basis was statistically significant but considerably weaker than the regional comparisons.

4 Discussion

eDNA promises to provide reliable answers on marine diversity for marine and fisheries resource management studies, without posing a risk to dangerous species, due to the non-invasive approach. The objective of this study was to evaluate the performance of eDNA tools for revealing the abundance of

Gadus morhua in the North and Baltic Sea, based on the qPCR approach.

The results of this research provide a basis for future studies to use this non-invasive tool. Significant differences were found by comparing the performance of the primers previously published and the ones designed in this study, and the type of primer used, as well as the computational model, used to predict the relationship between eDNA and the number of fishes per Ha. In addition, the three sampling positions at the same station were comparable, although the samples from the middle station were significantly better to provide more eDNA than both others positions, (according to Anova test, the f-ratio value is 6.5003. The p-value is.004168. The result is significant at $p < 0.05$). Also, the models used in this study were significantly different, with Gaussian process regression (GPR) applied to logarithmic data offering the best correlation between observed and predicted data.

TABLE 5 Modeling results with tests for various combinations of covariates on cod eDNA concentrations at fishing positions where eDNA levels were above the level of quantification.

Method Name	Model No	Variable	Error (MAE)	Regression (%) Predicted vs True	Comments
Gaussian process regression (GPR)	GPR_1	• Copies/L • kg/h	59.4	94%	Exponential, Model till 20 copies per reaction
Gaussian process regression (GPR)	GPR_2	• Copies eDNA/L • kg/h	74.5	92%	Square Exponential, Model till 20copies kg/
Gaussian process regression (GPR)	GPR_3	• Copies eDNA/L • kg/h	0.03	94.7%	Optimized GPR
Gaussian process regression (GPR)	GPR_4	• Copies eDNA/L • CPUE/ha	0.002	96%	Optimized GPR
Neural Network	NN_1	• Copies eDNA/L • kg/ha	6.04	89%	Wide NN
Neural Network	NN_2	• Copies eDNA/L • kg/ha	7,21	84.5%	Trilayered NN
Bayesian Fixed	Bay_1	• Copies eDNA/L • kg/ha		0.05	
GLS regression	GLS_1	• Log copies/L	0.5	92%	Log copies
OLS	OLS_1	• Log Copies/L	0.6	16%	Log CPUE
OLS	OLS_2	• Sqrt Copies/L	0.2	24%	Sqrt CPUE
R2	R2_1	• Log Copies eDNA/L		40%	Log10 CUPE/ha
R2	R2_2	• Log Copies eDNA/L		28%	Log kg/ha

GPR, Gaussian process regression; GLS regression, generalized least squares (GLS); OLS, ordinary least squares (OLS); R2, coefficient of determination, denoted R^2 or r^2 and pronounced "R squared"; NN, Neural Network; CPUE, Catch per unit effort; Ha, hectare; MAE, mean absolute error.

Predicted vs True: regression between the outputs of the developed model basing on eDNA (predicted) and the catch results by trawl bottom (True).

4.1 Atlantic cod qPCR test/primer development

Four primer pairs targeting the mitochondrial genes mt-ATPase6, mt-ND5, mt-COI, and mt-CytB were designed in silico and validated in-situ. All designed qPCR assays were found to be specific to *Gadus morhua* in the North and Baltic Sea regions, with high sensitivity and specificity. Although the primers may give a positive signal for other untargeted species, this occurs only after a Ct value higher than 36, which is equivalent to 16 copies per reaction or less, which means that every signal after Ct 36 is a false positive signal. This false positive signal may be only due to primer breaks or incomplete hybridization due to thermic exchange during PCR cycles.

The GmCytb-LNA and Cod_ND5 pair was found to be highly specific to *Gadus morhua* with the Environmental Thermo-Fisher polymerase/master mix and the KAPA master mix than with other polymerases. This highlights the importance of polymerase choice in eDNA studies. (Knudsen et al., 2019) determined the LoQ value of their *Gadus morhua* primers to be 669 copies per reaction, while the commercial qPCR (Technique, Bibby Scientific, United Kingdom) has an LoQ value of around 771 copies per reaction. With an LoQ value equivalent to 16 copies per reaction, the GmCytb-LNA pair, coupled with TaqManTM Environmental Master Mix 2.0, has proven to provide more sensitive and reliable results than previously available or published *Gadus morhua* primers/kits.

This means that environmental DNA studies are more accurate and possible with the GmCytb-LNA pair than with other primers.

4.2 eDNA and Stock assignment

A study of the variation in the number of eDNA copies was carried out to determine the best horizontal position to sample the water. Based on the data obtained (Figure 3), we observed that samples taken at the middle/center of the station would cover 46% of the total copy number obtained by qPCR, whereas samples taken at the beginning and end of the station would only cover 27%. The center of the station was, moreover, more consistent with the trawling data than the other two sampling points. We note here that only one station had more significant samples taken at the beginning of the station than at the middle or end of the station.

Despite the large number of studies that have dealt with the subject of eDNA, on different organisms, there are still crucial open questions that need further consideration, especially in the field of fish stock assessment in the sea. These not only include the quantitative aspect, but also the richness of mathematical models, which can offer solutions to accelerate the use of eDNA methods in the field of standard fish population assessment and monitoring, being a non-invasive method.

The comparison of eDNA data with trawling data showed that simple regression models were less effective in establishing a

significant relationship between the number of eDNA copies and the amount of fish caught or the CPUE number, where the regression percentage did not exceed 40%. On the contrary, the regression models based on neural networks and the Gaussian process allowed obtaining a high percentage of correlation between the predicted results of the model and those obtained from the trawling, especially when the predictor is the value of CPUE, coupled with a standardization by \log_{10} . Therefore, the GPR-based model (GPR_4), predicting the CPUE based on the copy number per liter, achieved a degree of correlation in the order of 95% with an error rate in the order of 0.002 (Figure 4). In the same line with our results, the outputs of modelling by (Lacoursière-Roussel et al., 2016), showed that a simple regression is only able to offer a maximum of 40% regression between the trawling data and the predictive. In addition, the works of (Tillotson et al., 2018; Moushomi et al., 2019), showed that generalized least squares (GLS) regression was able to provide a 90% regression,

which is observed in our results, however, the error rate is of the order of 0.5 which may introduce a bias in the predictive data. The autoregressive integrated moving average (ARIMA) model invented by (Thalinger et al., 2019) also offers significant results between predicted and observed, however, this model focuses on studying the relationship between target eDNA concentration and fish numbers *via* time series modelling. Salter et al. (2019) found that their qPCR test could detect cod only at catch densities higher than 200 kg/h. Furthermore, data published by (Mahon et al., 2013) suggested that positive detection increased with the relative abundance of fish species in the Chicago area waterway system. In the same context, but in a controlled environment, (Doi et al., 2015) found a positive correlation between fish biomass and eDNA concentration in two experimental ponds.

In the present study, we were able to predict the stock abundance of cod in the North Sea and the Baltic Sea using eDNA tools from the samples. The results were similar to those

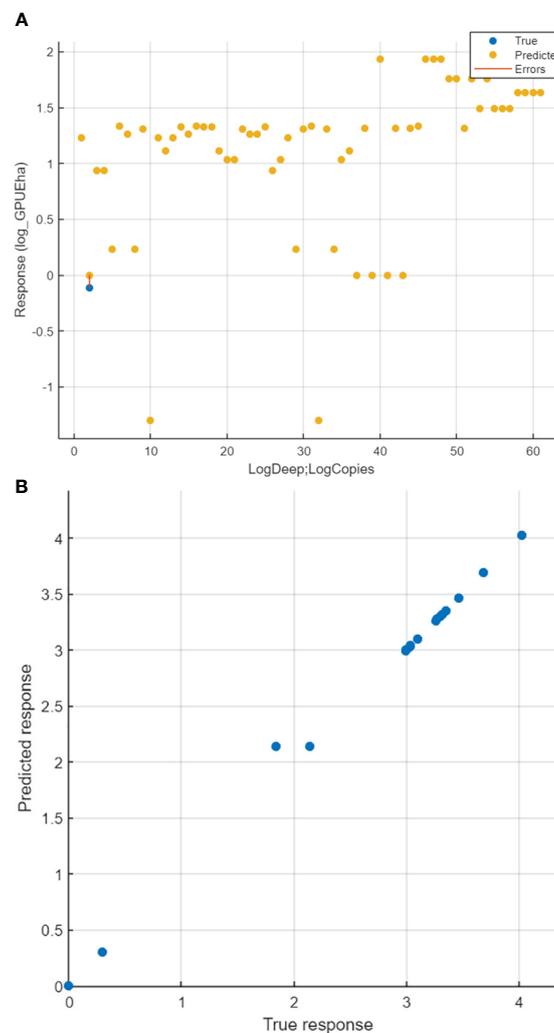


FIGURE 4

Comparison between output of Catch results and output of GPR model for eDNA copies. ($R = 96\%$, Error MAE = 0,002). (A) co-Plot of the real data from trawl (blue) and the predicted basing on the eDNA model (yellow), the x-axis present predictive values of Log Depth (log Deep) of sampling and number of DNA copies (Log Copies); most of data were in predicted as it was observed. (B) The correlation between predicted data by the model (Predicted response) and the real data collected from trawl (True response).

obtained by trawling and CPUE for cod. Therefore, the compatibility between the trawl and eDNA data, supports also that eDNA can be used as a tool to reveal the cod stock situation in real time. In addition, as the half-life of eDNA in water samples is significantly short - around 48h- (Maruyama et al., 2014; Collins et al., 2018; Harrison et al., 2019), which makes the effects of eDNA accumulation in water through time negligible, and then eliminates the assumption that the amount of eDNA due to the long-term fishery repertoire of stations. But only for short times prior to trawling (maximum 48h). The issues of transport from other areas *via* currents are important to study, but the results from the three sampling positions for each station show that transport by current was not significant in our study. The sampling sites in the middle of the stations are counted as the most eDNA rich sites in *Gadus morhua* compared to the stations within the boundaries. The results obtained at the different stations show that the intermediate samples were richer in DNA copy numbers than those taken at the beginning or end of the station. To the best of our knowledge, there are currently no studies explaining these results. In this respect, we suspect that it is a question of random chance or that the ship created a secondary current along the flight path inside the station, causing an accumulation of DNA in the middle. However, this interpretation has been abandoned, as the amount of eDNA in the samples from the end of the station were often lower than in the center and the starting point, which contradicts this proposal and therefore negates it. The true reasons remain unknown, and subject to further studies in different locations and times of year, to examine their results and compare them to the current results, as well as to understand the impact of spatio-temporal variation on the validity of the current results.

5 Conclusions

Our results support the idea that eDNA can be used in the assessment of commercial fish stocks species to estimate the abundance of marine species, and elaborate a new non-invasive complementary method to the conventional methods currently used. Although a total replacement of the reference methods in the short term is not envisaged, a complementary integration of eDNA tools and especially of our eDNA method, will be a plus to put these technical tools on the right track of implementation and familiarization for monitoring and stock assessment teams in the short and medium term. This allows also to standardize and determine the effect of biotic and abiotic conditions on the performance and predictive capacity of fish stock assessment by eDNA from marine waters for other marine species.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material. All data are available upon request to YK (yassine.kasmi@thuenen.de) or to RH (reinhold.hanel@thuenen.de).

Author contributions

BM and TB: Filtration and DNA Extraction. EE, TB, and YK: qPCR test development, optimization and Molecular Biology analysis. AE and YK: Bioinformatics and computational modeling. BM, EE, GD, MB, PN, SK, and TM, Water sampling of water from North Sea and Baltic Sea, Fisheries data. CS, EE, LC, RH, and YK: Conception of research and establish the methodology. LC, CS, and RH: Funding, Supervision, Orientation, and Coordination. RH: Correction of manuscript. YK: First draft manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2023.1058354/full#supplementary-material>

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Supplementary Material

Title: Atlantic cod (*Gadus morhua*) assessment approaches in the North and Baltic Sea: a comparison of environmental DNA analysis versus bottom trawl sampling

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1. Method of Calculate the number of copies per L

$$X \text{ (copies/L)} = (Y \cdot b)/(c \cdot a) = Y \cdot (20/(5 \cdot 5)) = 66.7 \cdot Y \text{ copies/L}$$

eDNA copy numbers in the original seawater samples (X, copies/L) were calculated based on the volume of filtered seawater (a: 5 L), the re-suspended volume of purified DNA (b: 50 µL), the amount of purified DNA added to each qPCR-reaction volume (c: 5 µL/qPCR reaction), and the measured copy number in the qPCR-reaction, calculated by our qPCR system software based on the standard curve (Y, copies/qPCR reaction) according to the equation (Eq.1) (Knudsen et al., 2019).

2. Calculation of LoQ and LoB

In real-time PCR (qPCR), false positives can be caused by various sources of molecular biological noise. Establishing a false-positive threshold for the quantification of nucleic acids is essential for the performance of a robust and reliable dPCR assay. Establishing the analytical performance indicators of an assay, including the limit of blank (LoB) that can also be referred to as the Negative Control, and limit of detection (LoD), is fundamental and must be done according to precise procedures. The LoB is the upper limit of the target concentration that is considered acceptable in a blank sample. The LoB is then used to calculate the LoD, the target concentration limit above which the presence of the target can be asserted and quantified with a given statistical confidence. In all assays we experienced cross-species amplifications of untargeted Gadid species after the Ct value 37. Therefore, we adopted a strategy based on the concept of analytical sensitivity addressing three analytical values as described in (Ficetola et al., 2016; Forootan et al., 2017; Knudsen et al., 2019; Barra et al., 2020).

To determine LoB, LoD and LoQ values, a qPCR assay was performed with a dilution series (from 1 million copies per reaction down to 1 copy per reaction) for the target species in 9 technical replicates and 9 technical replicates at 1 million copies per μL for each non-target species.

The majority of blanks and non-target species were negative. However, for the few replicates that were positive, the signal did not appear until after cycle 36, as the copy number per reaction for non-target species was 5 million DNA copies per reaction (1 million per μL), whereas at cycle 36 we obtained a positive signal amplification of 10 copies per reaction in the target species assays, which is equivalent to 2 copies per μL . LoB, LoD and LoQ values were then determined as defined below.

- The Lower Limit of Blank (LoB) corresponding to the lowest DNA copy number that can be detected with 95 percent confidence above the concentrations of the blank or untargeted species.
- The Limit of Detection (LoD) corresponding to the number of DNA copies that can be detected with 95 percent confidence, but which cannot be quantified under the experimental conditions.
- The Limit of Quantification (LoQ) defined as the smallest number of DNA copies, which can be measured and quantified with a defined precision and accuracy under the experimental conditions by a given method (Armbruster and Pry, 2008). The LoQ will never be below LoD (A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything, 2017). The LOQ defined as the smallest amount of the analyte, which can be measured and quantified with defined precision and accuracy under the experimental conditions by the method under validation (Armbruster and Pry, 2008). An alternative definition is that the LOQ is the lowest amount or concentration of the analyte that can be quantitatively determined with an acceptable level of uncertainty. The LoQ will be never under LoD (Kralik and Ricchi, 2017)

A Ct value for the LoQ (Ct_{LoQ}) is calculated as:

$$Ct_{LoQ} = Ct_{LoD} - 2(\sigma_{Ct_{LoD}})$$

where $\sigma_{Ct_{LoD}}$ is the standard deviation of the Ct_{LoD} for the assay. The qPCR slope should be around -3.49.

The CtLoQ is used to calculate a concentration (copies/rxn) using the standard curve run with the dilution series:

$$\log_{10}\text{concentration (LoQ)} = (\text{Ct}_{\text{LoD}} - 40.958) / \text{slope}$$

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Supplementary Material

Title: Atlantic cod (*Gadus morhua*) assessment approaches in the North and Baltic Sea: a comparison of environmental DNA analysis versus bottom trawl sampling

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1. Tables

Table S1: List of accession numbers used to design the primers. These sequences were used to elaborate the first primers, then a validation was performed according to the Primer development section. The sequences were downloaded between May and July 2021.

Species	Accession Number	Database
Complete mitochondrial Genome		
<i>Gadus morhua</i>	X99772.1	NCBI
	HG514359.1	NCBI
	LS999100.1	NCBI
	MN122852.1	NCBI
	KX266969.1	NCBI
	NC_002081.1	NCBI
	AM489716.1	NCBI
	EU877712.1	NCBI
	KX267069.1	NCBI
<i>Arctogadus glacialis</i>	AM919429.1	NCBI
	NC_010122.1	NCBI
<i>Arctogadus glacialis</i>	DQ356935.1	NCBI
	AM919428.1	NCBI

<i>Boreogadus saida</i>	MH035536.1	NCBI
	NC_010121.1	NCBI
<i>Chaetodontoplus mesoleucus</i>	NC_026521.1	NCBI
<i>Eleginus gracilis</i>	MH061051.1	NCBI
<i>Eleginus gracilis</i>	MH061079.1	NCBI
<i>Gadiculus argenteus thori</i>	AP018148.1	NCBI
<i>Gadus chalcogrammus</i>	AB182302.1	NCBI
	MH035590.1	NCBI
	NC_004449.1	NCBI
<i>Gadus macrocephalus</i>	KY296294.1	NCBI
	NC_036931.1	NCBI
<i>Hucho bleekeri</i>	HM804473.1	NCBI
<i>Lota lota</i>	KC844053.1	NCBI
<i>Melanogrammus aeglefinus</i>	DQ020497.1	NCBI
	NC_007396.1	NCBI
<i>Merlangius merlangus</i>	NC_007395.1	NCBI
<i>Microgadus proximus</i>	DQ356944.1	NCBI
<i>Micromesistius poutassou</i>	FR751401.1	NCBI
	NC_015102.1	NCBI
<i>Physiculus japonicus</i>	AP004409.1	NCBI
<i>Pollachius pollachius</i>	NC_015097.1	NCBI
<i>Pollachius virens</i>	DQ356945.1	NCBI
	NC_015094.1	NCBI
<i>Theragra finnmarchica</i>	AM489718.1	NCBI
	AM489719.1	NCBI
Cytb sequences		
<i>Gadus morhua</i>	Identifier_MT00283	Aquagene
<i>Trisopterus minutus</i>	Identifier_Sp345	Aquagene
<i>Pollachius virens</i>	Identifier_MT01544	Aquagene

<i>Pollachius pollachius</i>	Identifier_MT04178	Aquagene
<i>Boreogadus saida</i>	Identifier_UAM-Fish-47926-370845	Aquagene
	AB091097.1	NCBI
<i>Gadus chalcogrammus</i>	Identifier_UAM-Fish-6586-372184	Aquagene
<i>Eleginus gracilis</i>	Identifier_UAM-Fish-8745-358404	Aquagene
<i>Trisopterus luscus</i>	DQ174081.1	NCBI
	EU492067.1	NCBI
<i>Molva molva</i>	DQ174071.1	NCBI
<i>Molva macrophthalma</i>	DQ174070.1	NCBI
<i>Gadiculus argenteus</i>	DQ174042.1	NCBI
<i>Trisopterus esmarkii</i>	EU492145.1	NCBI
	EU492305.1	NCBI
<i>Raniceps raninus</i>	AJ517497.1	NCBI
<i>Micromesistius poutassou</i>	EU492137.1	NCBI
<i>Microgadus tomcod</i>	KP644319.1	NCBI
<i>Brosme brosme</i>	DQ174037.1	NCBI
<i>Enchelyopus cimbrius</i>	DQ174040.1	NCBI
<i>Phycis blennoides</i>	DQ174072.1	NCBI
<i>Micromesistius australis</i>	AB262083.1	NCBI
<i>Microgadus proximus</i>	DQ174066.1	NCBI
	DQ174067.1	NCBI
COI sequences		
<i>Arctogadus glacialis</i>	HM421733.1	NCBI
<i>Boreogadus saida</i>	Identifier_UAM-Fish-47926-370845	Aquagene
<i>Eleginus gracilis</i>	Identifier_UAM-Fish-8745-358404	Aquagene
<i>Gadiculus argenteus</i>	KJ709532.1	NCBI

<i>Gadiculus argenteus thori</i>	LC146704.1	NCBI
<i>Gadus chalcogrammus</i>	Identifier_UAM-Fish-6586-372184	Aquagene
<i>Gadus macrocephalus</i>	JF952739.1	NCBI
<i>Gadus morhua</i>	Identifier_MT00283	Aquagene
<i>Melanogrammus aeglefinus</i>	KP975763.1	NCBI
<i>Merlangius merlangus</i>	KP975779.1	NCBI
<i>Microgadus proximus</i>	JQ354229.1	NCBI
<i>Microgadus tomcod</i>	KF930129.1	NCBI
<i>Micromesistius australis</i>	HQ882700.1	NCBI
<i>Micromesistius poutassou</i>	KJ709820.1	NCBI
<i>Pollachius pollachius</i>	Identifier_MT04178	Aquagene
<i>Pollachius virens</i>	Identifier_MT01544	Aquagene
<i>Raniceps raninus</i>	KJ128598.1	NCBI
<i>Theragra finnmarchica</i>	AM711630.1	NCBI
<i>Trisopterus capelanus</i>	KJ709669.1	NCBI
<i>Trisopterus esmarkii</i>	KJ205240.1	NCBI
<i>Trisopterus luscus</i>	KJ205244.1	NCBI
<i>Trisopterus minutus</i>	Identifier_Sp345	Aquagene

Table S2: List of untargeted species used for primer screening and validation.

Species	Source (Thünen Aquagene tissue samples)
<i>Brosme brosme</i>	SP630
<i>Chelidonichthys lucerna</i>	SP213
<i>Clupea harengus</i>	Ch1
<i>Enchelyopus cimbrius</i>	MF44
<i>Engraulis encrasiolus</i>	MA82
<i>Helicolenus dactylopterus</i>	SP310
<i>Lota lota</i>	SP509

<i>Melanogrammus aeglefinus</i>	MF26
<i>Merlangius merlangus</i>	MF1
<i>Merluccius merluccius</i>	MA36
<i>Micromesistius poutassou</i>	SP703
<i>Molva molva</i>	MF105
<i>Pollachius virens</i>	MF37
<i>Scophthalmus maximus</i>	SP173
<i>Trachurus trachurus</i>	MA31
<i>Trisopterus luscus</i>	MA159
<i>Trisopterus minutus</i>	SP344

Table S3: Sampling stations.

Station Number	Sampling Position	Sampling Depth [m]	Date
28	54°26,11/7°13,28	29	16.07.2019
28	54°25.15/7°13,92	32	16.07.2019
28	54°24,25/7°14,67	30	16.07.2019
51	56°41,78/5°20,78	53	24.07.2019
51	56°40,86/5°19,96	53	24.07.2019
51	56°40,00/5°19,00	53	24.07.2019
70	58°48,86/2°24,08	103	27.07.2019
70	58°47,90/2°24,81	104	27.07.2019
70	58°46,96/2°25,27	102	27.07.2019
76	60°21,53/2°29,40	91	28.07.2019
76	60°22.34/2°30.38	85	28.07.2019
76	60°22,91/2°31,96	84	28.07.2019
85	60°25,13/2°39,92	100	30.07.2019
98	58°49,10/2°27,12	105	01.08.2019
98	58°48,30/2°25,57	102	01.08.2019
98	58°47,58/2°24,28	103	01.08.2019
N 01	54°15,526N/007°30,338E	35,8	02.12.20
B 01	54°33,127N/010°48,236E	13,63	05.12.20
B10	54°49,377N/013°55,848E	35,75	06.12.20
B11	54°47,456N/013°52,488E	32,96	06.12.20
B 09	55°07,674N/018°10,216E	58,13	07.12.20
B25	58°45,029N/021°48,705E	40,6	08.12.20

Supplementary Material

Pori	61°13,828N/019°43,243E	63,2	09.12.20
B22	56°27,793N/020°27,551E	35,6	12.12.20

7. Chapter VII:

Detection and quantification of two flatfish species in the Sylt outer reef: Development of a qPCR assay and forecast model based on eDNA copies

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7.1. Abstract

Assessing fish diversity and abundance is crucial for effective conservation and management strategies, particularly in ecologically sensitive areas such as the North Sea. Bottom trawling, as the most common sampling method in fisheries science, is increasingly viewed critically even for scientific purposes and is already banned in sensitive zones. Therefore, it is crucial to rapidly establish non-invasive methods for solid biodiversity assessments.

In this study, a quantitative multiplex PCR (qPCR) environmental DNA (eDNA) analysis was tested for the detection of common dab (*Limanda limanda*) and European plaice (*Pleuronectes platessa*) in the North Sea. The concordance between the detectability of qPCR eDNA results and bottom trawling as the reference method was around 90% with a specificity of qPCR of 100% and a sensitivity of 82% for both species. For biomass estimation the outputs of a newly developed GLM and Nelder mead model showed correlations with bottom trawl catches of 80% and 74% for plaice and dab, respectively.

The model validation and performance were assessed using data from a separate sampling mission in a different area of the North Sea. For plaice, the Root Mean Squared Error (RMSE) and Mean Absolute Error (MAE) were 26% and 28%, respectively, while for common dab, these values were 48% and 34%, respectively.

These results demonstrate the potential of eDNA as a promising non-invasive tool for biodiversity as well as biomass assessments, comparable to traditional survey methods in terms of detection and species richness, although further optimization steps are certainly required for a stand-alone use.

Keywords: common dab (*Limanda limanda*), European plaice (*Pleuronectes platessa*), bottom trawling, environmental DNA (eDNA), quantification, North Sea.

1 Introduction:

Marine biodiversity monitoring is a key component of ocean observation programs to detect and follow climate- or other impact-driven changes in coastal or offshore ecosystems that may also affect marine living resources. Especially in the fields of fisheries biology and fish ecology, fishery independent abundance or biomass data are needed, to evaluate the stock status of exploited species.

There are numerous methods to quantify fish, most of them applying some kind of fishing gears and techniques, often specific for certain species or fish groups (Aguzzi et al., 2020; Fischer, 2013; Leonart & Maynou, 2003). Fish trawls are a common catch method to quantify the abundance of fish, as they allow often to sample a number of different fish species simultaneously and they are capable to sample large areas or volume of water, which makes them very efficient in collecting fish (Groeneveld, 2000; ICES, 2020; Kaartvedt et al., 2012). One advantage of catching fish in order to quantify abundance or biomass is obviously the possibility to take also biological data directly from the fish, such as length, weight, or tissue samples for all kind of subsequent analyses. However, there are also numerous disadvantages of trawl surveys, e.g., it is limited to species for which the gear is selective, and trawl surveys are expensive and time consuming. Besides that, in recent years also practical reasons hampered the performance of trawl surveys, especially in the North Sea: The growing sector of offshore wind energy uses vast marine areas (Stelzenmüller et al., 2020), in which it is often no longer possible to conduct trawl surveys (ICES, 2020, 2023). The destructive nature of trawling gears on the sampled organisms as well as on habitats is often not desirable or tolerable, especially in marine protected areas, of which more and more were and will be established (Cuetos et al., 2022; Hilborn et al., 2023; Petetta et al., 2021; Sala et al., 2023). Besides that, also the ethical debate about animal welfare became more important in recent years (Coghlan, 2022; Garcia et al., 2007; Mason, 2023; Rijnsdorp et al., 2016; RuMOHR & Krost, 1991; Witbaard & Klein, 1994). The aforementioned points illustrate that there is clearly a growing demand for alternative, non-invasive methods to quantify fish abundance, biomass and diversity.

The use of environmental DNA (eDNA) in marine research enables species detection from simple water samples, and thus offers a non-invasive method for monitoring (Rishan et al., 2023; Rourke et al., 2021; Stat et al., 2017). Furthermore, it provides information that complements conventional biodiversity monitoring methods, thereby contributing to a better understanding of ecosystems and distribution patterns of organisms (Bista et al., 2017; Handley

et al., 2020; Lacoursière-Roussel, Rosabal, et al., 2016; Milhau et al., 2019; Stat et al., 2017). While many studies have already shown the growing potential of eDNA to monitor the presence of marine organisms (Benoit et al., 2023; Karlsson et al., 2021; Kasmi et al., 2023), the approach of the qPCR using eDNA in order to quantify the biomass of marine organisms or fish is at its beginning (Benoit et al., 2023; Karlsson et al., 2021, 2022; Kasmi et al., 2023).

Recent studies focusing on Atlantic cod have demonstrated a positive correlation between trawl data and eDNA, in which the regression between eDNA copies from qPCR and Atlantic cod biomass (kg) was 0.79 ($P = 0.003$) using GLM (Salter et al., 2019). In the same line, Kasmi et al., (2023) found a positive correlation between qPCR output and CPUE using gaussian process regression. Furthermore, efforts to estimate juvenile Pacific salmon (*Oncorhynchus spp.*) biomass by qPCR from water eDNA revealed a positive correlation between biomass and eDNA copy numbers (Benoit et al., 2023). However, the results revealed also the influence of eDNA shedding rates on the eDNA concentrations which reduces the significance of this correlation (Benoit et al., 2023). In another study, the link between Atlantic herring (*Clupea harengus*) and Atlantic mackerel (*Scomber scombrus*) bycatch biomass and eDNA from three herring catches was found to be consistent in terms of quantity and in reflecting the variety of species within each catch, although of the presence of certain differences in the total estimates of mackerel biomass (Urban et al., 2024). A key aspect of all studies using qPCR is the importance of establishing consistent methods for determining and interpreting the limit of detection (LOD) and limit of quantification (LOQ) for single-species quantitative PCR (qPCR) eDNA studies (Klymus et al., 2020). For numerous species, these values are still too high (Knudsen et al., 2019; Salter et al., 2019), which request the development of more sensitive assays.

In the present study, eDNA analysis using a quantitative multiplex PCR (qPCR) test was used, to directly compare the catch by unit effort (CPUE) obtained by bottom trawl catches of common dab (*Limanda limanda*) and European plaice (*Pleuronectes platessa*), two highly abundant fish species in the investigation area, the Sylt Outer Reef (SOR) in the South-Eastern North Sea, a Natura 2000 site characterized by a high diversity of sediment and habitat structures. In this work, a qPCR assay was developed for the first time to detect and assess the abundance of the two species. Resulting data were used to develop a simple computational model to predict the abundance of both species given the eDNA results.

2 Method

2.1 Trawl catches

Data of dab (*Limanda limanda*) and plaice (*Pleuronectes platessa*) were analyzed from catches taken during two surveys (WH457 and WH469) with the Fisheries Research Vessel “Walther Herwig III” (Table 1).

During WH457 fish data were sampled at twelve stations in June/July 2022 in the Natura2000 area Sylt Outer Reef (SOR) using a 3 m beam trawl equipped with a sole net and five tickler chains (Table 1). The codend mesh size was 40 mm (stretched). The towsing time was set to 15 minutes with a maximum speed of around 3 knots.

Fish data from seven stations in the southern and central North Sea were analyzed during WH467 in August 2023. Sampling of fish was conducted by means of the ICES standard bottom trawl „Grande Ouverture Verticale“ (GOV) with 20 mm stretched mesh size in the codend. GOV hauls were applied with 25-30 minutes towsing duration at 4 knots as described in the manual for the International Bottom Trawl Survey (IBTS) (ICES, 2020).

In total, 19 hauls were analyzed for the study. For each haul, the weight and numbers of dab and plaice were recorded. Biomass and abundance data were standardized to a towsing time of one hour (kg/km² and individuals/km²).

Table 1: Sampling locations for water and catch samples.

Station	Latitude (N)	Longitude (E)	Expedition
1	54°35.03N	007°37.11E	WH457
2	54°41.52N	007°20.16E	WH457
3	54°47.94N	007°32.71E	WH457
4	54°47.20N	006°44.91E	WH457
5	54°49.65N	006°39.16E	WH457
6	54°56.98N	007°02.23E	WH457
7	55°01.05N	007°00.21E	WH457
8	55°06.11N	007°20.08E	WH457
9	55°01.01N	007°47.92E	WH457
10	55°06.04N	007°38.89E	WH457
11	55°01.02N	007°24.04E	WH457
12	55°50.07N	007°40.43E	WH457
1/452	54°32.76N	010°47.02E	WH469
6/457	54°48.35N	013°10.91E	WH469
7/458	54°51.79N	013°18.77E	WH469
11/462	55°02.46N	018°23.53E	WH469

23/474	54°53.88N	011°25.35E	WH469
32/483	55°01.73N	006°18.08E	WH469
36/487	56°31.06N	002°52.50E	WH469

2.2 Water Sampling

Water sampling has been performed as described by (Kasmi et al., 2023). At each station, two 5-liter samples of water were collected. During the sampling process, Niskin bottles were utilized to collect the water. Moreover, two negative control samples were included in the sampling process. Subsequently, the collected water was immediately filtered through 0.45µM PES membranes in sterile funnel units (Sartorius, Göttingen, Germany) with a 2-port filtration system. The filters were stored in clean sterile tubes at -20°C until extraction. All Niskin bottles were thoroughly cleaned prior to water sample collection by rinsing first with fresh water and then with ultrapure water. Negative controls for all Niskin bottles used were performed by filtration of 0.5 L of ultrapure water rinsed through the cleaned bottles prior to the first sample collection. All filtration steps were performed in a pre-cleaned UV sterilization cabinet (Clever Scientific, Rugby, UK). Clean gloves were worn during each sampling step and all materials used, e.g., water sample containers or membrane tweezers, were sterilized by washing with 3% bleach and/or UV sterilization where possible.

2.3 Environmental DNA extraction

The eDNA extraction process, as outlined by Renshaw et al., (2015), begins with the placement of filtered and frozen PES membranes into a 2 ml tube. This tube is then filled with 700 µL of CTAB buffer, which consists of 2% CTAB (w/v), 1.4 M NaCl, 100 mM Tris, and 20 mM EDTA. The tube is then incubated at a temperature of 65°C for 10 minutes, with an agitation speed of 650 rpm. Following this, 900 µL of PCI Phenol/chloroform/isoamyl alcohol (mixed in a ratio of 25:24:1) is added to the tube, which is then vortexed for 5 seconds. The tube is then centrifuged at 15,000 x g for 5 minutes. After centrifugation, 700 µL of the supernatant is transferred to a new 2 ml tube. To this, 700 µL of chloroform is added, and the tube is centrifuged again at 15,000 x g for 5 minutes. 500 µL of the supernatant is then transferred to a new 2 mL tube. The Monarch® cleaning kit from New England Biolabs, Frankfurt, Germany is used at this stage, with 1000 µL of binding buffer being added to the tube. The elution process is carried out into 40 µl of elution buffer. The DNA extracts obtained were stored at -20°C until

analysis. All DNA extraction steps were performed in a dedicated area for eDNA analysis at the genetics laboratory and under a chemical hood.

2.4 Quantitative assay (multiplex qPCR)

To develop the multiplex qPCR, a primer and probe design was performed by aligning the Cytochrome c oxidase subunit I (COI) gene reference sequences of the two species with the COI sequences of related (non-target) species. The purpose of this alignment was to identify regions of commonality between the both target species and at the same time to distinguish the two species from other related species, so that they could serve as potential sites for primers and as distinctive regions for probes.

2.4.1 Developing the qPCR assays

Referential COI sequences from two target fish species were used to design primer pairs and probes. COI sequences from related (non-target) species, genera and other families were also included to increase the genetic diversity of dataset and cover most of the species described in the literature to date; all sequences and associated genome accession numbers are listed in Table S1.

Nucleotide sequences were aligned using Bioedit, Version 7 . Conserved regions were then manually identified and checked for relevance. Short informative regions of 18-25 nucleotides were selected as potential primer sites, depending on the nucleotide polymorphisms, to be universal for both target and non-target species. To discriminate between the both target species, short informative regions of 20-40 nucleotides including specific nucleotide variations for Common dab (*Limanda limanda*) and European plaice (*Pleuronectes platessa*) were chosen as potential sites for hydrolysis probes within the amplicon. All potential oligos were analyzed for auto- and cross-dimer and hairpin formation, as well as general properties such as melting temperatures and G/C content, using various oligo-analysis web tools: Multiple Primer Analyser (Thermo Fisher Scientific Waltham, Massachusetts) and Oligo Analyse Tool (Eurofins Genomics Germany GmbH, Ebersberg).

As a result, LimaPleuCOI-F and LimaPleuCOI-R as a single primer pair, amplifying a 105 bp long PCR product, combined with PleuCOI-P and LimaCOI-P as specific hydrolysis probes for both targets, met all required qPCR assay design criteria (Table 2) and were ordered from Eurofins Genomics GmbH (Germany, Ebersberg, Germany).

Table 2: Oligo properties

Oligo name	Sequence (5'-3')	Length (bp)	Tm (°C)	GC content (%)	Wobbles	Modifications
LimaPleuCOI-F	TCTACCYCCATCSTTTCTKCTTC T	24	61	45,8	3	-
LimaPleuCOI-R	AGCGTGTGCTAGRTTCCAGCT AA	24	61,9	47,9	1	-
PleuCOI-P	GTATACGGTTCACCCTGTTCCC GCC	25	67,9	60	-	5'FAM 3'BHQ1
LimaCOI-P	GGTATACAGTTCAMCCAGTTCC AGCCC	27	67,2	51,9	1	5'HEX 3'BHQ1

2.4.2 qPCR optimization and specificity check

The initial screening for optimal primer concentration and annealing temperature was performed by standard PCR followed by gel electrophoresis. For this purpose, genomic DNA was extracted from two individual samples each of the target species Common dab (*L. limanda*) and European plaice (*P. platessa*) and closely related species American plaice (*Hippoglossoides platessoides*), and European flounder (*Platichthys flesus*) using Chelex100 (Walsh et al., 1991) as well as the Invisorb® Spin Tissue Mini Kit (Invitek Molecular, Berlin, Germany) as templates in different concentrations. The qPCR reactions were performed on a qTower3 G Touch RealTime PCR System (Analytik Jena, Jena, Germany). Two different qPCR polymerase mixes in combination with and without PCR additives were tested with the DNA samples mentioned above. Different probe concentrations and annealing temperature gradients were investigated until an optimal qPCR reaction and cycling setup was found that allowed efficient amplification of the target species even at low concentrations and ensured no false-positive amplification of non-target species. The specificity of both target probes was verified by analysing several genomic DNA samples of related non-target species extracted with Chelex100 with the final qPCR assay. The non-target species analyzed, in addition to the two species mentioned above, were Lemon sole (*Microstomus kitt*), witch (*Glyptocephalus cynoglossus*) and solenette (*Buglossidium luteum*). All PCR and qPCR reactions were set up in a dedicated amplicon-free area and each run included genomic DNA templates of both target species as positive controls, as well as a no template control to prove the absence of contamination during PCR.

2.4.3 Amplification efficiency and sensitivity of qPCR assays

To evaluate the multiplexed qPCR assays, complex target species DNA pools were prepared for use as templates. Identical concentrations of the two target DNAs were then combined into a single DNA pool. In addition, the DNA mixture was prepared at two different target species ratios (DNA *P. platessa*: DNA *L. limanda* = 10:1 & 1:10) using the dilution series of the individual target DNAs. Therefore, the final concentration of target DNA per species-specific qPCR reaction ranged between 0.009 and 23 ng/ μ l.

Singleplex qPCR was performed for each hydrolysis probe prior to multiplex qPCR to verify both probe functionality and potential interfering primer-probe interactions. In both single and multiplex qPCR, the DNA mixture was used as template at different concentrations. The quantification cycle (Cq) values per probe obtained from different concentrations of target DNA in the singleplex approach were then compared with the corresponding Cq values obtained from multiplex qPCR. Regression lines were generated by plotting Cq values against the logarithm of the target DNA concentration used for qPCR to determine reaction efficiencies. To assess the sensitivity of the qPCR assay, the linear dynamic range and the lower limit, defined as the number of gene copies detectable using the minimum target DNA concentration, were determined.

2.4.4 Standard curve and assay sensitivity

A standard curve experiment was performed using purified and diluted target amplicons as templates. Common dab and European plaice PCR products were amplified with the universal barcoding primer cocktail (VF2_t1-M13, FishF2_t1-M13, FishR2_t1-M13, FR1d_t1-M13) for the COI gene (Ivanova & Grainger, 2007; Ward et al., 2005). Amplicons were purified with Monarch DNA & PCR Cleanup Kit (New England Biolabs, Frankfurt am Main, Germany) and fluorometrically quantified by Qubit 1x dsDNA-HS Assay (Fisher Scientific GmbH, Schwerte, Germany). The purified amplicon was diluted 1:1.000.000 serving as a stock solution, and stored at -20 °C. By calculation with the amplicon length and Avogadro's constant a 10-fold dilution series starting with STD1 (10^6 copies per reaction) down to STD6 (10 copies per reaction) was prepared for each of the two targets. Nine replicates of each standard were analyzed to determine the amplification efficiency and detection limit for each primer/probe set, defined as the lowest copies per reaction with >90% amplification success (Bustin et al., 2009).

2.4.5 Application of the qPCR on eDNA

Detection and quantification of common dab and European plaice in eDNA from marine water samples was performed using the primer/probe combination and optimal reaction conditions developed in Section 1. The final qPCR multiplex assay setup resulted in a total reaction volume of 20 μ l, including 10 μ l of Applied Biosystems™ TaqMan™ Environmental Master Mix 2.0 (Fisher Scientific GmbH, Schwerte, Germany), 0.1U Applied Biosystems™ AmpErase™ Uracil N-Glykosylase (UNG) (Fisher Scientific GmbH, Schwerte, Germany), 3% DMSO (NEW England Biolabs, Frankfurt am Main, Germany), 400 nM of each primer, 200 nM of each probe, 5.5 μ l template and ultrapure water. All qPCR reactions were performed in white PCR strips with transparent covers in a qTower3 G (Analytik Jena, Jena, Germany) under the following reaction conditions: initial denaturation at 95°C for 10 minutes, followed by 40 cycles consisting of a denaturation step at 95°C for 15 seconds and a combined annealing/extension step at 61°C for 59 seconds. Data acquisition by scanning for color channels (FAM at 470 nm excitation and 520 nm detection, HEX_3 at 535 nm excitation and 580 nm detection) was activated at each combined annealing/extension step.

All eDNA samples were analyzed in triplicate, as were both amplicon standard dilution series. Genomic DNA samples used during the qPCR optimization phase were analyzed in duplicates with a maximum of 400 ng DNA per reaction.

2.4.6 Statistical analysis and modeling

Several generalized linear models (GLMs) and non-linear models were tested to assess the relationship between biomass and environmental DNA (eDNA) counts. Non-linear models were fitted using the Nelder-Mead method (Nelder & Mead, 1965). From a pool of 150 GLMs, a small selection of best models was made using the Akaike Information Criterion (AIC) (Akaike, 1974), the coefficient of determination R^2 and parameter's significance (all models are listed in the supplementary data (Table S2)). Two models were selected that appeared most promising in providing insight into biomass estimation from eDNA data. The relationship between eDNA copy number determined by qPCR and the catch per unit effort (CPUE) of dab and plaice from trawl catches in the standard study area was assessed using different combinations of covariates where eDNA levels were positive. The response variable (CPUE data) was standardized by swept area to allow comparisons between the modelled dataset (WH457) and the validation dataset (WH469). In this study the type and selectivity of the trawl gear was not taken into consideration. Therefore, to overcome the issue of using 2 different

nets, the unit kg/km² was introduced to make the data from both cruises comparable. This was necessary because three different nets were used in the cruises, each with its own specific characteristics. To account for this, we calculated the number of fish caught per hour (as each cruise had its own sampling time) and per km² or ha of net (to account for the differences in the nets). This means that while the results provide some insights, they should be interpreted with caution, especially that the selectivity of the net have a significant impact on the results, and further modeling studies should consider this factor.

Variable Filtering: The GLM response in our models was catch per unit effort (CPUE) and expressed in kg/km² and fish/km², for which both identity and logarithmic link functions were tested in the GLM models. The predictors included observed eDNA copies obtained through PCR per liter of water sampled (referred to as $eDNA_L$ in this paper) and the expected number of eDNA copies at the time of sampling (referred as $eDNA_{(t0)}$) (eq 01). Moreover, we incorporated abiotic factors, i.e., depth, pressure, temperature and salinity derived from CTD data. To reduce multicollinearity, we removed variables showing more than 70% correlation, retaining only two key variables for the final analysis: (1) **Depth of water sampling:** Expressed as a positive value indicating the sampling depth from the sea surface (where the surface is 0). (2) **Temperature:** Expressed in degrees Celsius. Given the high correlation between temperature and depth (approximately 70%), we included only one environmental factor in each model to assure adequate performance of the significance test for model parameters.

$$eDNA_{(t0)} = \frac{eDNA_L}{e^{-(T_n * R_n)}} \quad (\text{Eq. 1})$$

Where $eDNA_{(t0)}$ is the eDNA expected at the initial time t_0 , $eDNA_L$ is the eDNA observed in the PCR per liter of water, and R_n is the ratio of eDNA degradation at temperature (in °C) of state n at time T_n . The n indicates the different states in which the sample was subjected prior to extraction of eDNA, i.e sample preservation and filtration.

Model Selection: A small number of models from the original 150 was selected based on their correlation with observed biomass (i.e., the coefficient of determination R^2), low AIC values, and number of significant predictors. Additional important criteria to select the models were also their ability to correctly represent the relations between eDNA, fish biomass and environment, as well as the degradation of eDNA with environmental variables: The dependence of the response variable to each predictor was calculated by varying the target

predictor while fixing all others to their sample means. Afterwards, it was verified that the relationship between the response variable and the predictors was the expected one: (1) Fish biomass should monotonically increase with eDNA and have maxima at reasonable values of temperature and depth; (2) eDNA shedding increases with increasing temperature (McCartin et al., 2022; Robson et al., 2016; Strickler et al., 2015) and with decreasing depth (note that (Danziger et al., 2022; Merten et al., 2021; Nevers et al., 2018; Visser et al., 2021) have showed a link between eDNA and depth).

Some of the GLMs tested included the sum of predictors inside a logarithm (e.g., $\log(eDNA_L - temperature)$ or $\log(eDNA_L - depth)$, see Table S2 in the Supplement). Because the predictors have different units, mathematical formalism demands that one of those predictors is multiplied by a coefficient (e.g., $\log(eDNA_L - \alpha \cdot temperature)$). Therefore, any of these GLMs showing good model performance was explored further by replacing it with a similar non-linear model that included a coefficient inside the logarithm (keeping otherwise the same equation of the corresponding GLM). These models demand a special type of non-linear solvers, from which the Nelder-Mead (Nelder & Mead, 1965) is one of the most commonly used. Initial values for the models were estimated from the GLM, with coefficients α of environmental predictors inside the logarithm initially set to 1.

The Nelder-Mead algorithm is a robust numerical method widely used for finding the minimum of an objective function in a multidimensional space. As objective function for the algorithm we used the sum of squares between the model predictions and observations. Subsequently, we performed 1000 Monte Carlo simulations using random data within the standard deviation limits. Then the initial values obtained from the Nelder-Mead model were compared with pseudo coefficients from the Monte Carlo simulation to determine the significance of the model coefficients.

To validate the best model using the WH469 dataset, we employed two metrics: Root Mean Squared Error (RMSE) and Mean Absolute Error (MAE), expressed as percentages. Low RMSE and MAE values indicate good model performance. This process helps to ensure the reliability and effectiveness of the best model in predicting outcomes based on the WH469 dataset.

All analyses and data visualizations were conducted in R. The GLM was implemented using the GLM package and the `glm` function (Marschner et al., 2018), while Nelder-Mead

optimization was performed using the “nelder mead” package (Bihorel et al., 2015). Figures were generated using ggplot2 and plotR (Wickham et al., 2016).

3 Results

3.1 Quantitative multiplex assay for *common dab and European plaice*

3.1.1 Multiplex assay performance and sensitivity

A multiplex qPCR assay targeting the COI gene has been developed to detect and quantify common dab and European plaice in marine environments using environmental DNA.

The performance of the test was evaluated in terms of specificity, sensitivity, efficiency and practical application. The results showed that the probes developed are characterized by their high specificity for dab and plaice in the simplex assays, where the specificity was 100%, therefore no non-target species were found to be positive in this step. In the multiplex assays, no cross-detection between the probes and the two species was observed, which ensuring high specificity also during the multiplexing assay.

In terms of sensitivity, the serial dilution assays of purified DNA from common dab and European plaice showed promising results, i.e., the common dab probe has the ability to detect up to 100 copies/reaction, while the European plaice probe is able to detect 10 copies/reaction as the minimum quantifiable copy number; on the other hand, the maximum Ct-value for quantification is 37 for both probes, which demonstrates the ability of the assay to detect small amounts of DNA.

Both species were detected with a linearity of 98% and 97.6% at high replication rates. These findings demonstrate that the assay is able to detect positive samples with high accuracy, even at low DNA concentrations.

3.1.2 Application of the qPCR assay on the North Sea samples

The results showed the presence of dab and European plaice in the majority of the samples with positive trawl results, thus confirming the ability of the developed assay to detect both species from environmental samples within the same assay qPCR.

The multiplex qPCR and bottom trawl results were analyzed for 22 samples (11 stations) (Table 4 & 5). The eDNA results of 17 and 15 samples were positive for European plaice and dab respectively. The Ct for European plaice in these samples ranged from 21 to 38. Whereas,

common dab showed Ct values between 15 and 39. All negative samples were negative (Niskinbottle controls, negative extraction and qPCR without matrix). The copy numbers were determined according to the standard curves for each species of interest (Table 3 and Table 4). Overall, the test was found to be positive for 82% of the stations.

The results showed that both species could be detected even at low abundance (lower than 160 kg/km² for plaice and 190 kg/km² for dab) with a reproducibility rate in the 3 technical replicates of the same sample of 100% using the qPCR approach for eDNA analysis. This rate is reduced to 50% for less than 180 kg/km².

Table 3: Results of qPCR from European plaice

Station	Copy DNA per reaction	Copy DNA per sample	CPUE (Kg/ km ²)	CPUE (individual/km ²)
Negative Control	0	0	0	0
	0	0	0	0
1	0	0	184	3346
1	0	0	184	3346
2	0	0	629	20250
2	27	194	629	20250
3	17	126	197	2158
3	22	159	197	2158
4	26	190	114	1620
4	39	283	114	1620
5	0	0	192	8189
5	0	0	192	8189
6	38	273	583	16568
6	18	134	583	16568
7	13	94	472	12773
7	13	92	472	12773
8	11	77	370	14317
8	42	303	370	14317
9	7	54	367	11140
9	43	314	367	11140
10	14	98	381	7396
10	15	112	381	7396
11	16	113	445	10196
11	14	102	445	10196

Table 4: Results of qPCR from common dab

Station	Copy DNA per reaction	Copy DNA per sample	CPUE (Kg/km ²)	CPUE (fish/km ²)
Negative	0	0	0	0
Control	0	0	0	0
1	395	2874	828	14149
1	0	0	828	14149
2	1008	7329	2188	52247
2	260	1888	2188	52247
3	0	0	164	5723
3	680	4942	164	5723
4	743	5400	129	3037
4	778	5659	129	3037
5	315	2291	998	32649
5	1290	9380	998	32649
6	626	4550923	1319	49169
6	48	351	1319	49169
7	323	2351	1032	23027
7	1189	8645	1032	23027
8	0	0	158	5404
8	0	0	158	5404
10	0	0	689	27343
10	0	0	689	27343
11	1930	14036	1480	31985
11	1284	9337	1480	31985

3.1.3 Modelling the eDNA outputs to predict biomass

The relationships between eDNA concentrations and the biomass of dab and/or European plaice trawled across the standard study area were investigated using various combinations of covariations for eDNA concentrations at fishing stations with eDNA concentrations above the quantification threshold. The correlation between eDNA copy number at time of sampling (t_0) and the biomass of common dab and European plaice caught by the linear model were insignificant and weak. Attempts were made to normalize the data using logarithmic functions, of which the best regression obtained was 28% between eDNA copy number per L and biomass in kg/km². For CPUE/km², the best correlation obtained with log eDNA copies/L was 21%.

Based on our performance metrics (AIC and R²), as well as on our analysis of model realism, we selected two best-performing models. These models are designated here as Model 1 (eq.2)

and Model 2 (eq.3) for both European plaice and common dab (see Table 5 for the models' performance metrics):

$$\log(\text{CPUE}(eDNA_{t_0}, \text{Depth})) = (\alpha + \beta \cdot (\text{Depth})) + \gamma \cdot \log(eDNA_{t_0} - \delta \cdot \text{Depth}) \quad \text{eq. 02}$$

$$\log(\text{CPUE}(eDNA_{t_0}, Tp)) = (\alpha \cdot Tp + \beta \cdot (Tp^2)) + \gamma \cdot \log(eDNA_{t_0} - \delta \cdot Tp) \quad \text{eq. 03}$$

Table 5: Performance metrics of our two best models equations 2 and 3. AIC and R2 are calculated from the WH457 dataset used to build the model, while RMSE and MAE are obtained from the validation of the model using the WH469 dataset.

Model	Link	Plaice				Dab			
		WH457		WH469		WH457		WH469	
		AIC	R2	RMSE	MAE	AIC	R2	RMSE	MAE
1 (eq2)	log	28	0.80	0.28	0.26	66	0.74	0.48	0.36
2 (eq3)	log	58	0.24	0.75	0.76	75	0.54	0.73	0.68

Model 1 demonstrates superior predictive accuracy for both species, particularly evident in the case of plaice, as indicated by its lower AIC value (28) and higher R² (0.8) compared to Model 2. Similarly, in the case of the common dab, Model 2 displays relatively poorer performance, with a higher AIC (75) and lower R² (0.5) compared to Model 1 – (AIC=66 and R²=0.7). Moreover, Model 1 exhibits robust predictive capability even on independent validation datasets, with RMSE and MAE being less than 28% for plaice and 48% for dab, signifying acceptable error deviation compared to our other models tested. Consequently, the validation results align with the findings of the fitted models, reinforcing their reliability.

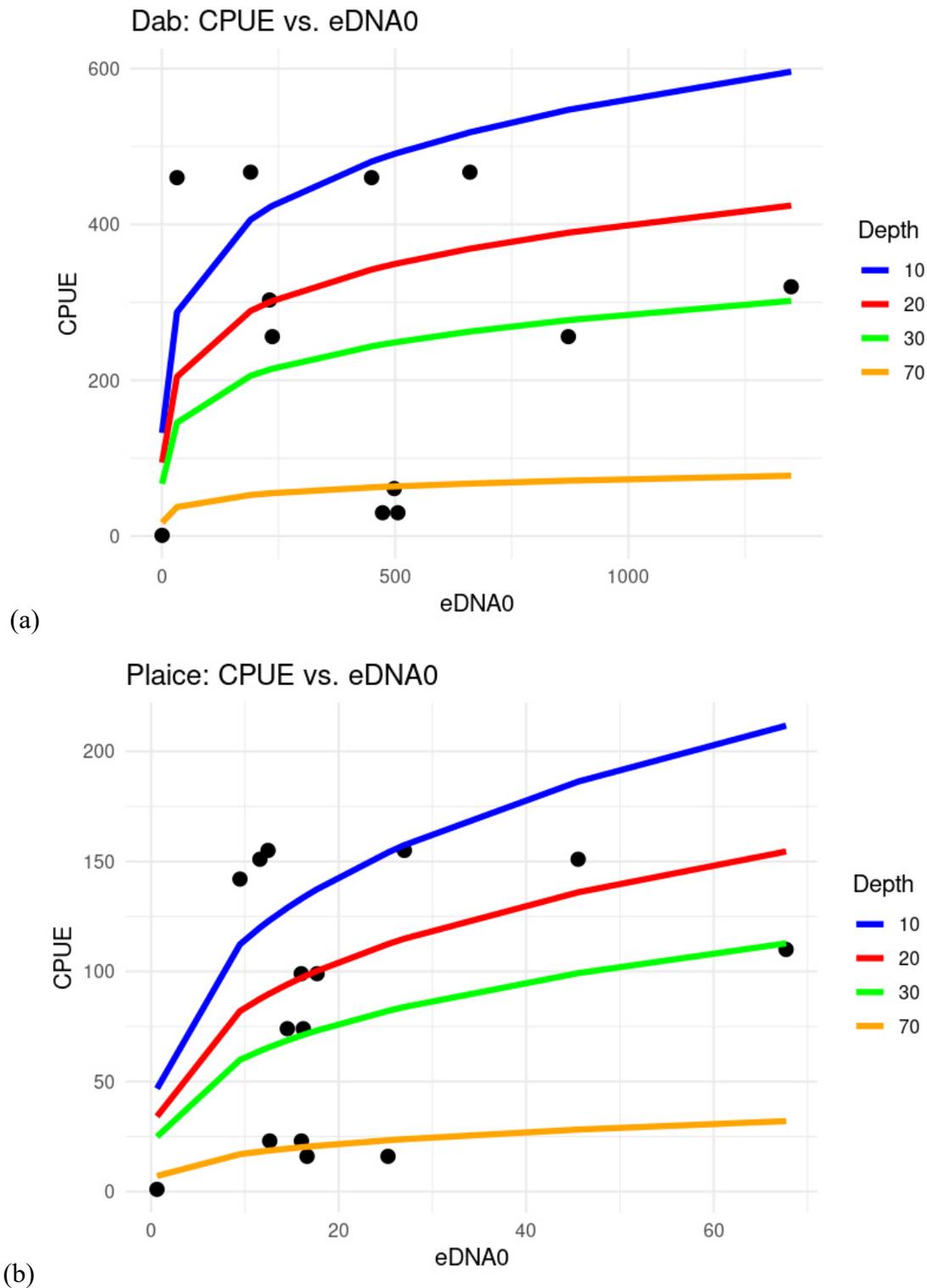


Figure 1 : Comparison of eDNA and Biomass in function of Depth changes of dab (a) and plaice(b) Datasets.

By modeling the relationship between abiotic factors and biomass, a significant correlation emerges between optimal abiotic conditions and biomass distribution (Figure 1). The Monte Carlo simulations indicate that the coefficients of both non-linear models were significant for both species with 95% probability (Figure 2).

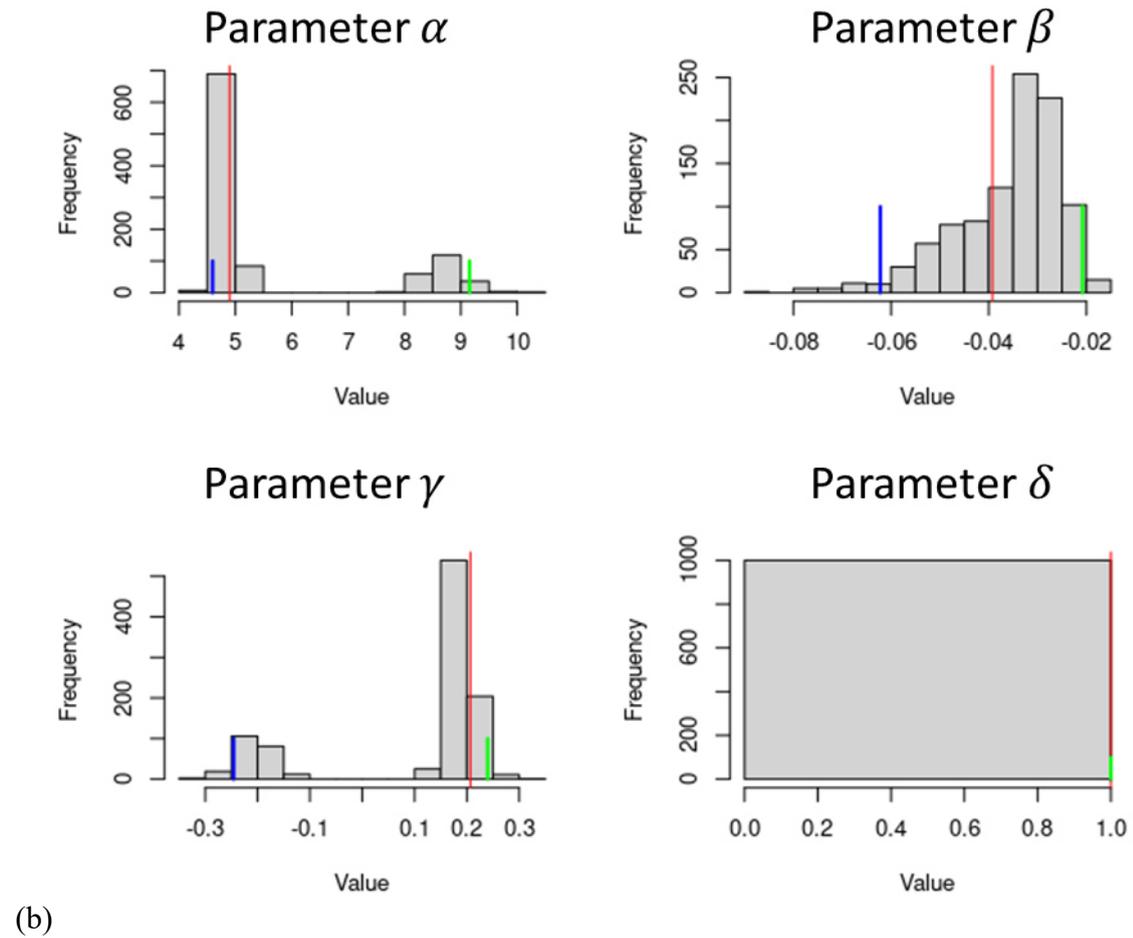
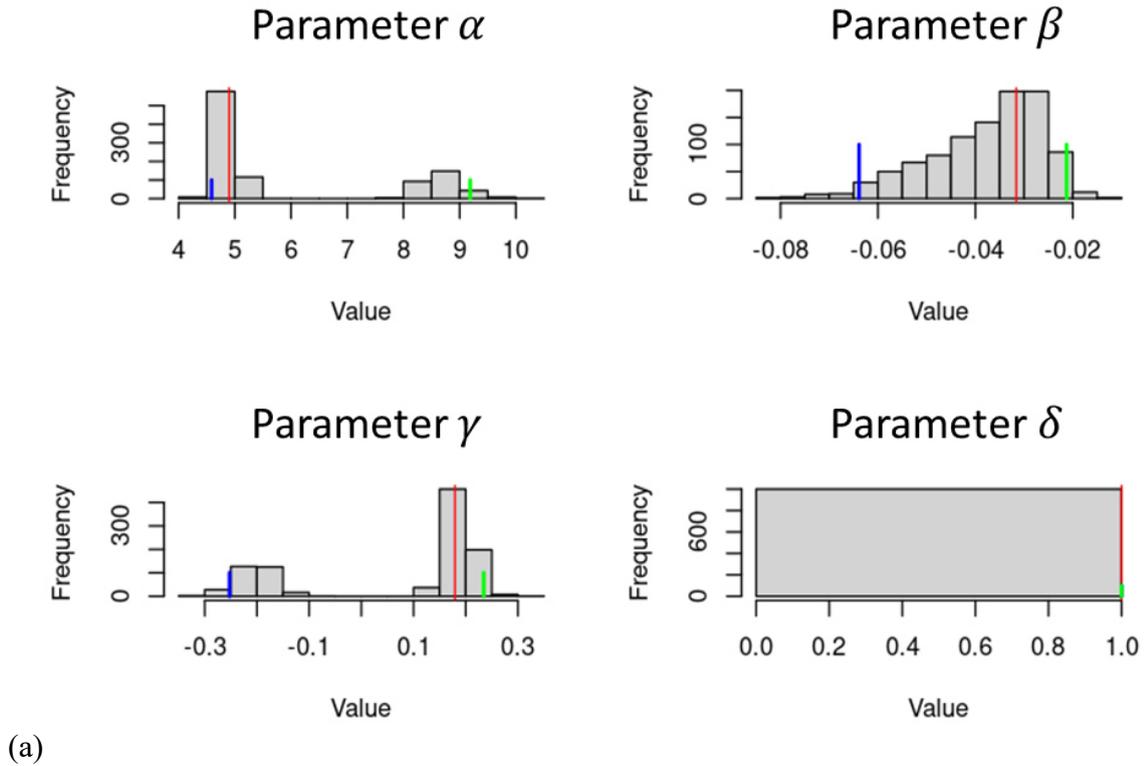


Figure 2: Frequency of Model 1 (Eq2) coefficients from a Monte Carlo simulation (1000 iterations). The red color is the initial model coefficient, green is quartile 97.5 and blue is quartile 2.5 corresponding to 95% confidence. The coefficient is significant with 95% confidence if the domain between the quartiles do not contain zero. For (a) plaice and (b) dab, respectively.

4 Discussion

In fishery science and management, knowledge of species -biomass is essential. The catch per unit effort (CPUE) serves as a proxy for biomass and typically requires physical sampling of fish species. During last years, the environmental DNA (eDNA) has been proposed as a novel tool for estimating biomass from aquatic environments, mitigating some limitations of traditional techniques (Deiner et al., 2017). The aim of this study was to construct a model that links the number of eDNA copies detected through quantitative Polymerase Chain Reaction (qPCR) to the biomass of the Common dab (*Limanda limanda*) and European plaice (*Pleuronectes platessa*). The results demonstrate the potential of using quantitative PCR (qPCR) methods for the detection and quantification of common dab and European plaice in the North Sea.

4.1 qPCR assay:

The findings underscore the effectiveness of environmental DNA (eDNA) as a tool for biodiversity assessment, showing compatibility with traditional survey methods such as trawling in terms of detection. The multiplex qPCR assay developed for detecting and quantifying common dab and European plaice in marine environments proved to be highly specific and sensitive, making it a valuable tool for monitoring and assessing the abundance of these species. The high specificity observed in this study is crucial for the precise identification of species. The probes designed for both common dab and European plaice demonstrated 100% specificity in simplex tests, ensuring exclusive detection of the target species. Furthermore, no cross-detection occurred between the probes and the both target species in the multiplex assays, indicating the robustness of the assay in distinguishing closely related species.

In terms of sensitivity, the serial dilution experiments proved the ability of the test to detect low DNA copy numbers. Thus, the common dab probe could detect up to 89 copies/reaction of 5 L, while the European plaice probe had a minimum quantifiable copy number of 10 copies/reaction of 5 L. These results indicate that the assay is able to detect even small amounts of DNA, suggesting its sensitivity to low population densities or rare occurrences of these species. For

instance, another qPCR assay developed for the detection and quantification of European plaice only (Knudsen et al., 2019) shows a lower sensitivity rate with around 667 copies per L.

The detection of both species, even at low abundance (down to 160kg/km² for plaice and 190 kg/km² for dab), demonstrates the sensitivity of the test in catching rare species or species occurring with low densities. On the other hand, 100% reproducibility was achieved for samples with abundances above 160 kg/km², highlighting the potential challenges of reliably detecting species present at extremely low densities. For the remaining 18%, the negative signal of the presented multiplex qPCR assay is explained by (1) the limit of quantification of the assay (160 kg/km² for European plaice and 190 kg/km² for common dab), although detection below these limits may be possible under certain circumstances; (2) the influence of long DNA filtration in some stations or the efficiency of the assays in some reactions (Li et al., 2018; Sassoubre et al., 2016; Takahashi et al., 2020). Therefore, increasing the volume of water samples to 10 L per sample may be more effective or developing more efficient filtration method and also sampling from various period of the year, as well as increasing the number of samples taken from each station.

4.2 Biomass estimation and modeling

Our modelling efforts revealed a non-linear relationship between the concentration of eDNA at the time of sampling and the CPUE obtained by the trawl of dab and European plaice within the study area. This relationship was obtained using GLMs and non-linear models fitted with the Nelder-Mead method.

Although attempts to normalize the data using logarithmic functions resulted in slight improvements in correlation, the correlation coefficients remained relatively modest, peaking at around 28% for the correlation between eDNA copy number per liter and CPUE, and 21% for eDNA copy number per liter and CPUE. This suggests that reliance on eDNA copy number alone may not be sufficient to accurately estimate fish biomass from that. These findings are consistent with those of previous studies, highlighting the complexity and inherent variability of factors influencing eDNA concentrations in aquatic environments (Karlsson et al., 2021, 2022; Stat et al., 2017, p. 20).

Furthermore, previous research by Kelly et al., (2014) found a lack of correlation between eDNA copy number and biomass using ordinary least squares (OLS) models. To improve regression rates, Kasmi et al., (2023) explored artificial intelligence techniques using Gaussian Process Regression (GPR) models. While GPR has shown to be promising, caution is needed

due to the risk of overfitting resulting from the high flexibility of the technique together with the limited sample size.

The models developed provide a mathematical framework for estimating biomass levels by integrating eDNA and some environmental predictors (eq. 4, figure 1). Both models utilize two interrelated equations to illustrate different aspects of the relationship between eDNA, environmental conditions and biomass, expressed as follows:

$$\log(\text{Biomass}(\text{environmental DNA}_{(t_0)}, \text{environmental factor})) = g(\text{environmental factor}) + h(\text{environmental DNA}_{(t_0)}, \text{environmental factor}), \text{ (eq. 4)}$$

where $g(\text{environmental factor})$, depicts the impact of abiotic factors on biomass, such as depth and temperature, and offers insight into how these factors shape biomass within ecosystems. The second summand, $h(\text{eDNA}_0)$ models the interaction between eDNA concentration, and biomass, thus exposing the relationships between these variables and also the possible degradation of eDNA at source as function of the environment. The biomass is modelled logarithmically as a function of eDNA₀ concentration and depth to account for non-linear dynamics (eq. 4).

Depth is used as a proxy for unmeasured but influential environmental factors (eq. 2). The coefficients (α , β , γ , δ) in the equation indicate the strength and direction of the relationships, providing insight into how changes in eDNA concentration and depth affect the acquisition rate at sea. The inclusion of the interaction term (δ) highlights the subtle relationship between eDNA concentration and depth, suggesting that there are depth-dependent differences in the correlation between eDNA and biomass. This comprehensive model provides a valuable framework for understanding the complex dynamics of biomass estimation in aquatic ecosystems, facilitating insights relevant to environmental studies and resource management strategies.

Model 1 (eq. 4), which included abiotic variables along with eDNA copy numbers at t_0 (eDNA₀), such as sampling depth and water temperature, significantly improved the correlation between observed trawl data and model outputs. In fact, correlations reached up to 90% for models incorporating eDNA₀ and sampling depth. The inclusion of additional variables allowed for a more comprehensive analysis and improved predictions of fish abundance. Similar performance has been observed in previous studies predicting species distributions based on eDNA data (Kasmi et al., 2023; Lacoursière-Roussel, Rosabal, et al., 2016; Urban et al., 2023). Maes et al. (2024) reported high correlations between eDNA and biomass in

mesocosm tanks, with R^2 values reaching 91% for sole and 72% for plaice. These efforts also showed a correlation of 88% between eDNA and plaice density in samples from the southwestern North Sea samples (Maes et al., 2024). Urban et al., (2024) provide further evidence for the predictability of eDNA, fish biomass and its applicability in fisheries monitoring. The incorporation of abiotic factors illustrates the multifactorial nature of eDNA distribution (Lacoursière-Roussel, Côté, et al., 2016).

Indeed, it's essential to recognize the limitations of this study. One significant limitation is the difference in the nets used in the training data and the validation dataset. Additionally, the geographic location of the sampling can also be considered a constraint in the validation step of this model. These factors could potentially influence the results and should be taken into account into further modeling studies by incorporating the sensitivity of model as a variable into the model. However, we were unable to perform it due the limited period covered by this study.

5 Conclusion

This work highlights the potential of combining qPCR analysis with statistical modelling techniques to improve the estimation of fish abundance using eDNA data. These results contribute to the growing body of knowledge on the use of eDNA as a valuable tool for monitoring and assessing fish populations in marine environments. By integrating molecular data with environmental factors, this model provides a more holistic understanding of the factors influencing fish populations and their distribution in the North Sea. Such predictive models can contribute to the sustainable management of fisheries resources, assisting decision-making processes and informing conservation strategies.

6 Supplementary data

Table S01: List of species and samples used to develop the qPCR assay

Species	NCBI Acc.Nr.	Aquagene database ID-Nr.
<i>Limanda limanda</i>	MN539944.1	
	KF930052.1	
	KJ204963.1	
	JN312183.1	
<i>Pleuronectes platessa</i>	KY018784.1	MG13
	JN312179.1	MG14
	KJ205134.1	
	MG253491.1	
<i>Hippoglossoides platessoides</i>	JN312191.1	Sp178
	KJ204920.1	MF031
<i>Platichthys flesus</i>	KM287030.1	MF013
	KJ768280.1	MF014
<i>Solea solea</i>		MF0050
		MF061
<i>Microstomus kitt</i>	JN312170.1	MG5
	KJ205058.1	MG6
<i>Glyptocephalus cynoglossus</i>	KJ204915.1	
	KC015419.1	
<i>Hippoglossus hippoglossus</i>	KJ204925.1	
	KC015482.1	
<i>Buglossidium luteum</i>	KJ204741.1	
	JN312474.1	
<i>Lepidorhombus boscii</i>	KX782797.1	
<i>Lepidorhombus whiffiagonis</i>	KX782943.1	
<i>Engraulis encrasicolus</i>	JF493424.1	
<i>Helicolenus dactylopterus</i>	JF493629.1	
<i>Merlangius merlangus</i>	JN168880.1	
<i>Clupea harengus</i>	KC015291.1	
<i>Agonus cataphractus</i>	KJ204645.1	
<i>Brosme brosme</i>	KJ204733.1	
<i>Sprattus sprattus</i>	KJ205205.1	
<i>Chelidonichthys lucernus</i>	KJ709497.1	MG28
		MG29
<i>Eutrigla gurnardus</i>	KX782790.1	
<i>Phycis blennoides</i>	KJ709582.1	
<i>Micromesistius poutassou</i>	KJ709820.1	
<i>Mustelus asterias</i>	KJ709832.1	MG31
<i>Sardina pilchardus</i>	KJ768297.1	
<i>Anarhichas lupus</i>	KP975535.1	
<i>Melanogrammus aeglefinus</i>	KP975763.1	

<i>Molva molva</i>	KP975793.1	
<i>Lota lota</i>	KX145590.1	
<i>Scomber scombrus</i>	MT456185.1	
<i>Callionymus lyra</i>		MG18
<i>Callionymus lyra</i>		MG19
<i>Mullus surmuletus</i>		MG22
<i>Mullus surmuletus</i>		MG23

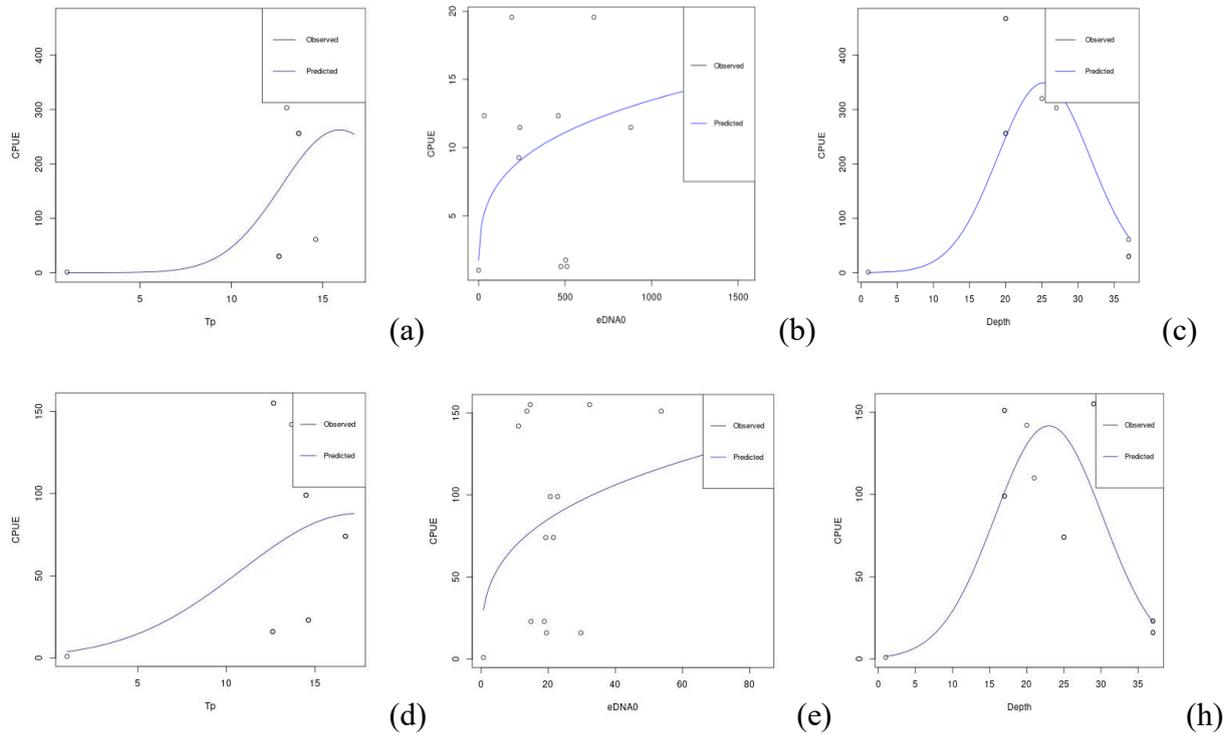


Figure S1: Relationships between eDNA, temperature, depth and CPUE for dab (a-c) and plaice (d-h). Dots: observations; curves: model (Equation 141).

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Supporting Data:

Codes, Scripts and other supporting data are available in [10.5281/zenodo.13234556](https://zenodo.org/doi/10.5281/zenodo.13234556)

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Ethical

No ethical permission is required.

Conflict:

The authors declare no conflicts of interest regarding the content presented in this paper.

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8. General Discussion:

This thesis focused on an evaluation of eDNA-based methods to provide accurate qualitative and quantitative information on fish diversity in the North and Baltic Seas in comparison with established reference methods like bottom trawling, while also discussing their possible implementation in EU marine and freshwater monitoring.

In the context of declining aquatic resources and their economic and social contribution, the assessment of limnic and marine biodiversity is fundamental to detect disturbance factors, perceive community responses and set regulations for their sustainable use (Bezerra et al., 2019; Katona et al., 2023; Lam et al., 2020; Leal Filho et al., 2019). Moreover, the quantification of biodiversity is crucial for effective ecosystem management. In this regard, eDNA analysis appears to be a promising non-invasive tool for marine monitoring (Cadrin, 2020; Deagle et al., 2006; Evans et al., 2017). However, the eDNA tools are not yet at a stage where they are sufficiently mature to be implemented in fisheries assessments and a number of questions remain to be answered (Duhamet et al., 2023; Skelton et al., 2022; Zaiko et al., 2018). These include the comparability of eDNA results with reference methods, and, as part of it, the ability and fidelity of public databases to provide accurate, up-to-date and reliable taxonomic information as well as widely compatible pipelines, especially those developed in the context of microbiological surveys. Another important but less explored issue relates to the ability of eDNA tools to provide reliable and reproducible quantitative information, which raises the question: Is it possible to estimate abundance based on eDNA? And what factors influence these models?

In an attempt to find answers to these questions, the five core chapters of this thesis contribute by using biological methods and numerical tools (**Figure 8-1**). In that respect, the aim of chapter 3 was to set up a fish-specific database. Chapters 4 and 5 deal with the question of whether eDNA can provide qualitatively reliable information on the presence/absence of fish species via metabarcoding.

While Chapter 4 also addresses the questions of whether metabarcoding/NGS outputs are capable of providing quantitative or semi-quantitative information on the abundance of species in the sea, chapters 6 and 7 specifically deal with the possibility of using eDNA to provide quantitative information and the determination of environmental factors to optimize the predictability of eDNA data derived from qPCR outputs.

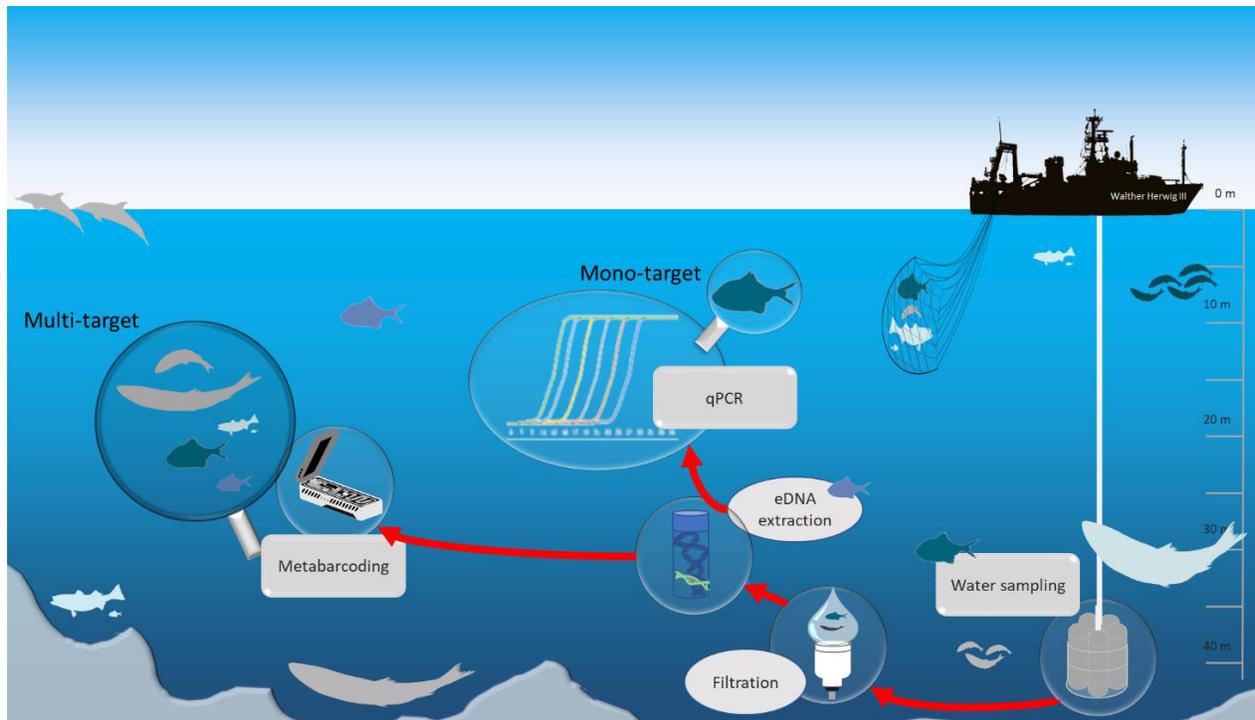


Figure 8-1: Thesis workflow. In this thesis, two eDNA methods, namely qPCR and metabarcoding, were applied. The results obtained from both methods were compared with bottom trawling data collected in parallel with the water sampling. The comparison was conducted both qualitatively and quantitatively.

8.1.Applications of eDNA analyses for marine biodiversity monitoring

In order to gain a comprehensive understanding of the potential of eDNA-based methods to unveil fish diversity in the Baltic and North Seas, two distinct approaches were used: a mono-target, species-specific one approach on qPCR, and a universal method using metabarcoding. In chapters 4 to 7, the applied methods demonstrated a noteworthy capacity to provide information on the occurrence of species. For the mono-target, species-specific qPCR-based method, Atlantic cod (chapter 6), common dab and plaice (chapter 7) were detected at various stations, including some

where no specimens of the species were found in the concomitant trawl catches. At other stations, however, eDNA was unable to detect the species in spite of fisheries observations. This inability to detect the target species was mainly attributable to the low number of captured individuals, suggesting detection limits as an important factor. It varied from species to species, but was generally less than 10 kg/km². In the case of some species, such as Atlantic cod, the detection limit encountered was 0.8 kg/km². For all stations above such a detection limit, the eDNA results were in line with the trawl data. Likewise, metabarcoding results, using both short-read approaches with Illumina as the reference method (chapter 5) and Nanopore as a relatively novel emerging long-read sequencing method (chapter 4), showed high agreement between eDNA and trawl data. Nevertheless, the metabarcoding technique revealed an overall higher biodiversity, including rare species and even mammals, such as harbour porpoise (*Phocoena phocoena*), at several stations, which were undetectable by bottom trawling. One of the difficulties of eDNA metabarcoding is the difficulty of distinguishing between closely related species, due to the short target reads and the high degree of similarity between species in certain families or genera. The use of the fish database developed and screened from other databases such as NCBI, ENA and AquaGene, presented in Chapter 3, has significantly reduced the false positive results and therefore significantly increased the accuracy of the metabarcoding method.

As a consequence, the work outlined in Chapters 4 and 5 provides empirical evidence and estimates of possible fish species richness in the two studied ecosystems. The knowledge resulting from these findings can help updating fish biodiversity mapping, including some rare and endangered species, an attempt to develop better strategies for stock management in fisheries and species recovery programmes in nature protection.

8.1.1. The reliability of eDNA as an indicator for marine biodiversity

Since the beginning of the new millennium, the decline of marine resources and the disruption of marine ecosystems has raised serious concern among scientist, media and the public (Greenstreet, 2008; Lawson et al., 2020; Nikolaou & Katsanevakis, 2023). These concerns have triggered a wide range of actions at international, regional and local levels around the world. As a result, numerous environmental laws have been drafted and enacted, and a number of international and local commitments have been made to protect and conserve marine biodiversity (Biermann et al., 2017, Kirkfeldt & Frazão Santos, 2021). A key component of these efforts has been the adoption of

methods for marine biodiversity, and in particular halieutic monitoring, as a key tool for assessing the impact of anthropogenic activities on marine resources (FAO, 1995; ICES, 2020). In this way, preventive and corrective decisions can be taken to ensure the long-term health of our oceans and the sustainability of resources for future generations.

eDNA-based methods have become a major hope for many stakeholders in the assessment of aquatic, particularly marine, resources, due to their non-invasive and comparatively inexpensive character (Compson et al., 2020; Zaiko et al., 2018). Consequently, the number of studies that have used eDNA to assess fish abundance has increased significantly, with over 40% of published eDNA studies focusing on biodiversity assessment (Compson et al., 2020). In addition, a significant percentage of government and policy-making bodies have published reports or scientific papers comparing eDNA with other assessment techniques (Compson et al., 2020), while others have even adopted eDNA as a reference method (Compson et al., 2020; Schenekar, 2022). The concordance between eDNA findings and fisheries trawl reference data was generally high for both, metabarcoding and qPCR in Chapters 4 to 7 of this thesis. This is consistent with earlier studies, that have demonstrated the ability of eDNA to provide good overviews about species distributions in aquatic environments (Bessell et al., 2023; Bessey et al., 2020; Doorenspleet et al., 2021; Evans et al., 2017; Schenekar et al., 2020; Terrasa et al., n.d.; Veldhoen et al., 2016).

In the field of metabarcoding, Illumina's short-read technology is considered the reference method for NGS sequencing due to its low error rate and as the first U.S. Food and Drug Administration (FDA)-cleared next-generation sequencing (NGS) system for in vitro diagnostic (IVD) applications (Song & Florea, 2015; Stoler & Nekrutenko, 2021). Overall, the concordance of short read outputs with bottom trawl fisheries reached 80%. However, while eDNA analyses in seawater even increased the number of detected species by 32% compared to bottom trawling, the success rate for sediment eDNA was low (Chapter 5). Similar results were observed for long reads using Nanopore technology (Chapter 4), proofing both methods to be suitable for marine fish diversity monitoring as shown previously (Closek et al., 2019; Evans et al., 2017; Maiello et al., 2022; Nakagawa et al., 2022; Zou et al., 2020).

On the other hand, the development of highly sensitive and specific single and multiplex qPCR primers has significantly lowered the limit of detection for target species in both assays compared to conventional primers (chapter 6 and 7). While the detection limits in earlier studies were around

700 copies per reaction (Knudsen et al., 2019; Pont et al., 2023; Salter et al., 2019; Yu et al., 2022), the primers developed in this thesis are characterized by a significantly lower limit of detection down to 10 copies per reaction for cod and dab, and 100 copies for plaice. This led to a substantial increase in the detectability of fish abundance using qPCR methods, with minimum detection limits of 0.8 kg/km² for Atlantic cod and approximately 10 kg/km² for plaice.

8.1.1.1. Alignment of long-read results with reference methods.

Advances in long-read high-throughput sequencing (HTS) technologies have not only reduced the cost of sequencing, but also significantly reduced the error rate. One of these new emerging technologies is Nanopore sequencing, which, due to its relatively low cost and small size, has made it possible to sequence libraries outside genetic labs, like e.g. aboard research vessels (Chen et al., 2023). Chapter 7 demonstrates the ability of MinION sequencers to provide an accurate insight into marine diversity with high confidence, similar to short read sequencing methods. However, the number of studies using these technologies in marine biodiversity assessments is yet low compared to the short read technologies (Davidov et al., 2020; Doorenspleet et al., 2021; Egeter et al., 2022). Until recently, long read sequencing was considered an emerging technology with comparably high error rates, until the new Q30 Nanopore chemistry with an error rate of less than 1% was released in 2023 (Banerjee et al., 2024; T. Zhang et al., 2023). Average e-values and bit scores for MinION sequencers are 97%, equivalent to Illumina sequencing (Truelove et al., 2019).

8.1.1.2. Detectability differences between eDNA results and bottom trawl catches

The results of this thesis proofed that in many instances eDNA can increase the detectability of aquatic species diversity compared to fisheries-based methods. This advantage has been observed for both qPCR and metabarcoding (Chapters 4-7). At many sampling sites, eDNA was able to detect the presence of a larger number of species, including rare or endangered species, that were not detected by standard bottom trawling. A crucial factor in the enhanced ability to detect marine biodiversity from eDNA is its long persistence in the water with a half-life of around 48 h (Andruszkiewicz, Sassoubre, et al., 2017; Barnes et al., 2014; Harper et al., 2019; Steiner et al., 2019), while traditional methods such as bottom trawling allow only snapshot pictures (Afzali et al., 2021; Aguzzi et al., 2020; ICES, 2020; Stat et al., 2019). eDNA can persist in the aquatic environment for days after the organisms have left the sampled area (Barnes et al., 2014; Dejean et al., 2011; Hendricks, 2022; Ramírez-Amaro et al., 2022; Salter, 2018). This long persistence

allows species to be detected even after they have left the survey area, making eDNA a powerful tool for monitoring aquatic biodiversity. Furthermore, eDNA-analysis has the advantage of being able to detect species that are difficult to capture using standard methods, particularly highly mobile, rare or cryptic species that show net avoidance behaviour (Feng et al., 2023; Wilcox et al., 2013). This ability not only allows the detection of a wider range of species, including rare and low-abundance species, but also provides a more accurate snapshot of biodiversity in aquatic ecosystems.

8.1.2. Limitations of eDNA-analyses

In addition to still open questions regarding the limitation of the persistence of eDNA in seawater, there is also a risk of too long persistence, that could result in the same group of fishes be detected multiple times in an eDNA analysis, leading to a potential overestimation of species abundance or distribution (Scriver et al., 2023). This scenario can occur through prolonged persistence of eDNA together with drift. In this respect, the use of sediment samples could be a way out. However, sediment-bound eDNA was found to be highly persistent in previous studies, ranging from thousands to millions of years (Anderson-Carpenter et al., 2011; Corinaldesi et al., 2011; Dell'Anno & Corinaldesi, 2004). The function of marine sediments as species archives could not be corroborated in Chapter 5 of this thesis, highlighting the need to learn more about physical and chemical parameters influencing DNA molecules.

One of the main challenges of eDNA metabarcoding are often observed false positive results (Rishan et al., 2023; Stat et al., 2019; Zinger et al., 2019) due to the short fragment sizes amplified e.g. by MiFish primers and the high similarity between closely related congeneric species or incorrect reference sequences in international databases (Miya, 2022). Various methods have been proposed to overcome this issue (Blackman et al., 2023; Doi et al., 2023; Rishan et al., 2023; Stat et al., 2019; Zinger et al., 2019). An alternative solution was to develop a pipeline constructed within the ARB environment for screening and selecting accurate sequences from existing databases, presented in Chapter 3. The incorporation of the recently developed Mare-MAGE database into our workflow has notably reduced the incidence of errors associated with the annotation of artifact species, due to its incorporation of a dedicated database tailored to the North and Baltic Seas.

A further significant limitation to the use of eDNA is that the genomic information, mainly the 12S gene, of many species is not yet available in public databases, thereby prohibiting their detectability (Blackman et al., 2023; Claver et al., 2021; Hestetun et al., 2020). Despite the North Sea being one of the best studied marine ecosystems and significant sequencing efforts in the past years, more than 50% of the native fish species have no entry for mitochondrial 12S rDNA in public databases. This is also due to the fact that most sequencing efforts only focus on a few genetic markers, mainly COI or Cytochrome b, which may have resulted in limited detectability rather than sequencing at least multiple genes or the entire mitochondrial genome.

8.2. Biomass estimation from eDNA

Different stakeholders, like politicians, fisheries managers and fishers, rely on accurate stock assessments for decision making. In this context, chapters 6 and 7 focused on the potential of eDNA to provide quantitative information on fish stocks. Using machine learning (Chapter 6) and generalized linear models (GLMs) (Chapter 7), qPCR results demonstrated high predictability for biomass estimates. Furthermore, the modelling work in Chapter 5 highlighted the potential of metabarcoding data to provide quantitative information. However, the correlation between eDNA reads and biomass is not always linear. It rather depends on the initial amount of eDNA present in the original samples, which can be influenced by various abiotic factors such as filtration time and water conservation conditions prior to filtration. Moreover, physical parameters like sampling depth were found to be crucial for explaining divergences between eDNA results and fisheries catches, as discussed in chapter 7. Depth may act as a proxy for other non-measurable or non-targeted abiotic and biotic factors influencing eDNA quantity and quality. This emphasises the complexity of the relationship between environmental variables and eDNA results and highlights the need for a comprehensive understanding and careful interpretation of eDNA data for stock assessments.

8.2.1. Biomass estimation using species-specific eDNA analyses

Mathematical modelling has proven to be a valuable tool for explaining the complexity of biological systems. In this thesis, mathematical modelling on qPCR results was applied to estimate single species fish biomass. A correlation was observed between biomass, depth and temperature, highlighting their role for interpreting eDNA results. Nevertheless, the relationship between eDNA

reads and biomass was found to be relatively weak. These results are of crucial importance, providing insight into the impact of environmental factors on the presence of eDNA in the samples collected, along with the biomass (Stewart, 2019; Strickler et al., 2015; Tsuji et al., 2017). Therefore, the predictability of models estimating biomass as a function of eDNA is increased when the abiotic effect is taken into account (Chapter 5) (**Figure 8-2**).

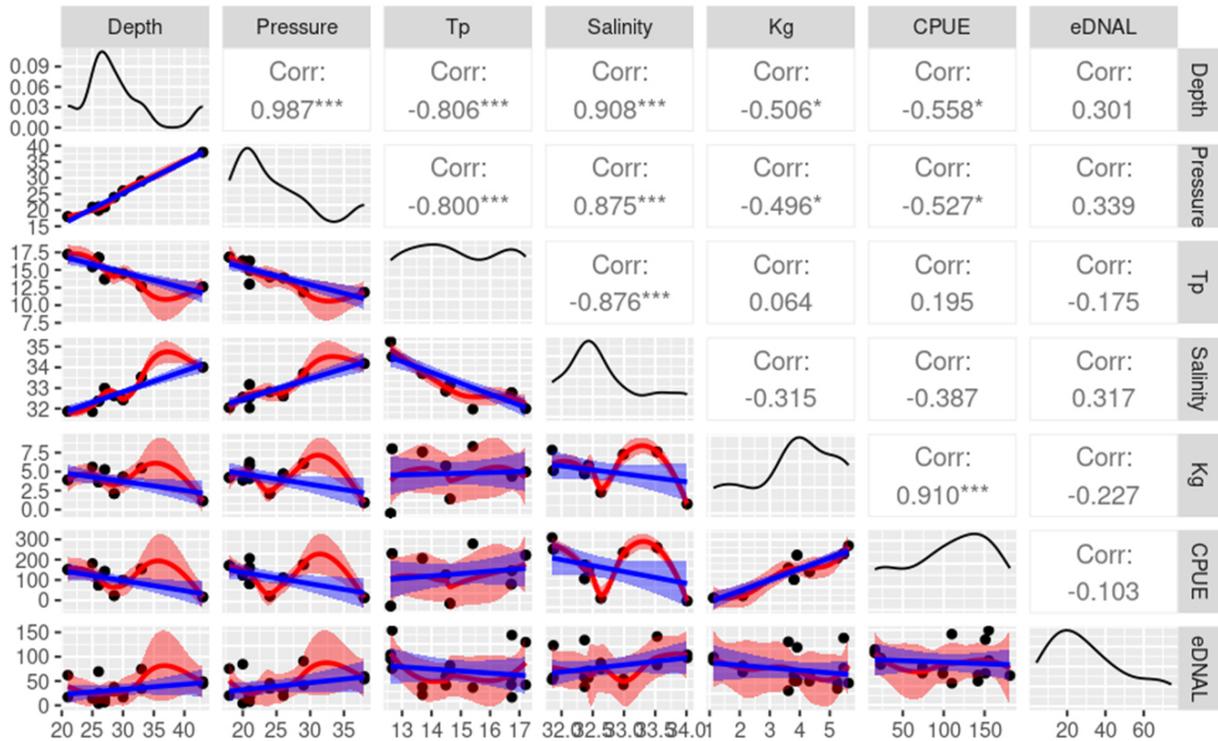


Figure 8-2: Correlation between biotic and abiotic factors in data collected. Temperature (Tp), kilogram (Kg), eDNA copies per L (eDNAL), catch per unit effort (CPUE), Depth of water sampling. The Corr., the value of correlation.

The concept of eDNA has been incorporated at time of sampling (T_0) with the objective of assessing the initial amount of DNA present in the water at the time of sampling. This approach enables the quantification of DNA losses that occur during the storage and filtration of water. As illustrated in **Figure 8-3**, there is a significant correlation between the duration of storage at 4°C as well as the duration of filtration, which can vary from a few minutes to a day depending on water composition, and the loss of DNA (Dell’Anno & Corinaldesi, 2004; Greaves & Wilson, 1970; Moushomi et al., 2019; Murakami et al., 2019; Saito & Doi, 2021). This highlights the importance

of physical parameters for eDNA processing, the subsequent qPCR or high-throughput sequencing (NGS) and the estimated biomass models, particularly in relation to the amount of eDNA measured at T_0 .

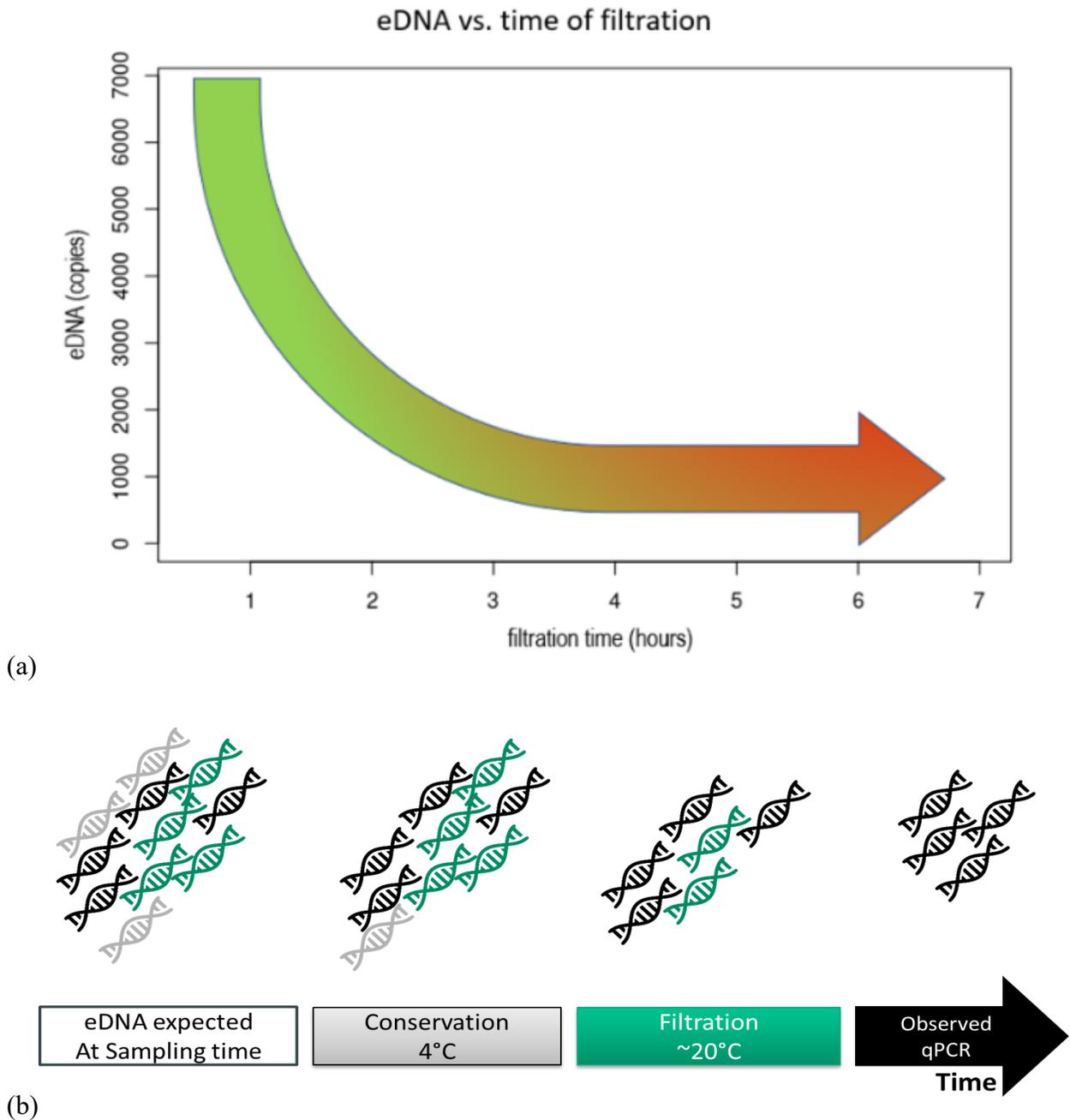


Figure 8-3: Effect of conservation and filtration time on eDNA loss. (a) Relationship between eDNA degradation and filtration time. (b) Schematic view of eDNA losses over time.

A Gaussian process regression (GPR) approach was implemented as a machine learning method to establish relationships between eDNA reads and bottom trawl catch data, also incorporating the effect of sampling depth. Unlike simple regression models, a GPR provides flexibility and the ability to capture non-linear relationships among variables, a critical issue for modelling complex phenomena (Das et al., 2018; Schulz et al., 2018; Williams & Rasmussen, 1995). As a result, a correlation of around 95% was achieved with a negligible error rate of around 0.002 (Chapter 6).

Nevertheless, in spite of the promising performance of GPR in the present case, it is important to also recognise certain limitations of this method. First of all, GPR can be sensitive to the choice of parameters and the selection of model hyperparameters, leading to inconsistent results and the need for careful optimisation (Banerjee et al., 2013; Das et al., 2018; Pleiss & Cunningham, 2021). Therefore, there is a high risk of overfitting, particularly when the model is too complex for the sample size, which may lead to degraded performance on new or unseen data. Moreover, the model is challenging to interpret, which hinders comprehension of the underlying mechanisms governing the relationship between eDNA and fish biomass (Krauth et al., 2017; Swiler et al., 2020). Likewise, GPR necessitates the availability of high-quality data and a sufficient quantity of samples to ensure the reliability of the results, which can occasionally prove challenging to obtain in complex and variable aquatic environments.

In light of the aforementioned limitations, it was necessary to also use more robust and simple modelling methods in order to fully exploit the potential of eDNA for monitoring fish populations. A non-linear relationship was established between eDNA reads and biomass using generalised linear models (GLM) and non-linear models using the Nelder-Mead method. Incorporating abiotic variables as well as eDNA₀ (environmental DNA copy number at T₀) together with sampling depth and water temperature, the model showed a significant improvement in the correlation between the observed and predicted fisheries catch data. Correlations reached up to 90% in the case of models incorporating eDNA₀ and sampling depth. Integrating additional variables enabled a more comprehensive analysis and improved predictions of fish abundance, an approach also supported by previous studies predicting species distributions based on eDNA (Lacoursière-Roussel et al., 2016; Maes et al., 2024; Urban et al., 2023, 2024), highlighting the predictability of eDNA in fisheries monitoring. However, discrepancies were observed between eDNA and fish density, highlighting the multifactorial complexity of eDNA distribution.

$$\log(\text{CPUE}(eDNA_{t_0}, \text{Depth.})) = (\alpha + \beta \cdot (\text{Depth})) + \gamma \cdot \log(eDNA_{t_0} - \delta \cdot \text{Depth}) \quad (\text{eq 1})$$

The model's core equation highlights the impact of environmental factors on biomass, represented by the function $g(\text{factor}, \text{environmental})$ illustrated in the equation 1 as the log of a static corrective factor α plus $(\beta \cdot (\text{Depth or temperature}))$ in eq 1, (chapter 7),. Depth seems to play a crucial role as an indicator of the underlying environmental conditions influencing the distribution and abundance of fish populations. Indeed, depth can affect the availability of food resources, water temperature and chemical composition, all of which shape the structure and dynamics of aquatic ecosystems. Therefore, depth was used as an indicator of unmeasured but influential environmental factors. The inclusion of the interaction term (δ) highlights the delicate relationship between eDNA concentration and depth, suggesting that there are depth-dependent differences in the impact of eDNA on biomass (Canals et al., 2021; Duhamet et al., 2023; Jeunen et al., 2020; Schade et al., 2022). At the same time, the term $h(eDNA_0)$ in the model represents the interaction between eDNA concentration and biomass, reflecting the ability of eDNA to provide information on fish population density. This interaction makes it possible to incorporate the potential degradation of eDNA in the environment and its impact on the reliability of biomass estimates. This highlights the complexity of this relationship and the need for an integrated approach to understand the underlying mechanisms.

8.2.2. Biomass estimation using eDNA metabarcoding

NGS metabarcoding is a method widely used in marine science to study the diversity and abundance of organisms. However, its quantitative application is still a major challenge. By applying the GLM model developed for qPCR to metabarcoding data, a positive correlation was found between biomass and the number of reads (Chapter 5), highlighting the possibility of exploiting metabarcoding data to extract quantitative information on fish stocks. Nevertheless, a variable associated with the sequencing process should be taken into account in the creation of future models, particularly the integration of competitive processes between species during sequencing and the depth of sequencing. The methodology employed in RNAseq data analysis could be a potential solution to the issues of standardising the data (Bell et al., 2019).

8.3. Implementation scenarios

eDNA has demonstrated a considerable potential to uncover marine biodiversity, surpassing reference methods such as bottom trawling, whilst offering a higher discrimination potential than other non-invasive techniques. However, it is still at an immature stage before it can be adopted independently for biodiversity monitoring and fish stock assessments. As a result, in the short term its implementation will require a combination with other non-invasive techniques, such as video surveillance and acoustics, or be used as a complementary and corrective method to standard fisheries techniques.

In the medium to long term, environmental genomic techniques, in particular eDNA combined with eRNA, could provide the information needed for stock assessment. This also requires the careful consideration of mathematical and statistical models for deriving biomass and density estimates from eDNA reads. A successful implementation of such techniques not only requires adequate technical and methodological lab tools, but also standardized sampling, bioinformatics and computational analysis, emphasizing the importance of investing in staff training at different levels.

To achieve short- and long-term solutions and ensure the successful implementation of eDNA in fish stock assessment, it is necessary to create collaborations and synergies between scientists, marine resource managers and fishing industry stakeholders. This can be done through working groups and research consortia, as well as by integrating eDNA into fisheries management guidelines and protocols established by regional and international organizations.

9. Perspectives and prospects

The focus of this thesis was to evaluate the performance of different eDNA-analyses as non-invasive methods for assessing fish stocks. While the methodology has overall made significant progress, there are still a number of aspects that require solutions before it can replace traditional, catch-based assessment techniques.

In the short term, integrating eDNA-analyses with other non-invasive methods such as underwater acoustics and visual observation is imperative. While eDNA offers valuable qualitative insights into species presence and geographical distribution, accurate biomass estimates remain a challenge. With this thesis, the potential of eDNA as a source for quantitative information was demonstrated, whether through artificial intelligence algorithms or GLM and Nelder Mead models. However, it is crucial to acknowledge the limitations of these approaches, given also the constraints imposed by the limited sample size and the sampling design across different project phases due to technical and budgetary restrictions. Considering the substantial difference in the number of stations studied compared to Mathon et al. (2022), surpassing 1200 stations, it is essential to be mindful of the inherent shortcomings of our study.

Given the documented persistence of eDNA in marine environments, which can exceed 24 hours in water and up to millions of years in sediments, future studies should not primarily focus on comparing biomass observed by reference methods with that of eDNA. Instead, new eDNA-based computational and statistical approaches, independent of fisheries catches, should be considered. Furthermore, the potential impact of additional abiotic factors must be considered and tested in order to enhance the robustness of eDNA-based approaches. A multidisciplinary baseline study, integrating data from tank experiments, microcosms, and aquaculture studies, could facilitate the development of reliable models.

Therefore, the move towards using random DNA repeat patterns to identify species and distinguish between individuals of the same species from eDNA is a promising advance. The development of sequencing tools, particularly long reads, has made it possible to sequence long fragments with

very low error rates. If we can exploit and develop methods to extract and sequence these motifs, followed by post-sequencing analysis to distinguish between species or characterise motifs specific to a given species, it may also be possible to estimate the number of individuals belonging to that species from eDNA. This approach opens up new possibilities for more accurate and efficient assessment of fish populations by exploiting the full potential of eDNA. By combining these advances with cutting-edge sequencing techniques and sophisticated bioinformatic analyses, we may be able to meet the current challenges of monitoring fish populations and provide vital information for a sustainable management of aquatic resources.

Further developments in NGS technology, particularly long read sequencing and a significant reduction in price over time, also offer the possibility of studying mitochondrial metagenomics. This could reduce the risk of incorrect sequence annotation, as the target sequences in 12S or COI are often not exceeding 200 nucleotides, while closely related species often just differ by one or two nucleotides in these target genes when using e.g. Mifish 12S primers. Sequencing the entire mitochondrial genome could allow better discrimination between species, while opening up the possibility of using these approaches to even genotype individuals in future applications.

The quantitative use of next-generation sequencing (NGS)/metabarcoding eDNA data, rather than relying solely on qualitative applications, is a key aspect of this thesis. The amount of data generated by any NGS application is substantial, but often remains under-utilised. Currently, NGS data is mainly used to infer relative species richness. However, by exploiting and reusing this data for tasks such as genotyping, quantification and subpopulation identification, eDNA analysis could become a much more powerful and valuable tool.

Effective monitoring and assessment of aquatic species and ecosystems involves several critical aspects beyond simple species identification and biomass assessment, including population dynamics, habitat assessment and interactions within ecosystems. While eDNA methods have proven useful, they are often insufficient to address these complex issues. Coding and non-coding RNAs offer an opportunity to overcome these limitations by providing an overview of biotic and abiotic interactions, and could allow to better understand the complex system, including the behaviour of animals. Non-coding RNAs (ncRNA), once considered transcriptional by-products, are now recognised as important regulators of gene expression and cellular processes, with diverse roles in development, immune response, behaviour and stress adaptation (Abo-Al-Ela, 2021; Chu

et al., 2021; Zheng et al., 2021). In marine organisms, ncRNAs play a critical role in mediating responses to environmental stressors, making them relevant to stock assessment efforts (Giroux et al., 2022; Hiki et al., 2023; Miyata et al., 2021). Integrating ncRNA analysis into stock assessment frameworks could offer several advantages, including serving as sensitive indicators of population health and facilitating the identification of population structure and connectivity patterns. By characterising population-specific ncRNA signatures, it may be possible to detect subtle genetic differences and migration patterns, thereby improving spatial management approaches and conservation priorities. Furthermore, the integration of ncRNA and coding RNA analyses provides a holistic understanding of population dynamics and adaptive responses to environmental change, elucidating molecular mechanisms underlying resilience and adaptation (Cao et al., 2023; Giroux et al., 2022; Miyata et al., 2021). Despite the promising potential, challenges such as methodological standardisation, data integration and longitudinal studies remain. Nevertheless, targeting RNAs will offer a deeper understanding of animal behaviour and complements the qualitative overview provided by eDNA analysis.

10. Conclusion:

In summary, the findings of this thesis provide solid support for the proposition that eDNA is a promising non-invasive alternative for stock assessment and marine species mapping compared to traditional invasive methods. In particular, it is non-invasive, capable of detecting a large number of species, and independent of the restrictions imposed by trawling. Nevertheless, further measures are needed before eDNA can be fully exploited in practice.

In this thesis, a significant step forward has been taken by directly linking eDNA data to the dynamics of fish populations. Despite the simplifications and assumptions involved in developing models, their validation by comparing them with observed data paves the way for more in-depth mathematical and experimental research in the future. These models have the potential to be extended to other species, thereby broadening the scope of research possibilities and prospects in the field of environmental DNA.

Altogether, the present thesis underlines the growing importance of environmental DNA as a tool for monitoring and managing marine resources, and highlights the potentially exciting opportunities for its integration into management and conservation practices. However, to fully realise this potential, continued research, development and awareness-raising efforts are imperative to overcome technical and operational barriers.

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Eidesstattliche Erklärung

Hiermit erkläre ich, Yassine Kasmi, dass ich die vorliegende Dissertation, abgesehen von der Beratung durch meinen Betreuer Prof. Dr. Reinhold Hanel, nach Inhalt und Form eine eigenständige Arbeit ist und nur mit den angegebenen Hilfsmitteln verfasst wurde. Die Dissertation ist unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft (DFG) entstanden. Diese Arbeit wurde weder ganz noch zum Teil an anderer Stelle im Rahmen eines Prüfungsverfahrens vorgelegt, veröffentlicht oder zur Veröffentlichung eingereicht. Mir wurde nie ein akademischer Grad entzogen.

Kiel, 1. Dez. 2024