Enhancing environmental monitoring in anthropogenically modified coastal habitats using eDNA and nsDNA metabarcoding

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Declaration

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

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Contributions to data chapters

Chapter 2

Alice Cunnington collected the samples throughout the year, with assistance from Erika Neave and Courtney May. The Albert Dock Canals and Rivers Trust and the West Kirby Sailing Club facilitated the sample collection. Alice Cunnington carried out the laboratory procedures, from extraction to sequencing, with initial guidance from Lynsey Harper. Bioinformatics pipeline was conducted by Alice Cunnington, with guidance by Peter Shum. Data analysis and figures were conducted by Alice Cunnington. The chapter was written by Alice Cunnington and reviewed by Erika Neave, Peter Shum, Rupert Collins, Craig Wilding, Kirstie Scott and Stefano Mariani.

Chapter 3

Alice Cunnington collected the samples with Kirstie Scott. Alice Cunnington conducted the laboratory procedures from extraction to sequencing, Bioinformatics pipeline was conducted by Alice Cunnington, with guidance by Peter Shum. The chapter was written by Alice Cunnington and reviewed by Kirstie Scott, Craig Wilding and Stefano Mariani.

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Chapter 5

Alice Cunnington collected the samples with help from Erika Neave, Robyn Mawdsley and Inverlussa mussel farm, with guidance from Iveta Matejusova. Alice Cunnington conducted the laboratory procedures from extraction to sequencing, Bioinformatics pipeline was conducted by Alice Cunnington, with guidance by Peter Shum. The chapter was written by Alice Cunnington and reviewed by Craig Wilding, Kirstie Scott and Stefano Mariani.

Abstract

Understanding the dynamic relationships that exist between species in urban coastal habitats is of critical importance as scientists, policymakers and the public work to address a range of environmental challenges. Developing robust and versatile environmental monitoring methods, refined and optimised for use in such coastal habitats, will be a necessary part of this. This thesis focuses for the most part on one technique - eDNA metabarcoding, where fragments of DNA shed by organisms are analysed - and explores its effectiveness and flexibility across a variety of coastal habitats. This thesis explores firstly fish assemblages in post-industrial Merseyside, using eDNA metabarcoding to produce a comprehensive list of teleost species, including species of ecological and conservation importance. Significant changes in fish community composition between habitats and across different seasons over a year-long period were revealed, demonstrating the method's potential for monitoring environmental impacts on fish communities. A multi-marker approach was then employed (COI and rbcL) to investigate eukaryotic communities in heavily urbanised waterways within Merseyside. It was found that both markers could discern distinct, localised communities while also identifying indicator species, including diatom species and potentially harmful algae. Exploring the use of both markers concurrently provides a comprehensive insight into ecosystem health and capitalises on the strengths, with implications for environmental monitoring. Expanding the scope of this research beyond eDNA metabarcoding, the role of beadlet anemones as natural environmental DNA samplers (nsDNA) for vertebrate biodiversity was also investigated. It was shown that using anemone nsDNA alongside water eDNA expanded the range of taxa detected, thereby enhancing the study's scope and revealing unique potential future applications for vertebrate monitoring. The role of a working mussel farm in supporting environmental monitoring through eDNA metabarcoding was explored. Using the mussel farm’s boat increased accessibility to detect fish communities, and our results highlighted extensive "molecular bycatch" as well as the interaction between land and sea in deep fjordic inlets. Although DNA extracted from the tissue of blue mussels were also analysed, this part of the study yielded no fish sequencing reads, revealing there are limitations to the use of natural sampler DNA in this context. Overall, this thesis emphasises the value of novel biodiversity-screening techniques when applied to coastal habitats particularly impacted by current and historical anthropogenic modifications. It highlights in particular the role that eDNA and nsDNA metabarcoding,

when used both in combination with other techniques or as standalone methods, have to play in expanding and enhancing toolkits for future environmental monitoring.

1. Chapter 1: Introduction

1.1 Importance of biodiversity monitoring in urban coastal environments

The importance of ecological monitoring in heavily urbanised coastal environments is growing steadily. These areas serve as crucial connections between terrestrial and marine ecosystems, rendering them particularly vulnerable to human-induced pressures (He et al., 2019). Coastal zones are some of the world's most densely populated areas, experiencing rapid population growth and urbanisation rates (Small and Nicholls, 2003). Human-made infrastructure, such as shipping docks, are a major contributor to these pressures (Bulleri et al., 2020). Such infrastructure can also facilitate the spread of invasive species (Bulleri and Chapman, 2010) and introduce pollutants, which can result in loss of biodiversity (Martínez et al., 2019). Efforts to mitigate and alleviate these ecological impacts by using natural habitat restoration methods, such as assisting seagrass growth, have shown promise (Orth et al., 2020). However, more methods are needed for coastlines where human-made urban infrastructure increasingly dominates the habitat (Strain et al., 2018). Despite the significant human pressures faced by coastal areas, these habitats are home to some of the most productive and functionally diverse flora and fauna on Earth, with estuaries containing exceptionally high biomass productivity (Kennish, 2002; Thrush et al., 2013). Teichert et al. (2018) outlined that in estuaries, alpha diversity (local level diversity) is generally lower than that of the continental shelf. However, overall gamma diversity (total species diversity) is higher, and the typical physiological and life-history adaptations of estuarine species make the biota functionally remarkable.

Considering the ecological richness present, monitoring these zones should be deemed essential, especially in light of the rapid changes occurring within these habitats (Webb et al., 2013). Coastal biodiversity plays a critical role in providing habitat for numerous species and supporting valuable ecosystem services, including shoreline stabilisation, nutrient cycling, commercial fisheries, and recreational activities (Barbier et al., 2011; Thrush et al., 2011; Alleway et al.,2019). Additionally, coastal ecosystems contribute to carbon sequestration and climate regulation, making their conservation crucial for mitigating climate change impacts (Howard et al., 2017; Hilmi et al., 2021). As such, developing effective monitoring strategies that can keep pace with the dynamic nature of coastal environments is essential for understanding and managing these invaluable ecosystems (Crain et al., 2009). These pressures are highlighted in Figure 1.1 (Melet et al., 2020).

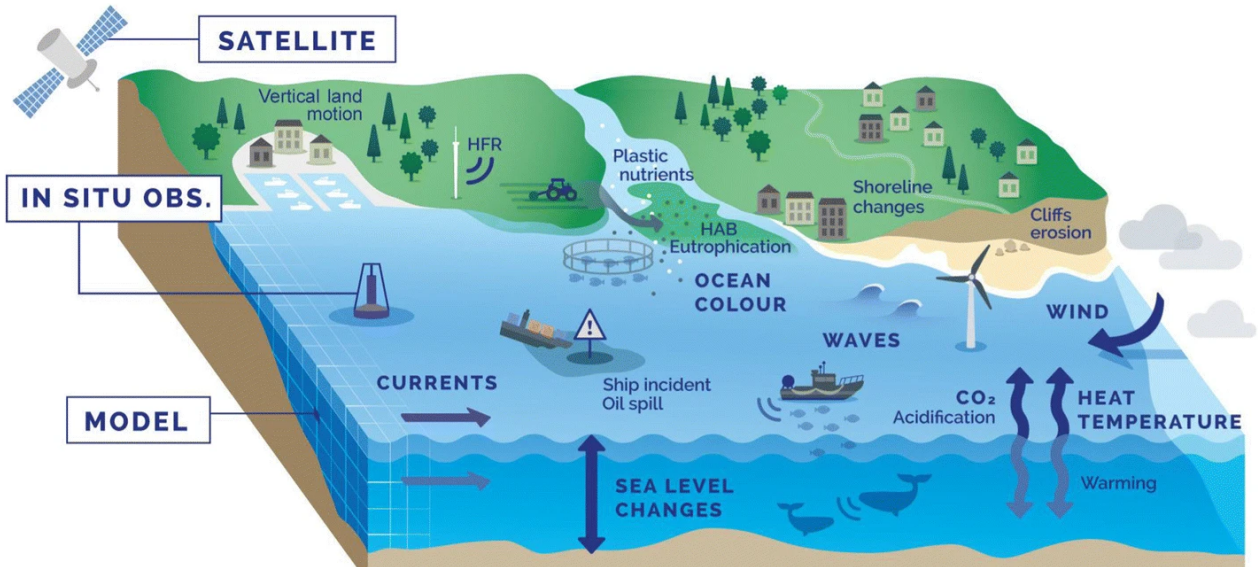

Figure 1.1 Diagram showing various hazards that occur in coastal zones.

Shows the various hazards and variables that occur globally across coastal zones (Melet et al., 2020).

In the UK, approximately one-third of the population resides within 10 km of the coastline (Hadley et al., 2009; Nicholls et al., 2013), underscoring the significant pressures exerted on coastal habitats. With one of the largest coastlines in Europe, the UK faces challenges exacerbated by industrial runoff (Hadley et al., 2009). An example of this is the severe pollution in the Mersey estuary from the 1930s to the 1980s caused by runoff from industrial sites (Gibson et al., 2007; Elliott et al., 2014). Urban renewal and restoration efforts in the Mersey estuary have included innovative actions such as biofiltration and artificial de-stratification (vertical mixing to eliminate layers of temperature), particularly focusing on rejuvenating Liverpool's dock basins (Hawkins et al., 2020). However, infrastructure such as Liverpool's Albert Dock not only shows evidence of anthropogenic impacts but may also pose challenges to fish communities due to its closed system, especially for species relying on vital migration routes between the Irish Sea and the River Mersey (Perkins et al., 2020).

Despite the importance of monitoring these environments in the UK, current practices often rely on antiquated techniques, largely unchanged since the early 1900s, and are primarily focused on targeted, impact-driven programmes such as fisheries or pollution monitoring (shown in Figure 1.2, Bean et al., 2017). Local impacts in freshwaters can have cascading effects on entire catchments, extending to coastal areas (Heathwaite, 2010). These changes

within freshwater catchments can lead to issues in estuarine and coastal systems, particularly in semi-enclosed estuaries, bays, and seas such as the Baltic, Adriatic, North, and Irish seas (Caddy, 2000; Geist and Hawkins, 2016). Conversely, problems at sea, such as overfishing, can adversely impact migratory fish populations crucial for freshwater ecosystems (Allan et al., 2005).

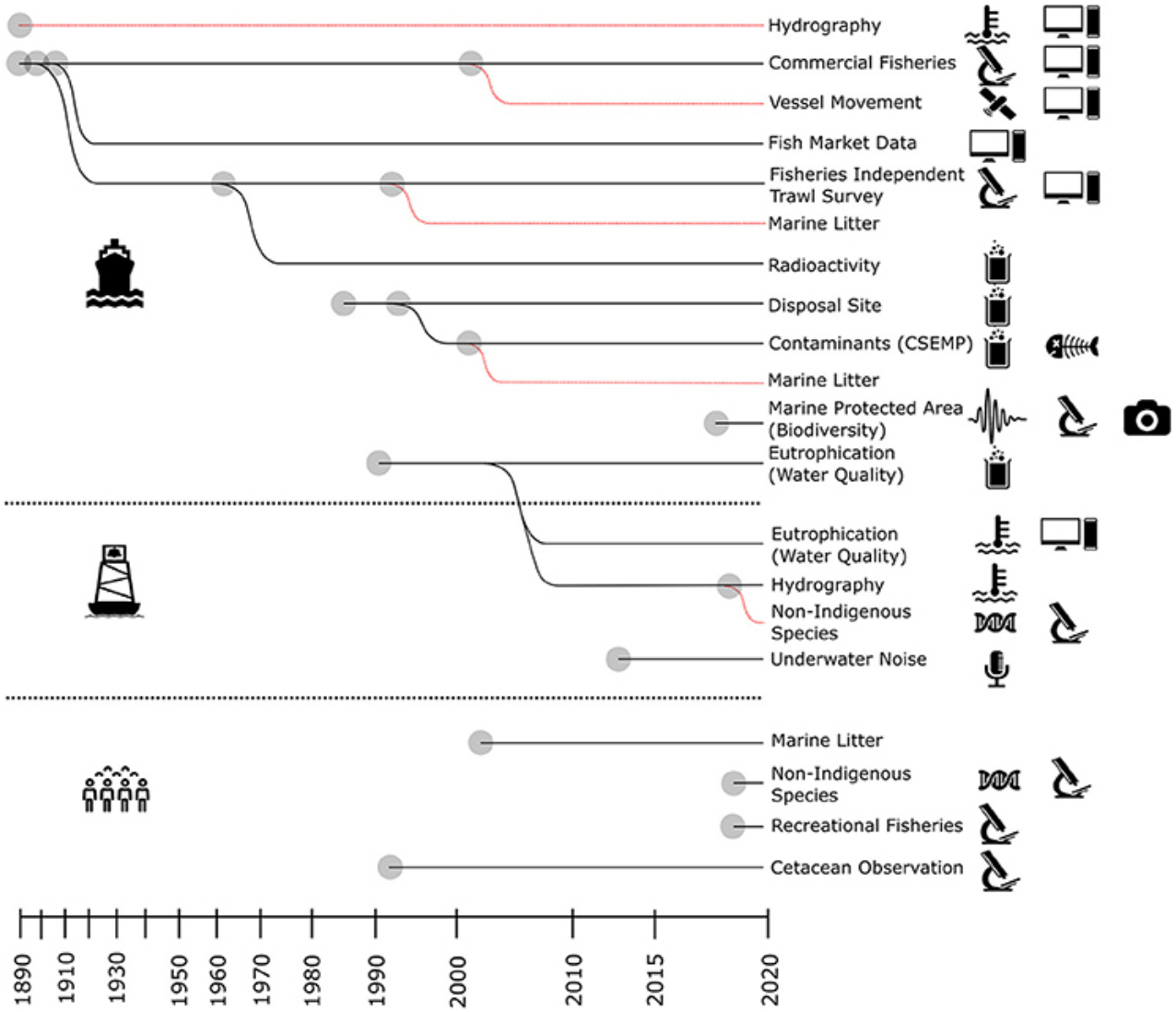

Figure 1.2 Diagram of environmental monitoring programmes in the UK.
Shows the range of monitoring programmes in the UK. Grey dots mark the start of a new surveys, whilst red lines show opportunistic sampling. (Bean et al., 2017).

Understanding fish communities in coastal regions holds significant practical importance, given their role as the predominant macrofauna (Martino and Able, 2003) and as indicators of

habitat well-being (Saenz-Agudelo et al., 2022). Legislation within the European Union and the UK mandates the monitoring of fish populations to assess the ecological condition of estuaries and coastal areas under directives such as the Water Framework Directive (WFD; EC, 2000) and equivalent UK legislation (Coates et al., 2007; Delpech et al., 2010; Harrison and Kelly, 2013) outlined by Gibson et al (2023). Common survey methodologies, including fish capture-based techniques such as trawling, seine, and fyke nets, are employed for this purpose (Gibson et al., 2023). Transitional and Coastal (TRaC) water fish surveys conducted by the Environment Agency focus on collecting data on juvenile fish abundance within UK estuaries, with the aim of evaluating an area's potential as a nursery site. These surveys primary use seine and fyke nets (Environment Agency, 2020).

Despite initiatives targeting fish in estuaries, crucial for marine ecosystem protection and management, the primary focus of legislation is commercial offshore species (Rees et al., 2020). Additionally, the limitations and environmental harm caused by trawling and seine netting hinder biodiversity and environmental management efforts, highlighting the need for refinement and the implementation of additional monitoring techniques (Rees et al., 2020). For freshwater management, the Water Framework Directive (WFD) mandates that water bodies achieve good quality status based on indicators such as the Trophic Diatom Index (TDI) and Mean Trophic Rank (MTR). However, it has been argued that the WFD's current approach primarily addresses eutrophication and requires adjustments to effectively assess ecological status (Voulvoulis et al., 2017; Moss et al., 2020).

1.2 Molecular techniques for enhancing coastal ecological monitoring

As outlined above, coastal fish monitoring in the UK typically involves relatively outdated techniques. Although these techniques will continue to play an important role in monitoring coastal fish populations, there is value in refining and strengthening methods used by marine environmental managers. Some methods, such as trawling, can be destructive, ant others are labour and resource intensive, requiring skilled taxonomists and/or research vessels for precise identification (Bean et al., 2017; Bouchet et al., 2018). In more recent years and research, molecular techniques have presented promising avenues to mitigate some of these biomonitoring obstacles and contribute to the efficacy of ecosystem monitoring shown in Figure 1.1 (Bean et al., 2017; Ruppert et al., 2019).

Environmental DNA (eDNA) can variously encompass genetic material such as whole cells, extracellular DNA, and even complete micro-organisms (Ruppert et al., 2019; Pawlowski et al., 2020), which are sampled from water, soil, air, or other environmental media. This DNA can originate from diverse sources such as bodily secretions, plant matter, decaying organisms, etc., and is subsequently processed through high-throughput sequencing via metabarcoding, enabling the identification of entire communities from a single source (Thomsen and Willersley, 2015). Despite being a relatively recent technique, eDNA demonstrates significant promise for biomonitoring and offers advantages over traditional surveys, particularly as it less destructive to the wider environment than traditional methods (as outlined in Figure 1.2 in section 1.1 in the introduction) (Zaiko et al., 2018).

eDNA presents a number of exciting opportunities. It offers universality by sequencing DNA fragments that can link to virtually every organism worldwide, allowing researchers to study the entire tree of life (albeit dependant on the availability of reference databases) (Bakker et al., 2019; Marques et al., 2021). In turn, this makes eDNA extremely powerful in biodiversity studies due to its ability to sequence DNA fragments which can link and identify a wide range of organisms, from endangered species such as killer whales to potentially harmful algae (Jacobs-Palmer et al., 2021; Suarez-Bregua et al., 2022). This enhanced species detection also requires a lower sampling effort, with researchers able to assess entire communities from just a single sample via metabarcoding (Ruppert et al., 2019). This can enable ecosystem-wide applications and shed light on communities and biodiversity. For example, eDNA studies have revealed interactions in functional ecology, even across large geographical areas (Rees et al., 2014; Ruppert et al., 2019).

Once a sample is collected, typically with ease through pushing water through a filter which "traps" eDNA, it can be processed using Next Generation Sequencing (NGS) technology. NGS allows for rapid DNA sequencing, providing higher throughput data at lower costs (Park and Kim, 2016). This approach is faster, requires less human power, and is more cost-effective compared to traditional survey methods for all marine taxonomic groups (Park and Kim, 2016; Ruppert et al., 2019). Analysing DNA fragments to link them to organisms does not require taxonomic expertise, bypassing the need for complex identification such as microscopic techniques for microalgal identification (Kutty et al., 2022). From a conservation science standpoint, one of the most notable advantages of eDNA is its non-destructive nature, as it involves collecting traces of DNA without disturbing the natural environment (Sahu et

al., 2023). Initiatives such as eDNA citizen science, which demonstrates the ease of being able to collect eDNA samples even on a global scale, highlight the method's ability to engage and involve a broader community (Bowers et al., 2021; Broadhurst et al., 2023; Sheard et al., 2024). This fosters a sense of participation in the stewardship of the natural environment, with the potential for a global impact.

In coastal ecosystems, the primary approach to acquiring environmental DNA (eDNA) involves sampling seawater, wherein this genetic material tends to exist in either intra-cellular (DNA contained within intact cells) or extra-cellular (DNA outside an organism) form (Barnes et al., 2014). Molecular markers, particularly mitochondrial DNA (mtDNA), are commonly used, targeting specific gene regions to identify various taxa (Deiner et al., 2017). After the collection of samples, eDNA is typically extracted from water using artificial filters such as Sterivex syringes, which force water through a filter to capture cells, organelles, or particular matter to which free DNA is bound (Kawakami et al., 2023). Subsequently, DNA is extracted from the filter paper by incubating it with lysis buffer to ensure cell lysis and DNA release. This method breaks down the outer cell membrane to release DNA. This step is often followed by purification to remove impurities that may interfere with downstream methods (Majaneva et al., 2018). Polymerase chain reaction (PCR) is then employed using primers specific to the target market genes. These could be mitochondrial (animals), ribosomal (eukaryotes) or chloroplast (plant), depending on the taxa of interest (Lear et al., 2018). Primers designed to detect multiple taxa from environmental samples often exhibit universality, annealing to DNA templates of broad taxa and amplifying DNA barcoding regions of multiple taxa simultaneously (Kumar et al., 2022) in a process termed “metabarcoding”. To facilitate sequencing on high-throughput platforms, library preparation is a crucial step involving procedures such as adapter ligation, fragmentation, magnetic bead-based clean-up, and dual indexing. Finally, the prepared samples are loaded onto a high-throughput sequencing machine for short-read sequencing analysis. These steps are outlined in Figure 1.3 adapted from Pawlowski et al. (2018).

Through a process broadly referred to as "bioinformatics," the billions of DNA fragments generated by PCR are processed by high-throughput sequencing machines and analysed in the “dry lab” workflow (Figure 1.3). This involves several key steps: i) quality checking, ii) partitioning among the original samples, iii) subdividing into putatively different taxa (MOTUs), and iv) assigning them taxonomically to the most likely organism. These

bioinformatic pipelines may vary, but for all of them, it is crucial to filter sequences based on the expected range to eliminate singletons and dereplicate sequences (Forster et al., 2019). This step ensures the removal of low-quality reads and adapter sequences, thereby enhancing the accuracy of downstream analysis by using only high-quality data (Gibson, 2022). Chimeras, artificial DNA sequences formed during PCR amplification when multiple templates combine erroneously, must be removed to ensure accuracy and reliability, reducing the likelihood of false positives and misinterpretation of results.

Following this, Molecular Operational Taxonomic Units (MOTUs), which are typically reads grouped that differentiate between taxa based on similarity of genetic code, are clustered using a specified clustering threshold (Ruppert et al., 2019). This threshold determines the similarity threshold for grouping DNA sequences, with sequences above the threshold clustered together into the same MOTU, typically expressed as a percentage of sequence similarity (Marques et al., 2020). The clustering threshold may be adjusted depending on the taxa in question. Taxonomy assignment involves classifying DNA sequences and linking them to their originating organisms. Alignment algorithms compare sequences from the database to the samples and assign taxonomic labels, typically indicating a percentage of alignment (Mathon et al., 2021). MOTUs have been widely applied in eDNA studies, having originated in microbial ecology typically with the use of the 16S rRNA molecular marker (Huse et al., 2010; Hira et al., 2017; Marques et al., 2020). In recent years, Amplicon Sequence Variants (ASVs) have gained popularity as an alternative approach. While both ASVs and Molecular Operational Taxonomic Units (MOTUs) serve to classify DNA sequences into taxonomic groups, they differ in their classification methods (Diniz-Filho et al., 2024). ASVs offer single-nucleotide resolution, identifying even the smallest differences in DNA sequences (Callahan et al., 2017; Callahan et al., 2019). By contrast, MOTUs rely on clustering or threshold-based taxonomic grouping (Bonin et al., 2023), Once

the data is organised into a MOTU or ASV table with taxonomic classification and assigned sequencing reads, further analysis can proceed.

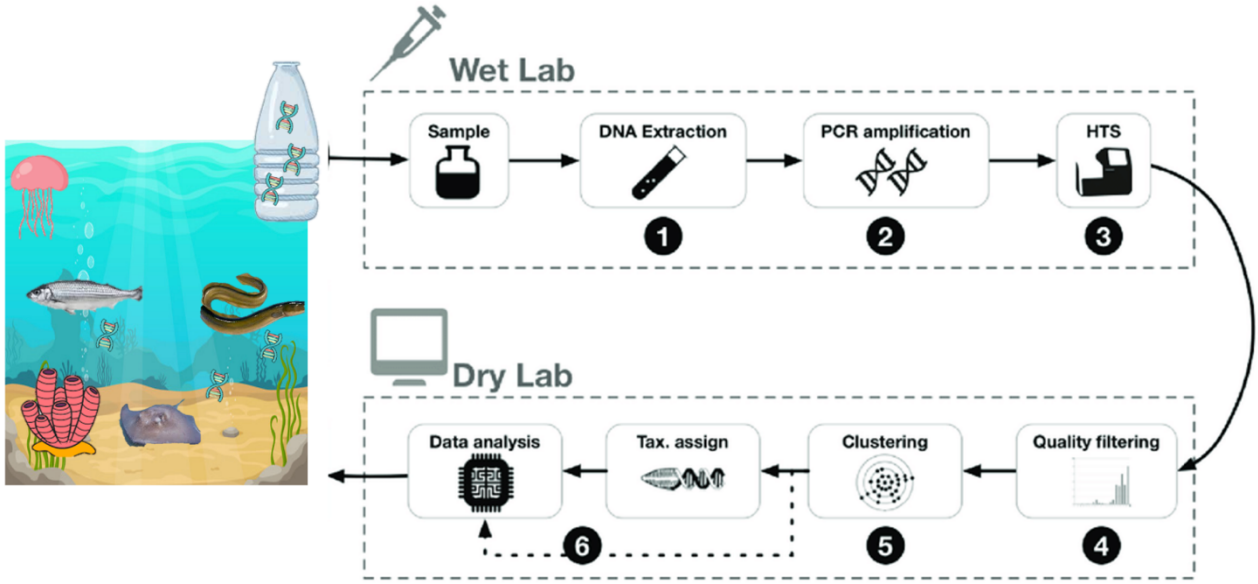

Figure 1.3 Diagram of the workflow of eDNA metabarcoding.
Illustrates the aquatic eDNA metabarcoding process, spanning from sample collection to dry lab results, adapted from Pawlowski et al. (2018).

An essential aspect of eDNA metabarcoding is the selection of appropriate molecular markers. These markers play a crucial role in amplifying DNA samples which contain a mix of organisms, facilitating subsequent high-throughput next-generation sequencing to distinguish species (Ruppert et al., 2019). Initially this technique was prominent in microbiology, however metabarcoding is now increasingly adopted for macroorganisms (Thompson and Thielen, 2023). Its wide-ranging applications at the ecosystem level include characterising biodiversity across extensive spatial scales (Ruppert et al., 2019). Despite the advantages of metabarcoding over traditional barcoding—which identifies single individuals at a time—in terms of speed, accuracy, and cost-effectiveness, obtaining comprehensive ecological insights necessitates the need for standardisation of taxonomic and molecular methodologies. The choice of PCR primers in eDNA metabarcoding studies is crucial, as it affects coverage, resolution, and potential biases across different taxa (Ruppert et al., 2019; Zhang et al., 2020). While mitochondrial cytochrome C oxidase subunit I gene COI is

typically used for metazoans and rbcL for plants, alternatives such as 12S or 16S may better accommodate different taxa (Maria et al., 2020). Effective metabarcoding primers must strike a balance: short enough to amplify degraded samples, specific enough to distinguish species at the individual level, variable enough to differentiate between species, and ensure specificity across a wide range of species (Zhang et al., 2018; Van der Heyde et al., 2022).

1.3 Exploring eDNA metabarcoding in coastal environments

Research into fish communities have played a key role in the development of eDNA metabarcoding for biodiversity assessment in coastal environments. Studies have shown that eDNA sampling can yield a greater number of fish species compared to traditional and destructive methods such as bottom trawling (Zou et al., 2020). It been shown to provide a comprehensive assessment of fish assemblages' functional traits, reflecting its effectiveness in describing functional structure (Fraija-Fernández et al., 2020; Aglieri et al., 2021; Marques et al., 2021). Additionally, Sigsgaard et al. (2017) demonstrated the utility of eDNA in capturing seasonal variation in marine fish communities, highlighting its importance for standardising long-term monitoring of marine biodiversity. The very anthropogenic changes (such as the construction of docks) that modify biota have also facilitated accessibility of coastal habitats, making citizen science initiatives more achievable, such as outreach to the public to collect seawater in Japan (Miya et al., 2022). This demonstrates the potential for large-scale eDNA surveys to capture regional to global fauna diversity. Overall, these studies underscore the potential of eDNA metabarcoding for broad-scale marine fish diversity monitoring, with the two-fold purpose of improving and enhancing environmental monitoring and creating unprecedented opportunities for community-based participatory activities.

Despite the strong tidal dynamism of coastal environments and the high potential for particle distribution, eDNA studies have shown that discrete assemblages can be identified at scales of a few kilometres (Jeunen et al. 2018) or even hundreds of meters (Port et al. 2016). Additionally, Kelly et al. (2018) observed that in an intertidal zone subject to tidal water movements, eDNA communities remained consistent across tidal cycles, indicating that eDNA is predominantly indigenous to the site rather than fluctuating with each tidal cycle. These findings reveal the significance of local eDNA production and persistence over external transport mechanisms. Regarding eDNA persistence, degradation rates can vary across different environments. Collins et al. (2018) discovered that eDNA degrades

approximately 1.6 times faster in inshore environments compared to offshore ones, with little influence from seasonal variations. This suggests that inshore eDNA detection could persist for around 48 hours, offering the potential to gather ecological data with high local fidelity.

The use of eDNA metabarcoding in coastal environments offers numerous advantages, primarily stemming from its non-invasive nature, minimising disturbances to the environment and reducing the need for prolonged or intrusive contact with habitats and species (Gibson, 2022). This method could require only a water or sediment sample, further mitigating disturbances. Additionally, eDNA metabarcoding proves to be both time and cost-efficient. With the expansion of this research field, more eukaryotic groups are being explored, providing valuable insights into biodiversity. Leray and Knowlton (2015) applied this approach to oyster reef biofilms, while Wangensteen et al. (2018) developed protocols for marine hard-bottom communities, revealing substantial eukaryotic diversity despite database gaps. Rivera et al. (2018) even explored diatom biofilms on sea turtle shells, demonstrating its novelty in studying larger vertebrates' movements. Overall, eDNA metabarcoding proves powerful for large-scale biodiversity studies, with potential for further enhancement through expanded reference databases and optimised primer sets.

1.4 Factors and limitations to consider when using eDNA metabarcoding

An important consideration to have in mind when conducting eDNA surveys in coastal regions is environmental factors and their effect on the transport of eDNA particles (Barnes et al., 2021; Hinz et al., 2022). In addition to tides, mentioned above, strong currents have the potential to disperse eDNA, while human-made infrastructures (such as shipping docks) may lead to its accumulation in specific areas (Jeunen et al., 2019). Further research is needed to gain a comprehensive understanding of the dispersal of eDNA in coastal habitats, but research has indicated that even though there is connectivity of waterways, eDNA methods are able to distinguish localised spatial communities (Jeunen et al., 2019). Temperature and salinity also play key roles in the stability and degradation of eDNA. Higher salinity levels may accelerate DNA degradation, reducing its persistence in the environment, whereas colder environments may preserve eDNA for longer periods (Joseph et al., 2022; Lamb et al., 2022). Additionally, biological processes of organisms, such as predation, fecundity, and metabolic rates, can affect the presence of eDNA (Hansen et al., 2018). Given the increasing anthropogenic influence on coastal habitats, environmental conditions may undergo

alterations, potentially impacting the presence of eDNA (Harrison et al., 2019). However, the dynamic nature of environmental factors in coastal zones underscores the importance of monitoring. Further research aimed at enhancing our understanding of eDNA dynamics in coastal areas is essential for improving the accuracy and interpretation of eDNA results.

The accuracy of identifying an organism from a sequence using reference databases depends on the availability and accuracy of the sequences in the databases, potentially biasing the results towards well-studied taxa (Ardura, 2019; Marques et al., 2021; Mathon et al., 2021). While these concerns are valid and should be considered during data analysis, it is important to note that, although false negatives may occur due to database limitations, false positives are less likely. This is especially true if methods to mitigate them, such as cross-checking taxa that are unlikely to be found in that location or rare species, are used (Fonseca et al., 2023). Negative and positive controls are important for detecting false positives, as a negative control is typically taken at each stage at which contamination could be introduced, including sampling in the field (filtering a field blank), extraction and PCR. The positive control which is typically introduced in the wet lab process to primarily identify the efficiency of the processes but also, much like the negative, it can track contamination. The sequencing data of these controls can be used to identify any false positives; if a species is in the controls, it is most likely derived from contamination from the field or lab (Ficetola et al., 2015).

To address these PCR biases, data normalisation is crucial (Kelly et al., 2019). Methods such as Hellinger transformation calculate the relative abundance of each taxon. Here, the square root transformation reduces the influence of dominant taxa (Skelton et al., 2023), therefore ensuring that the transformed data are standardised across samples and account for potential differences in sequencing depth (Shelton et al., 2023). However, these PCR biases and stochastic processes has led to debates about using sequencing read counts as a proxy for abundance (Fonseca, 2018; Bessey et al., 2020). As a result, some researchers continue to prefer interpreting metabarcoding data as merely presence absence (Compson et al., 2020; Coutant et al., 2021; Cuff et al., 2022). Although this reduces PCR bias issues, there is growing evidence that binary data may miss important ecological insights (Guri et al., 2024). Using read count data as a proxy for abundance has the potential to offer valuable ecological information, though it must be done with caution (Beng and Corlett, 2020). This method can help interpret species' functional traits and compare community composition and diversity across samples, enhancing an ecological study extending beyond just presence absence

(Aglieri et al., 2023; Andres et al., 2023; Sard et al., 2019). Mitigating bias can also be achieved by using multiple primer sets, though this increases costs and time, necessitating a trade-off between the taxa obtained, reliability, and cost (Rourke et al., 2022). To further mitigate PCR biases, sequencing depth is crucial; higher sequencing coverage per sample produces more reads, which can increase the number of taxa detected. However, it also raises the likelihood of detecting artefactual sequences (van der Loos et al., 2021; Shirazi et al., 2021). Despite challenges, with proper corrections, read count data can be a valuable tool in ecological analysis (Yates et al., 2023).

Although the use and application of eDNA in ecology have expanded rapidly, as with any emerging method, there remain limitations. While eDNA can enhance biodiversity assessments compared to traditional techniques, it may not always be the most suitable tool in certain contexts. For instance, it may struggle to provide detailed ecological information such as life history, age, breeding status, or conservation status (Evans et al., 2017). In such cases, traditional survey methods may hold an advantage and therefore be a more suitable choice (Rees et al., 2014). The persistence of DNA in the environment, while generally understood, can vary across ecosystems. Factors such as tidal transport may cause organisms to be detected far from their original location (Mauvisseau et al., 2022). Despite these limitations, the benefits of using eDNA metabarcoding in ecological studies, as discussed throughout this thesis, remain compelling.

1.5 Expanding the scope of eDNA sampling through natural sampler DNA.

Over the past decade, there has been rapid growth in the field of environmental DNA (eDNA) analysis, extending beyond the explored aquatic and soil/sediment analyses. Recently, studies have been exploring eDNA detection via more unconventional methods such as isolating eDNA from snow and air (Kinoshita et al., 2019; Franklin et al., 2019; Hellström et al., 2019; Tordoni et al., 2021; Roger et al., 2022; Lyngaard et al., 2022), and innovative artificial methods which use passive sampling (Maiello et al., 2022; Maiello et al., 2024; Albonetti et al., 2023; Bessey et al., 2021). Another emerging branch is natural sampler DNA (nsDNA), which involves harnessing eDNA that has naturally been collected by organisms or surfaces. This concept broadens the scope of biodiversity research, encompassing various organisms by using gut content of shrimps (Siegenthaler et al., 2019), or surfaces (e.g., cobbles) for

eDNA isolation and metabarcoding to unveil community compositions (Shum et al., 2019). With marine sponges being the most researched natural eDNA sampler (Mariani et al., 2019).

Marine sponges as natural eDNA samplers have attracted considerable attention due to their exceptional filtering abilities, surpassing artificial devices in water filtration efficiency (Mariani et al., 2019; Turon et al., 2020; Jeunen et al., 2022 and 2024). Sponge nsDNA offers several advantages over traditional aquatic eDNA sampling methods, including cost-effectiveness, low environmental impact, and adaptability to diverse habitats. Studies have successfully detected a wide range of marine fish and even mammals in regions such as the Mediterranean, Australasia, and Antarctica by extracting eDNA accumulated in sponge tissue (Mariani et al., 2019; Turon et al., 2020; Jeunen et al., 2022 and 2024). Research has demonstrated their potential in monitoring fish community composition between eutrophic areas and Marine Protected Areas (MPAs), using sponge extracts initially collected for unrelated purposes (Turon et al., 2020). Furthermore, even opportunistic archived sponge tissue has been effective in detecting fish communities and robust analytical protocols have been devised to maximise success in sponge nsDNA studies (Harper et al., 2023). This ultimately serves to expand the scope and reach of marine monitoring efforts (Neave et al., 2023).

Efforts have also been made to incorporate bivalves into eDNA surveys, given their abundance and accessibility in various habitats. Using a universal COI marker, it was shown that mussels recover similar taxonomic compositions to water eDNA samples, however richness was much higher in the water samples suggesting selectivity of the mussels filtering (Weber et al 2023). Jeunen et el. (2023) showed that mussel gill tissue yielded no successful eDNA detections when using vertebrate primers. However, challenges related to background signal and co-occurring organisms complicate analyses, necessitating further investigation into optimal sampling strategies and DNA extraction protocols.

To further research using coastal invertebrates as sources of nsDNA, Siegenthaler et al. (2019) collected shrimp samples from European estuaries, employing both 12S and COI markers. Their study successfully detected fish assemblages and retrieved a greater number of species compared to traditional net surveys. Similarly, Wells et al. (2021) analysed the gut diet of plumose anemones and unexpectedly captured a broader range of prey than previously

suspected based on conventional visual analysis, suggesting their potential as effective natural eDNA samplers.

There has been ongoing discussion regarding the terminology employed in eDNA studies, particularly the distinction between eDNA and community DNA. This debate has been further complicated by the extraction of genomic DNA directly from specimens, prompting questions about the scope of the eDNA concept. Pawlowski et al. (2020) proposed that the term eDNA should adhere to its original and broad definition, encompassing DNA from all organisms present in environmental samples, spanning both microbial and macrobial taxa. They recommend specifying the origin of the sample, such as water, soil, etc., and the targeted taxa: e.g. ‘fish eDNA’ or ‘arthropod eDNA’. It could be argued that ‘natural samplers’ further complicate this distinction, as they encompass a diverse range of naturally occurring organisms or surfaces, from marine sponges to cobbles, from which eDNA can be isolated and assessed via metabarcoding to discern community compositions. However, adopting Pawlowski et al.'s (2019) suggestion to specify the origin of the sample, such as "Shrimp eDNA" or "Sponge eDNA," could lead to unnecessary complexity in terminology. Defining these samples under the umbrella term’ nsDNA’ helps maintain clarity, even though it encompasses a broad range of naturally occurring organisms and surfaces. This standardised terminology can assist in delineating the scope of eDNA studies, particularly for environmental managers planning future biomonitoring surveys.

Overall, the expansion of eDNA analysis to include natural samplers presents promising opportunities for cost-effective and enhanced (through identification of potentially more taxa) biodiversity monitoring. While challenges remain in optimising laboratory workflows and data interpretation, ongoing research efforts aim to refine these methods and enhance their applicability in ecological studies.

1.6 Thesis aims and chapter rationale

This thesis aims to assess the viability of using environmental DNA (eDNA) to enhance biodiversity monitoring in coastal habitats, particularly focusing on urban areas with significant anthropogenic impact. These regions are often overlooked due to misconceptions about their lack of biodiversity (Jefferson et al., 2014). This thesis investigates several scenarios where the interplay of these novel biodiversity-screening techniques with current

and historical anthropogenic modifications of the coastal zone can help shape future, ambitious, yet cost-effective, strategies for urbanised coastal biomonitoring.

Chapter 2, aimed to detect temporal and spatial variations in fish communities in historically one of the UK's most anthropogenically disturbed coastlines, Merseyside, carrying out sampling at least twice per season over a year-long period.

In **Chapter 3**, based on previous experience on microalgae-based freshwater environmental assessment (Liu et al., 2020; Kutty et al., 2022) and comparison of molecular markers (Gui et al., 2015), whole-community and micro-eukaryote assessments were carried out using two mostly complementary barcoding regions (COI and rbcL) across six locations within Merseyside, with a particular emphasis on diatoms.

In **Chapter 4,** the novel field of natural sampler DNA was delved into, examining whether beadlet anemones (*Actinia spp.*) could serve as effective eDNA samplers to assess temporal and spatial vertebrate diversity in two UK coastal locations, using water eDNA samples as a baseline.

Chapter 5 further explores potential applications of natural sampler DNA by collecting mussels from a working mussel farm for eDNA vertebrate analysis, alongside observing temporal changes in fish communities through eDNA water samples.

2. Chapter 2: Temporal and spatial eDNA analysis of fish assemblages in post-industrial, urban coastal habitats

(Currently in review with *Environmental DNA*, with the following coauthors: Alice V Cunnington, Erika Neave, Peter Shum, Rupert Collins, Stefano Mariani)

Note: Please see the "Contribution to Data Chapters" section. As described, sample collection, wet and dry lab processes, bioinformatics, data analysis and writing of the manuscript was conducted by Alice Cunnington. Co-authors reviewed the manuscript.

2.1 Abstract

Urban coastal habitats experience substantial disturbances due to their proximity to human settlements and activities. Yet, despite the negative impact of urbanisation on coastal environments, industrial structures can also provide artificial habitats. These are often easily accessible to regular surveys, including water sampling for environmental DNA (eDNA) analysis, an emerging and powerful tool for monitoring biodiversity in aquatic ecosystems. In this study, we utilised eDNA metabarcoding to investigate temporal and spatial trends in fish assemblages within urban coastal habitats between the Dee and Mersey estuaries (United Kingdom), historically one of the most anthropogenically impacted post-industrial coasts in the world. Over a 12-month period, nine water sampling trips were conducted at two locations: the Albert Dock, in central Liverpool, and the Marine Lake, in West Kirby. Illumina sequencing was used to analyse PCR amplicons generated using the fish-targeting Tele02-12S metabarcoding region. Significant changes in fish community composition were observed across the various months. Fish communities significantly differed between the two sites, with the patterns of temporal changes also varying substantially between them. Seasonal appearances/disappearances of specific taxa (e.g. European eel, sand smelt, flounder, herring) shed light on important ecological and behavioural processes that may have management implications. Results also corroborate previous findings on the importance of 'molecular bycatch' (non-target sequences) in expanding our understanding of the anthropogenic influences on the natural environment. Overall, these findings emphasise the value of eDNA monitoring as a non-invasive, affordable, and sensitive approach for routine

monitoring of temporal trends in fish assemblages, facilitating the stewardship of resilient urban coastal zones and recognising interventions that could increase biodiversity.

Key words: eDNA, metabarcoding, coastal fishes, biodiversity, urban environment, European eel, sand smelt, coastal habitats, harbours.

2.2 Introduction

The world's oceans are under unprecedented pressures from numerous climatic and anthropogenic stressors (Bijma et al., 2013), with coastal habitats especially affected (Harley et al., 2006). These highly productive environments often include elements such as (but not limited to) seagrass beds, kelp forests and tidal flats, providing vital ecosystems for fish species at all stages of their life cycles, from spawning to nurseries, to adulthood (Henseler et al., 2019). The deterioration of these habitats has led to environments that can no longer support crucial nursery, feeding or reproductive functions which are vital for the fishery yield of species of transnational importance, as assessed by the International Council for the Exploration of the Sea (Seitz et al., 2014).

In many regions globally, human-made structures (e.g. shipping docks, seawalls, groynes and jetties) have significantly altered natural dynamics in coastal areas (Crain et al., 2009; Bulleri and Chapman, 2010; Pardal-Souza et al., 2017, Todd et al., 2019), and the introduction of pollutants have led to imbalances in nutrient cycles, resulting in the degradation of coastal fish habitats (Brown et al., 2018). In order to combat these negative outcomes, the development of tools to assess and monitor coastal fish communities accurately and effectively are crucial (Hoffmann, 2022). Industrial, human-made structures are also particularly suited to support these monitoring efforts as they often offer easy access to water for surveys than more remote sampling sites.

The rising popularity of environmental DNA (eDNA) as a research tool has provided invaluable ecological insights into the world's ecosystems (Deiner et al., 2017). Environmental DNA metabarcoding can reveal the trace DNA left behind by all organisms present in a given environment (Pawlowski et al., 2020), offering a non-destructive alternative to traditional survey methods that rely on the capture of organisms (Collins et al., 2022), and opening unprecedented opportunities for both routine and targeted biomonitoring (Taberlet et al. 2012). Additionally, eDNA analysis holds an advantage over less intrusive underwater visual censuses since it doesn't rely directly on expertise in morphological identification for precise species identification (Thomsen and Willerslev, 2015; Miya et al., 2022) and is less biased towards certain ecological traits (Aglieri et al., 2021).

Despite the rapid expansion of eDNA research and its promising advancements, there are limitations that must be considered. Although eDNA persistence in water is generally well understood, more research may be needed across a broader range of environments, especially urban human-made structures, where the interplay of factors such as temperature, pH, salinity, and microbes affect DNA stability in different ways (Collins et al., 2018). Additionally, eDNA cannot distinguish between live and dead organisms or different life stages, which can impact the scope of detection (Kamoroff and Goldberg, 2018). Also, biases can arise during PCR amplification, potentially inflating sequencing reads for certain taxa and skewing the results (Fonseca, 2018; Bessey et al., 2020). This has led to concerns about the use of sequencing reads from eDNA metabarcoding to accurately quantify species abundance. Some researchers address this by using presence/absence data to reduce bias (Compson et al., 2020), though this may overlook key ecological insights, limiting the method's full potential (Guri et al., 2024). Techniques such as data transformation and/or modelling (Shelton et al., 2022) can help mitigate biases, allowing read count data to offer valuable insights into species' functional traits, community composition, and diversity (Sard et al., 2019). Still, using read counts as abundance proxies requires caution, with appropriate acknowledgment of the biases involved (Cauvin et al., 2022).

eDNA studies have consistently demonstrated the effectiveness of eDNA metabarcoding in characterising fish diversity in marine ecosystems (Stat et al., 2017; Bessey et al., 2020), with some research showing that eDNA surveys can detect greater fish diversity than traditional methods (Thomsen et al., 2016; Port et al., 2016; Zou et al., 2020; Liu et al., 2022), thereby improving fish diversity estimates across various ecological, spatial, and temporal scales (Collins et al., 2022; Port et al., 2016; Sigsgaard et al., 2020). Furthermore, eDNA metabarcoding analysis has been shown to successfully detect a broader spectrum of functional traits within coastal fish communities (Aglieri et al., 2020), and has proven effective in detecting rare fish species that are often challenging to find using conventional sampling methods (Rees et al., 2014; Boussarie et al., 2018; Oka et al., 2020). Such versatility, universality and effectiveness of the eDNA approach may soon turn it into a staple tool used routinely by environmental managers.

This study investigates fish communities in two urban coastal habitats constructed in the nineteenth century: the commercial docks in the city of Liverpool and the recreational marine lake in West Kirby, Northwest England. Samples were collected multiple times per season,

over a 12-month period, during which eDNA data revealed diverse assemblages and different phenological patterns of resident and migratory species. The main hypotheses were that: i) seasonal eDNA metabarcoding analysis would be sensitive to temporal changes in urban coastal fish assemblages; ii) the eDNA approach would be able to reveal spatial differences in ichthyofauna that reflect the historical and environmental features of urban infrastructures, thereby enhancing local-scale ecological heterogeneity. The findings showcase how these new, low-impact molecular technologies can inform the public and local authorities on the diversity of coastal marine organisms that thrive alongside us in urban settings, and how accessible multipurpose human-made structures may become convenient platforms for biodiversity monitoring.

2.3 Methods and Materials

2.3.1 Field Collection

Sampling was conducted at least twice per season (spring, summer, autumn, and winter) in two distinct locations in Northwest England, United Kingdom. The first location, the Royal Albert Dock, opened in 1846, has long been the centre of Liverpool's maritime industries, and it remains a major tourist destination, experiencing high levels of human activity. The water in this area is enclosed within high dock walls (approximate depth: 5 meters) but is connected to the estuary of the river Mersey through a sluice gate, allowing water to mix during boat entry or exit.

The second location, West Kirby Marine Lake, is a Victorian period structure built in 1899. It has remained popular for its recreational aquatic activities such as sailing, kayaking, windsurfing and walking. Here, there is also a sluice gate, which is regulated to allow tidal water to enter the lake twice a day at high tide and be retained inside the lake when water retreats at low tide (approximate depth: 1.5 meters) which determines frequent hydrological changes, resulting in a more open coastal environment.

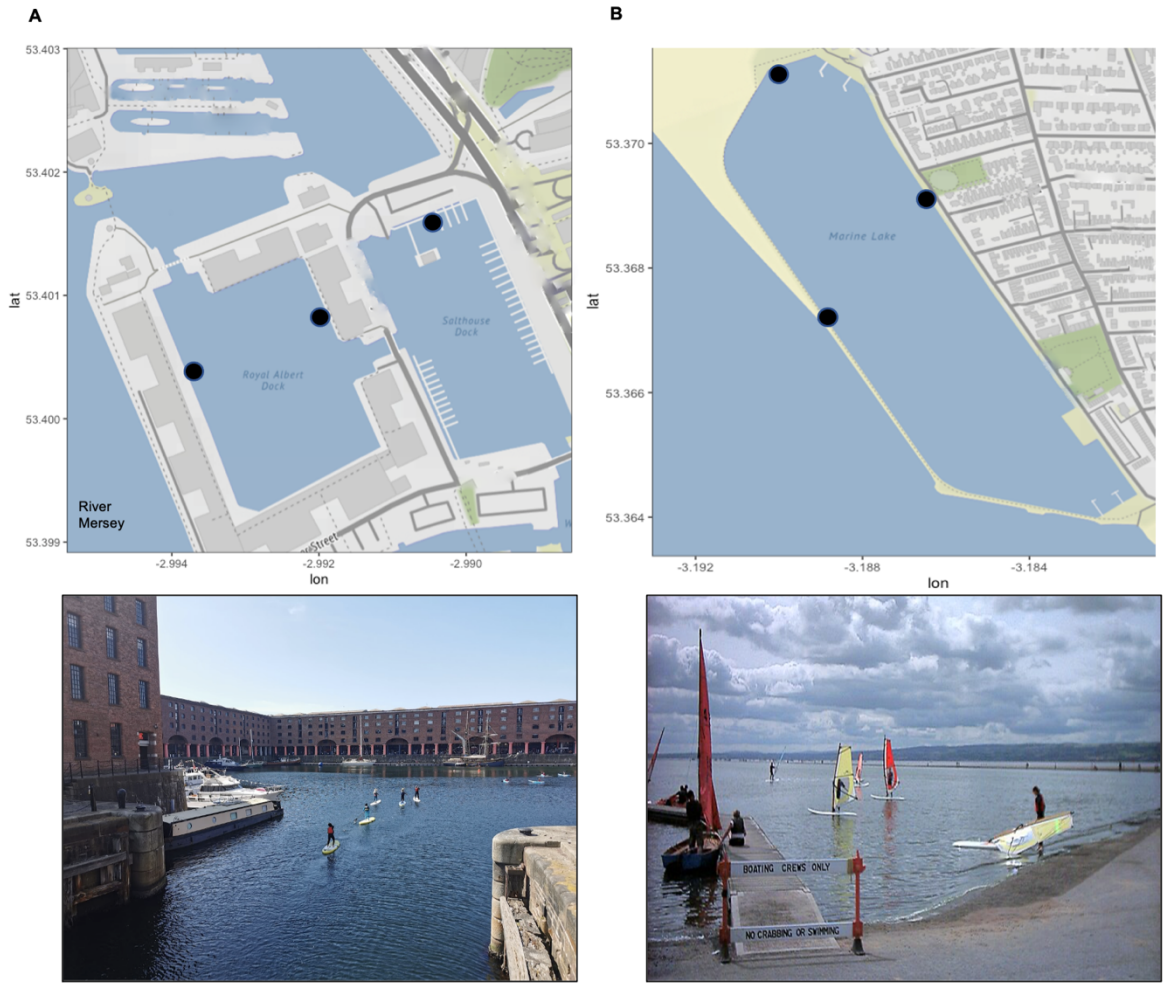

Figure 2.1: Map of the sampling location (A) Albert Dock and (B) Marine Lake, West Kirby.

Map showing the two sampling locations: (A) Albert dock, Liverpool, UK and (B) Marine Lake, West Kirby, UK (Wikimedia commons, 2009). The points represent the three different sampling sites within each location.

In each location, three sampling sites were selected (Figure 1). For the Albert Dock, the coordinates for each site were: 53.400964 -2.992001, 53.400185 -2.993644, 53.401701 -2.990286. For West Kirby the coordinates for each site were: 53.370971 -3.189751, 53.367476 -3.189022, 53.368756 -3.186189. At each sampling location, two Sterivex filters (0.22 µm, PES membrane) were collected from each of the three sites. A Sterile syringe was used to pass three litres of water through each Sterivex filter in the field, amounting to a total of 18 litres of water per location, per sampling event.

Prior to sampling, all field equipment was sterilised with 10% bleach followed by 70% ethanol. Laboratory grade gloves were worn and regularly changed after filtering each sample. To monitor contamination at each site, a field blank was taken, in which purified water was pushed through a Sterivex filter, with blanks treated identically to other samples throughout the collection and extraction process. Sterivex filters were placed inside two sterile bags, and immediately stored on ice. All samples were stored at -20°C in the lab until further processing.

2.3.2 DNA extraction

All laboratory work, including DNA extraction, library preparation, and sequencing, was conducted by Alice Cunnington at Liverpool John Moores University for all chapters included in this thesis.

DNA extraction from Sterivex filters was based on the mu-DNA protocol by Sellers et al. (2018). Firstly, the eDNA filters were removed using pincers and the filter paper was removed using sterile dissecting scissors and forceps. Filters were cut up into small, ~20 mm^2 pieces. Half of the filter was used in DNA extraction with the other half archived at -20°C to allow for future testing if needed (Nguyen et al., 2020). 750 µL lysis solution, 250 µL lysis additive, 20 µL Proteinase K (concentration 100 µg/mL) per sample were combined in a 1.5 mL Eppendorf tube to make the lysis master mix, which was added to each sample. These were placed on a thermomixer for 12 hours at 55°C at 650 rpm and then centrifuged at 10,000x g for 1 minute at room temperature. The supernatant was transferred to new tubes and 0.3x volume flocculant solution per sample was added and then placed on ice for 10-30 minutes. Samples were then centrifuged at 10,000x g for 1 minute and the supernatant transferred to 2 mL Eppendorf tubes. A 2x volume of the tissue binding buffer was added and vortexed. The sample was then transferred to a spin column and centrifuged at 10,000x g for 1 minute. This step was repeated until the entire volume had passed through the filter. 500 µL of wash solution was added to each sample and centrifuged. This was repeated twice. 100 µL of elution buffer was added directly to the spin column which was then incubated at room temperature for 5 minutes. The sample was then centrifuged at 10,000x g for 1 minute and the supernatant retained.

2.3.3 Target amplification and library preparation

PCR amplification was performed in triplicate for each sample, using the Tele02 primers (F: 5′-AAACTCGTGCCAGCCACC-3′, R: 5′-GGGTATCTAATCCCAGTTTG-3′), which amplify a ~167 bp fragment of the 12S rRNA mitochondrial region (Taberlet et al., 2018) and are designed to target teleost DNA (Miya et al., 2015). Positive controls were put in place for each PCR batch. Iridescent shark catfish (*Pangasianodon hypophthalmus*) was used as a positive control DNA at 0.05 ng/µl, choosing this organism as it is a tropical freshwater fish, and has no close relatives in UK coastal waters. The positive control was used in separate reactions from the eDNA amplification and was used to confirm that the PCR, sequencing and bioinformatic steps were functioning as expected. Primer pairs were uniquely indexed for each sample to enable demultiplexing for downstream bioinformatic analysis. The 20 µL reaction mix included: 10 µL Myfi mix (2x), molecular grade water 5.84 µL, Bovine Serum Albumin (BSA) 0.16 µL, 1 µL of each primer (10µM) and 2µL of eDNA template. The thermocycling profile comprised: 95°C for 10 min; 40 cycles of 95°C for 30 s, 60°C for 45 s, 72°C for 30 s; and a final extension of 72°C for 5 mins. PCR products were run on 2% agarose gels (150 ml 1X TBE and 3g agarose powder) stained with SYBR safe (1.5 uL). 1.1 uL of loading dye and 1.1 uL of loading dye mixed that is loaded onto the gel. Ladder was loaded with a mix of 1.1 uL of ladder with 1.1 uL of loading dye. Gel was run at 100V for 40mins. PCR replicates were then pooled. Samples were purified at a 1:1 ratio with 30 µL Mag-Bind TotalPure NGS magnetic beads and 30 µL of the three pooled PCR products from each sample. The 1x ratio binds the target tele02 fragment, whilst the second 0.6x ratio targeted larger secondary products. The concentration of each purified PCR was quantified using a Qubit 4 Fluorometer dsDNA HS assay kit and pooled in equimolar amounts. The pooled PCR product, containing all samples at equimolar concentration, was quantified using a Tapestation 4200 using the high sensitivity D1000 assay. Then, a further 1x bead clean-up was performed on the pooled PCR product. Illumina libraries were made using the NEXTFLEX Rapid DNA-Seq Kit 2.0 (PerkinElmer), using 1 µg as starting concentration of the pooled PCR product following the manufacturer's guidelines with library amplification.

For 1 µg of input DNA, the adapter concentration was 25 µM. Thorough mixing of the reaction was essential for optimal results, due to the viscosity of the NEXTFLEX® Ligase Enzyme 2.0. 50 µL of End Repaired & Adenylated DNA was combined with 44.5 µL of NEXTFLEX® Ligase Buffer Mix 2.0, 2.5 µL of NEXTFLEX® Barcoded Adapter, and 3.0 µL of NEXTFLEX® Ligase Enzyme 2.0. The mixture was pipetted up and down 15 times to

ensure thorough homogenisation, and the tubes were visually inspected to ensure adequate mixing. Adapters were not premixed to avoid excess adapter dimer formation. The strip tubes were then sealed and incubated in a thermocycler at 20°C for 15 minutes, with the heated lid turned off, followed by a 4°C hold.

A 1x bead clean-up was performed on each sub-library. New strip tubes were placed on a tube rack and the beads were vortexed for 20-30 seconds to ensure even resuspension. The required volume of beads was aliquoted, vortexed again for 20-30 seconds, and added at a 1x ratio (based on the sample volume) to each PCR tube. The mixture was pipetted up and down 10 times, vortexed, and briefly spun down for 2 seconds to collect residue. The mixture containing DNA and beads, was incubated at room temperature for 5 minutes.

The tubes were placed on a magnetic stand to bind the Mag-Bind® TotalPure NGS beads to the side of the tube. After waiting approximately 2 minutes for the solution to clear, the cleared supernatant was carefully removed, ensuring the bead pellet on the tube's side was not disturbed. Beads were washed with 80% ethanol, and this was step was repeated twice. Beads were resuspended with a final elution of 25 uL and transferred cleared supernatant was 23 uL.

For PCR amplification, for each sub-library, 23 µL of Adapter Ligated DNA was combined with 25 µL of NEXTFLEX® PCR Master Mix 2.0 and 2 µL of NEXTFLEX® Primer Mix 2.0. The mixture was thoroughly mixed by flicking the tubes and then spun down. The strip tubes were sealed and incubated in a thermocycler under the following conditions: 30 seconds at 98°C, followed by xx cycles of 15 seconds at 98°C, 30 seconds at 65°C, and 30 seconds at 72°C, then 2 minutes at 72°C, with a final hold at 4°C.

A 1x bead clean-up was performed on each library. New strip tubes were placed on a tube rack, and the beads were vortexed for 20-30 seconds to ensure even resuspension. The required volume of beads was aliquoted and vortexed again for 20-30 seconds to ensure proper mixing. A 1x volume of Mag-Bind® TotalPure NGS beads (based on the volume of the sub-libraries) was added to each PCR tube. The mixture was pipetted up and down 10 times, vortexed to mix well, and briefly spun down for 2 seconds to collect any residue on the side of the tube. The DNA/bead mixture was incubated at room temperature for 5 minutes. The tubes were then placed on a magnetic stand to bind the Mag-Bind® TotalPure NGS beads to the side of the tube. After waiting approximately 2 minutes for the solution to clear, beads were washed with 80% ethanol and resuspension of beads with 23 uL of elution buffer.

Tapestation was conducted on the library. The library and 20% PhiX control were quantified by qPCR using the NEBNext Library Quant Kit for Illumina (NEB). The Master Mix and Primer Mix were prepared by adding 500 µL of NEBNext Library Quant Primer Mix to the bottle of NEBNext Library Quant Master Mix (7.5 mL). The NEBNext Library Quant Dilution Buffer (1X) was prepared by making a 1:10 dilution of the 10X buffer in molecular-grade water. For each sub-library being quantified, 1.3 mL of 1X buffer was made by mixing 1170 µL of molecular-grade water with 130 µL of 10X buffer. An initial 1:1000 dilution of each sub-library was prepared in the 1X buffer by adding 1 µL of each sub-library to 999 µL of 1X buffer. Two additional dilutions were then prepared to create 1:10000 and 1:100000 dilutions for qPCR analysis. Each DNA standard and each sub-library dilution were run in triplicate. A total of 16 µL of the master mix was pipetted into each reaction tube, along with 4 µL of 1X buffer, standards, or sub-library dilutions. All standards and sub-library dilutions were briefly vortexed and spun down before pipetting into the reaction tubes.

The sub-libraries were diluted to 1 nM and pooled at equimolar concentrations and the final pooled library was quantified using Qubit before proceeding to the next qPCR. After diluting the sub-libraries to 1 nM and pooling them at equimolar concentrations, the final pooled library and the PhiX intended for sequencing were quantified. qPCR was then performed on the 1 nM library and the PhiX diluted to 1 nM.The final library and PhiX control were diluted to 85 pM and loaded onto an Illumina iSeq 100 Reagent v2 (300-cycle).

2.3.4 Bioinformatics and downstream analysis

The bioinformatic process was based on the OBITOOLS pipeline (Boyer et al., 2016). FASTQC was used to assess sequence quality scores. ILLUMINAPAIREDEND was then used to merge the paired-end reads and remove alignments with low (<40) quality scores. NGSFILTER was used to de-multiplex samples. To remove sequences that were not in the target base pair range, we filtered the sequence lengths to 120-200bp by using OBIGREP. We dereplicated the samples using OBIUNIQ. Chimeras were then removed using the uchime-denovo chimera search function in VSEARCH (Rognes et al., 2016). Molecular operational taxonomic unit (MOTU) clustering was implemented using SWARM with "-d 3" (Mahé et al., 2014; Maiello et al., 2022; Cai et al., 2022). Taxonomic assignment per sample was carried out using ECOTAG with a 12S reference database which was constructed using 'ecoPCR' in silico against the EMBL database (Release version r143 October 2023). The taxonomic assignments included seven levels of biological classification along with a percentage indicating the likelihood of

accurate classification. After taxonomic assignment, non-native and unexpected taxa were cross-checked by manual BLAST against the NCBI nucleotide database (NCBI, 2024). MOTUs that could not be assigned to genus or species level were also cross-checked manually against the NCBI nucleotide database.

All downstream analyses were conducted using R version 4.2.2. The package DECONTAM in R was used on the raw MOTU output (Davis et al., 2018). MOTUs were filtered by retaining assignments with >97% identity match for biological classification. Prior to downstream analysis, the data were transformed using the Hellinger approach, which converts species abundances from absolute to relative values followed by square-rooting for standardisation (Legendre & Legendre, 2012). Alpha diversity indices (richness and Shannon) were calculated using the PHYLOSEQ and VEGAN package (Mcmurdie and Holmes, 2013; Oksanen et al., 2013). ANOVA was used to test for significant differences among seasons and locations.

To visualise spatial (Albert Dock vs West Kirby) and temporal (seasons) differences among eDNA samples, we used nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distance. We tested for differences between locations and seasons using permutational multivariate analysis of variance (PERMANOVA, 999 permutations) on pairwise distance matrices using the function *adonis* in VEGAN. Finally, to identify whether there were any species significantly associated with certain months, we used an indicator species analysis in R using the *multipatt* function in the INDISPECIES package (Cáceres et al, 2016).

2.4 ***Results***

Samples were sequenced over one Illumina iSeq100 run alongside an unrelated eDNA project. The samples from this study made up 89% of the overall sequencing run. There were 134 samples altogether: 108 eDNA filters, eighteen field blank controls, three extraction blanks, three PCR blanks, and two positive controls which yielded 1,579,456 of raw sequencing reads, with a mean of 14,625 reads per sample. To minimise false positives and contamination, low abundance OTUs (< 5 reads) were removed from downstream analysis. All human reads were removed (which made up 66% of the total raw reads). There were only low abundances of human reads found in the controls (<100), but we also removed 102 reads of blackmouth catshark *Galeus melastomus* from downstream analysis across real samples, as

this was a known contaminant from a different project. Six samples from the Albert Dock (all from the sampling trip in September 2021) contained no sequencing reads and were removed. After the removal of the above reads and quality filtering, 533,342 reads were retained for downstream analysis across 102 samples.

From the Albert Dock, we recovered 31 taxa altogether: 16 fish, 9 birds, 5 mammals and 1 sea star. From West Kirby, we recovered 24 taxa altogether: 15 fish, 5 birds, 3 mammals and 1 sea star (Fig 2.2).

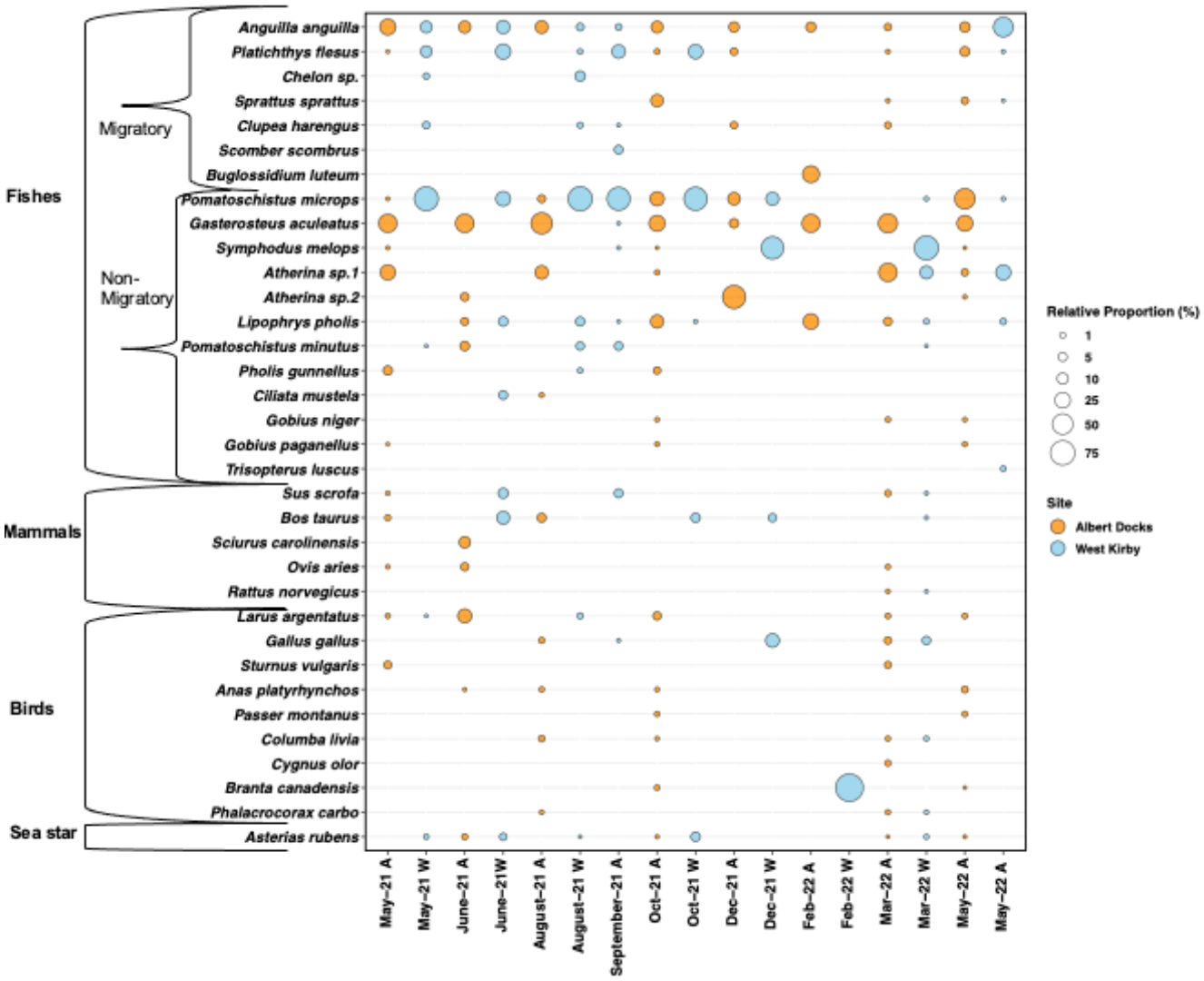

Figure 2.2**: **Bubble plot of proportional read counts of species detected.

Proportional read counts of 34 species detected at the Albert Dock and West Kirby throughout the different sampling seasons starting from Spring 2021 until Spring 2022. The

size of the bubble indicates the proportional read counts that species represented in a sample. Fish species are grouped by migratory or non-migratory.

In the Albert Dock, the three-spined stickleback *Gasterosteus aculeatus* and European eel *Anguilla anguilla* were detected throughout all seasons. In West Kirby, the assemblage was dominated by the common goby *Pomatoschistus microps*, which amounted to 72.1%, 68%, and 66.1% of the proportional read counts in Spring 2021, Summer 2021, Autumn 2021 respectively (Figure 2.2). Though also frequent in the Albert Dock, the common goby was never dominant here, except for May 2022, with a greater variety of other species present throughout the year (Figure 2.2). Two species of sand smelt (*Atherina* sp.) were detected across locations and in most seasons and are likely to represent *A. presbyter* and *A. boyeri*, but due to uncertainties around their taxonomy and the reference sequence data, they were precautionarily recorded as “sp. 1” and “sp. 2” (see Discussion 2.5). As *Chelon labrosus* and *Chelon ramada* share an identical 12S reference, making it impossible to differentiate between the two, we have documented this taxon as "*Chelon* sp.". The critically endangered European eel was identified in both locations: across all seasons in the Albert Dock, whilst absent in Winter in West Kirby (Figure 2.3).

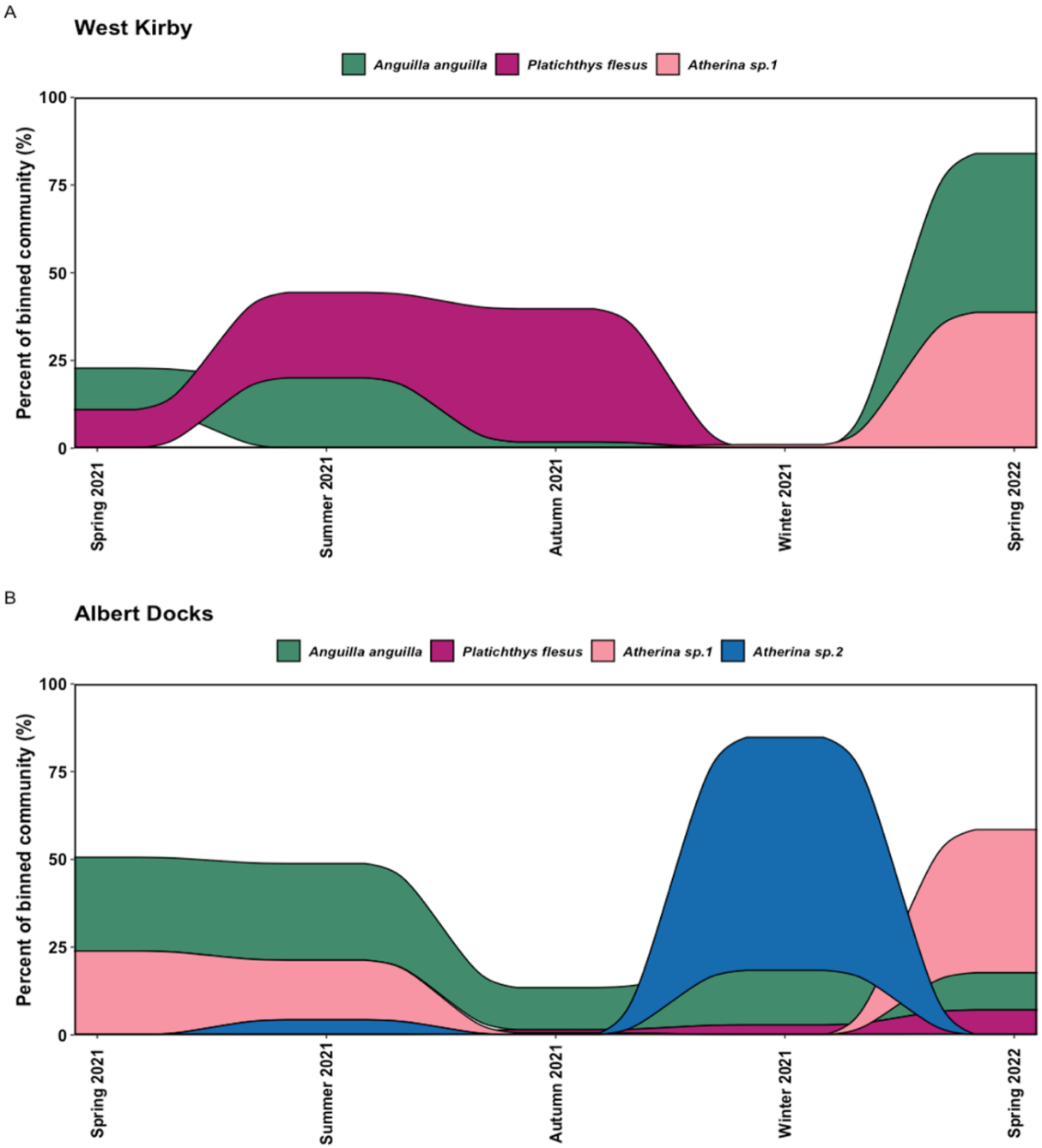

Figure 2.3: Alluvial plot showing the percentage of binned community among seasons between (A) West Kirby and (B) Albert Dock.

Figure shows: (A) Percentage of the binned community for three different species: Anguilla anguilla, Platichthys flesus and Atherina sp. 1. (B) For the Albert Dock only, Atherina sp. 2 is also displayed. The figure shows the change from Spring 2021 to Spring 2022.

In both locations, diversity drops pronouncedly during the coldest months (Figure 2.4) (Shannon Index, ANOVA F=14.22; P< 0.001). The diversity is consistently higher in the Albert Dock than West Kirby throughout all seasons (Figure 2.4) (Shannon Index, ANOVA F= 5.352; P< 0.001). European eels were detected in the Albert Dock throughout the year, whereas in West Kirby they show two main peaks, in Spring 2021 and Spring 2022 (Figure 2.4).

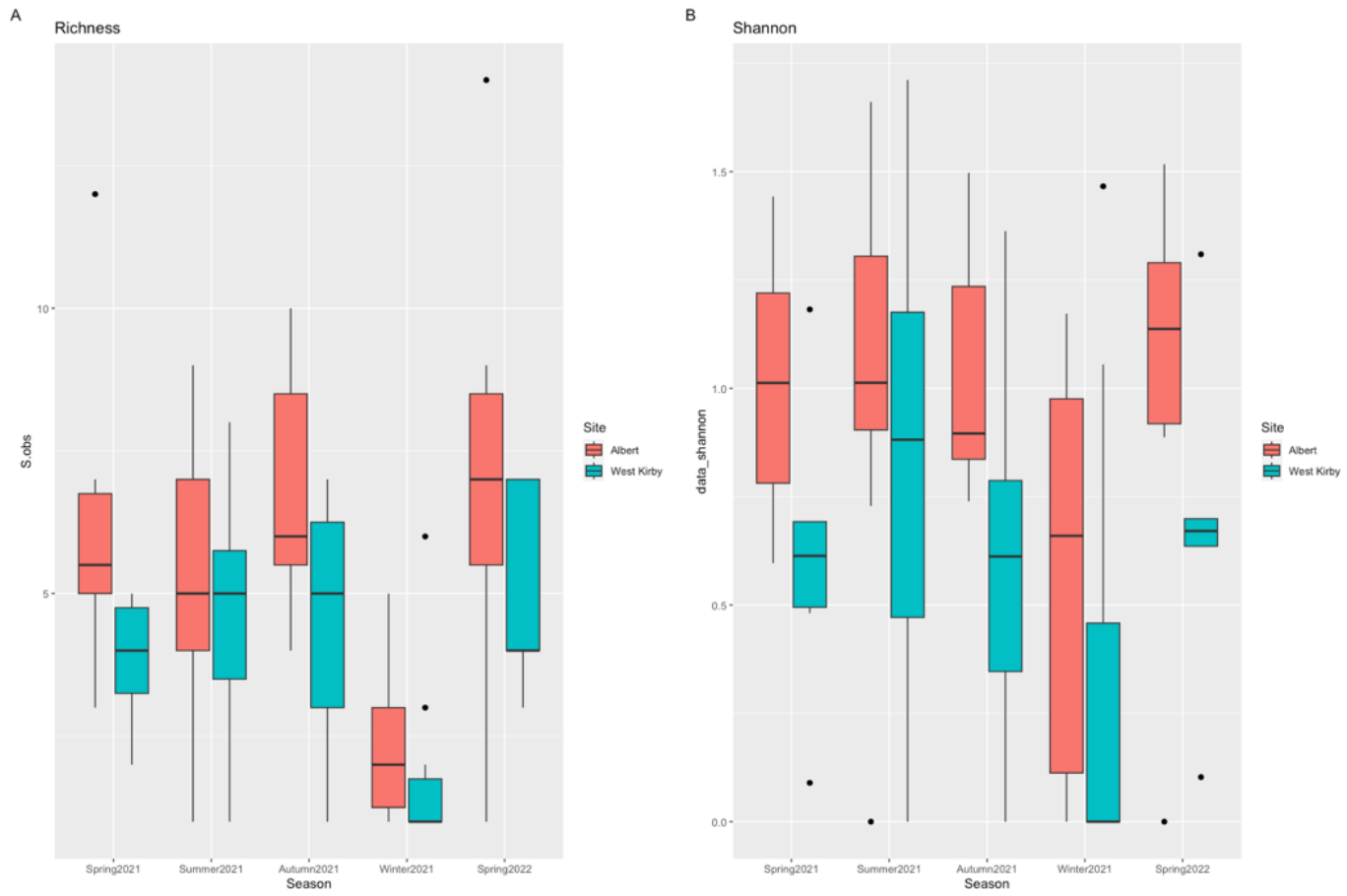

Figure 2.4: Boxplots of (A) Species richness and (B) Shannon alpha diversity.
Species richness and Shannon index alpha diversity between Albert Dock and West Kirby locations for each season. The plots include error bars indicating the variability or dispersion of the data points for each season measured.

There was a significant difference in community composition detected between the two sites (PERMANOVA F= 6.3667 P= 0.002) (Figure 2.5). Indicator species analysis showed that two fish species were statistically more abundant in the Albert Dock: three-spined stickleback (P= 0.0123) and the *Atherina* sp. 1 (P=0.0314).

When fish community structure is compared across the various seasons, we found significant temporal (seasonal) differences (PERMANOVA F= 1.7082; P= 0.037) (Figure 2.5). The interaction between season and location is also significant (PERMANOVA F= 4.5276; P= 0.002). A PERMANOVA was conducted separately for each site to evaluate whether seasonal differences in fish community structure were more pronounced at one site compared to the other. Both Albert Dock (PERMANOVA F = 2.5644, P = 0.001) and West Kirby (PERMANOVA F = 2.5814, P = 0.001) were significantly affected by season.

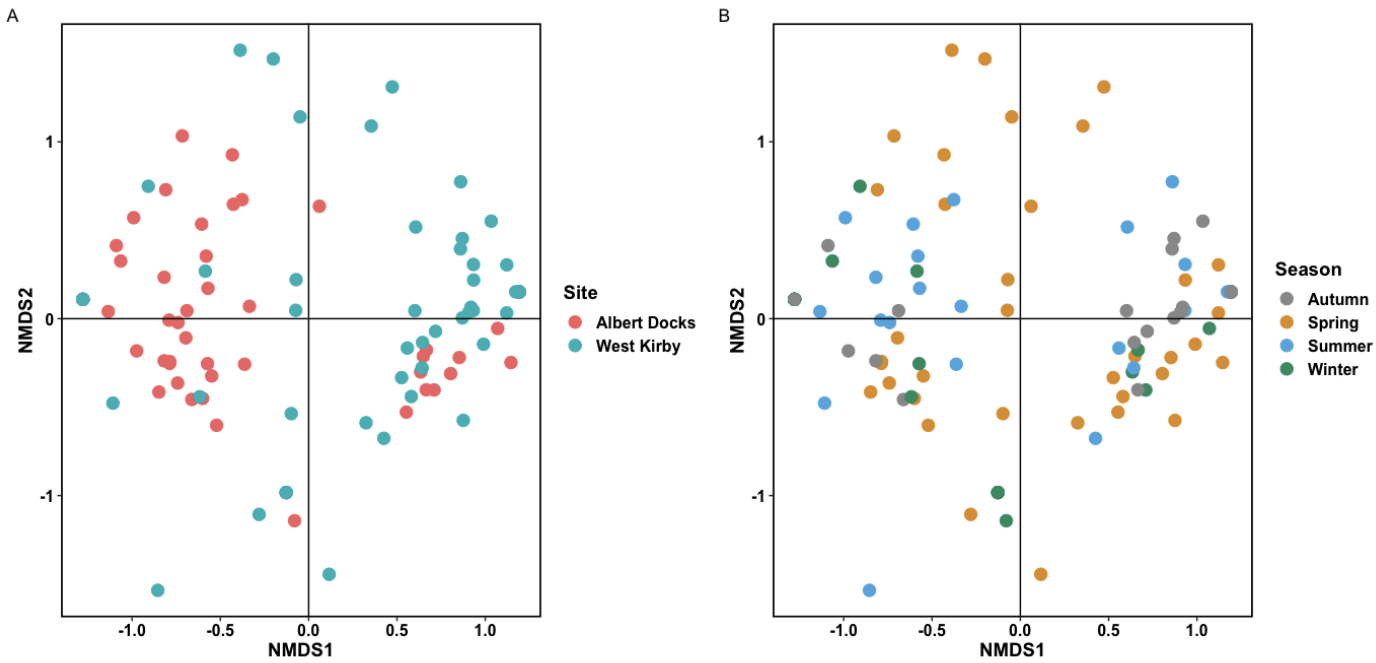

Figure 2.5: Non-metric multi-dimensional scaling of all eDNA samples between (A) season and (B) sites.

A non-metric multi-dimensional scaling (NMDS; Bray-Curtis dissimilarity) showing all eDNA samples collected from Albert Dock and West Kirby and annotated by season, (A) Colours depict the two different sites. (B) Colours depict the four different seasons.

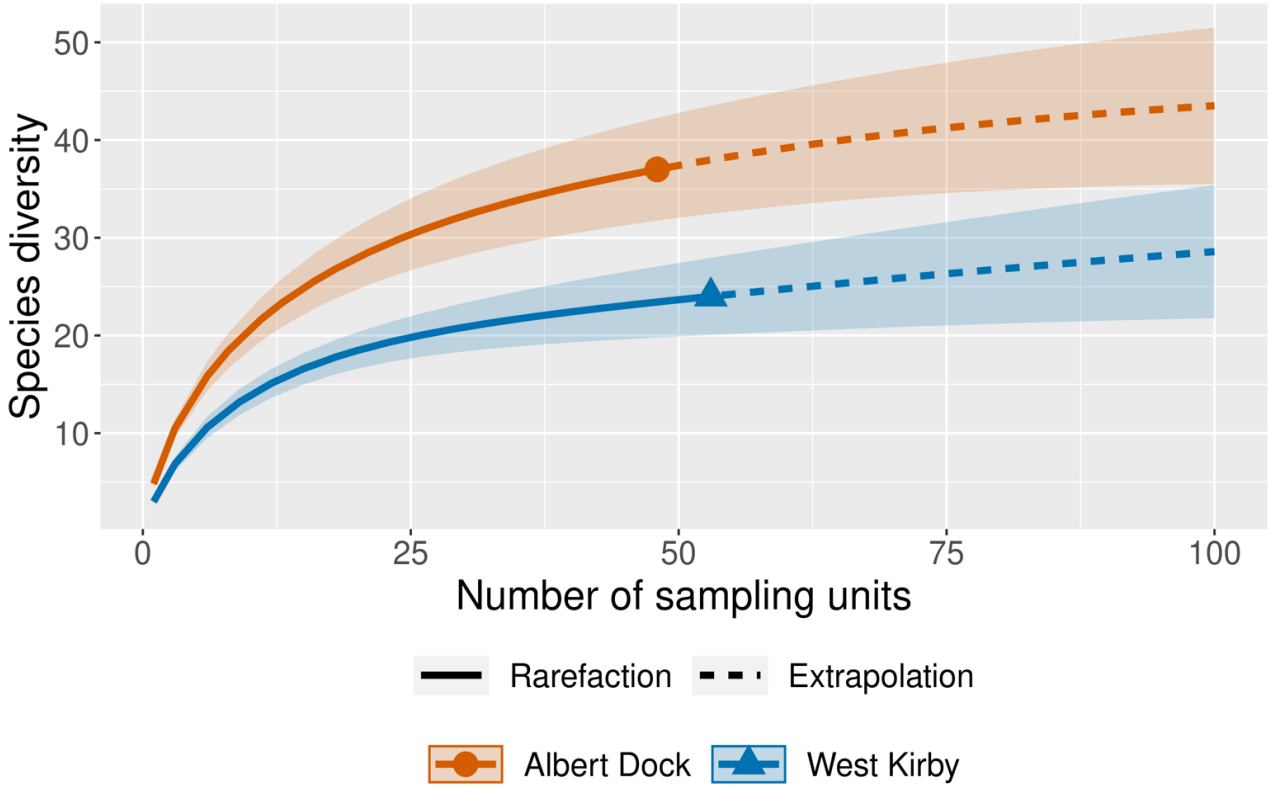

Figure 2.6: Rarefaction curve between Albert Dock and West Kirby.
The dashed line represents the extrapolated estimate calculated with R package iNEXT (Hsieh et al., 2016) and the shaded section shows the 95% confidence interval for the estimation.

2.5 Discussion

The importance of sampling in coastal urban habitats is increasingly apparent, particularly due to the escalating pressures of pollutants, infrastructure construction, and multiple, commercial and recreational human activities. Coastal marine fish populations are in decline (Strain et al., 2017; Cowley et al., 2022), a phenomenon aggravated by the human-made developments and activities, which drive ecological changes across a variety of habitats (Bulleri & Chapman, 2010). Artificial docks such as those in West Kirby and Liverpool, are commonplace in estuaries and coastal waters worldwide (Kennish 2002, 2016; Kelty and Bliven 2003) and are vital for various industrial and recreational activities; with shipping docks often associated with adverse environmental impacts, (i.e. pollution and invasive species; Todd et al., 2019). Nevertheless, these areas can also play a role in providing habitats and supporting aquatic communities through alterations in environmental conditions (Logan et al., 2022). Given the complex dynamics of urban coastal ecosystems, it is particularly

important to advance the development of efficient, scalable, and reproducible methods for detecting and monitoring fish communities.

By using eDNA analysis, 34 distinct taxa were identified across the two locations, supporting the growing evidence on the benefits of eDNA metabarcoding for assessing fish biodiversity in coastal and aquatic environments (Hallam et al., 2021; Kumar et al., 2022). The sampling effort appeared sufficient, as indicated by the curves nearing asymptotes for both locations (Figure 2.6), suggesting that a comprehensive snapshot of the diversity of species was captured. The observed fish species were consistent with the anticipated composition for these environments (Baldock and Dipper, 2023), although there is evidence suggesting that fish assemblages associated with artificial structures differ from those that inhabit more natural coastal environments (Clynick and Chapman, 2008), potentially leading to the enhancement of assemblage heterogeneity across these areas. Indeed, the two coastal urban infrastructures investigated exhibited significantly divergent fish communities. Despite the similarity in location, the nature of the infrastructures themselves may account for these differences.

The Albert Dock exhibited a consistently higher species richness and diversity overall, a difference that was maintained across all seasons. The consistently greater species diversity suggests that this habitat is more complex and ecologically stable compared to West Kirby. In the winter season (December 2021 to February 2022), the Albert Dock contained five migratory fish species (*Anguilla anguilla*, *Platichthys flesus*, *Clupea harengus*, *Lepidorhombus whiffiagonis*, and *Buglossidium luteum*) and four non-migratory fishes (*Pomatoschistus microps*, *Gasterosteus aculeatus*, *Atherina* sp. 2, and *Lipophrys pholis*). In contrast, West Kirby during the same winter period, had only two non-migratory fish species detected: *Pomatoschistus microps* and *Symphodus melops*. These findings underscore the diversity of resident and migratory species affiliated with the Dock in winter compared with West Kirby. This reinforces the notion that the deeper, sheltered dock waters may offer a more stable and complex habitat, compared to the marine lake's dynamic, shallower and more homogeneous substrate that undergoes daily exchange of water. The presence of unexpected migratory species in the dock during winter may be linked to the infrastructure, which could be trapping fish and altering their behaviour. Infrastructures may disrupt natural water dynamics and hinder the connectivity of marine populations (Bishop et al., 2017), but

on the other hand, it may also contribute to creating novel habitats and shelters that may enhance fish populations (Barwick et al. 2004; Guidetti et al., 2005).

Significant changes were found in fish community composition across the seasons, observing seasonal appearances and disappearances of specific taxa. The European flounder *Platichthys flesus* exemplifies this, migrating to warmer waters in winter, before spawning in spring. During summer, shallow coastal waters and estuaries become feeding grounds for its larvae, juveniles, and adults (Orio et al., 2017). In West Kirby, increased abundances of *P. flesus* in summer and autumn 2021, aligning with its expected behaviour. We also detected *P. flesus* were observed in all months apart from winter which is again, consistent with its expected migratory behaviour. However, in the Albert Dock, *P. flesus* was detected even in winter, indicating that the depth of the dock allows these fish to maintain a local population all year round. A similar pattern is also observed for Atlantic herring *Clupea harengus*, an emblematic pelagic species that has been exploited by European commercial fishers for centuries. Spawning occurs for approximately four weeks in autumn within the Irish Sea (Dickey-Collas et al., 2010), but pockets of winter-spawning contingents are also known (Burke et al., 2009). While *C. harengus* was observed in both locations during spring and autumn, winter detections were exclusive to the Albert Dock. These findings shed light on the impact of urban infrastructure on migratory patterns, and their role in detecting variations in the ecological and behavioural patterns of fish communities inhabiting the adjacent shelf seas.

Two distinct species of *Atherina* sand smelt were identified at the two study locations. There are two species of *Atherina* that are known to be present in the UK: sand smelt *Atherina presbyter* and big-scale sand smelt *A. boyeri*. *A. presbyter* is a common UK coastal species, known for its seasonal migrations within the Atlantic Ocean (Palmer, 1983). By contrast, *A. boyeri* is believed to be present only in specific UK locations, primarily in more southern regions (Bowers et al., 1964). Populations of *A. boyeri* found further north in the UK are typically situated in urban coastal areas. In this study, two distinct sand smelt species were identified through two different DNA reference sequences in the NCBI GenBank database, indicating their existence in urban coastal areas within the Irish Sea. However, due to historical taxonomic misidentification and ambiguities (Ardura, 2019) which are also represented in DNA reference databases such as NCBI, were not able to determine at this point which of the two divergent sequences identified in this study belongs to *A. presbyter* or

A. boyeri. It is however noteworthy that *Atherina* sp. 2 is detected only in the Albert Dock which suggests potential differences in ecological preferences between the two species. Given that *A*. sp. 2 is observed only in the Albert Dock, it raises the possibility that human-made coastal habitats can serve as artificial havens for ecologically significant traits. The occurrence and/or abundance ratio between these species could also plausibly reflect trends in rising water temperatures (given *A. boyeri*'s preference for warmer water). Similar patterns are being documented in commercial fish species, where warm-water species appear in UK waters alongside a decline in cold-associated species (Simpson et al., 2013; Townhill et al., 2023). Although further research will be required to comprehensively understand distribution and adaptation of sand smelts, it is possible that these and other species may in the future contribute to devising new indicators for environmental monitoring. Once the current ambiguities with reference sequence data are resolved, environmental DNA analysis could be particularly well suited to monitoring the distribution of sand smelts due to the difficulty identifying them to species level in the field.

Even though urban infrastructures are often associated with having negative impacts on the environment, our results reveal their potential significance as crucial refuges for species of conservation importance. Long-term surveys indicate that mussel biomass and consequent biofiltration rates in the Albert Docks are essential for maintaining water quality, offering habitat for other species and supporting relatively stable ecosystems and constituting vital refuge for fish (Firth et al., 2024). The European eel was found to be abundant in both locations. Notably, in the Albert Dock, eels were detected in every month sampled, while in the marine lake, there were no detections in winter 2021. The eels in these habitats have featured on BBC documentaries (i.e. Springwatch, 2016), reinforcing that the species have been inhabiting this area as a vital refuge for several years. These findings demonstrate the presence of European eels in both areas, which is particularly noteworthy. Although it is not unusual to observe European eels in the UK, it is however valuable that they have been detected, given the population decline that contributed to its critically endangered status (IUCN., 2023). There has been a recent push to add eDNA analysis among the methods to assess their occurrence and abundance in UK habitats reaching beyond an academic setting (Hillsdon, 2023; Horston, 2023; Rodriguez, 2023). The substantial presence of this species in both locations is both encouraging and concerning (especially the detection of eels all year round in the Albert Dock). Indeed, while the existence of an important refuge for such an iconic endangered species in the middle of a bustling city is inspiring, the design and

functioning of the Albert Dock raises the possibility that these catadromous animals may become trapped within the structures of the dock during the time of their long reproductive journey (Verhelst et al., 2018). In contrast, the marine lake exhibits a fluctuation in presence and absence, which might be attributed to its location as a more exposed coastal habitat, enabling the ongoing use of migration routes. The presence of the European eel in these urban coastal areas carries significance for their conservation and underscores the need for assessing and monitoring the ecological and behavioural impacts of urban structures within these environments.

Among the detections, there were some unexpected deeper-sea species: blackmouth catshark (*Galeus melastomus*), sand ray (*Leucoraja circularis*), megrim (*Lepidorhombus whiffiagonis*), and greater fork-beard (*Phycis blennoides*). These species are not expected in our inshore coastal urban study area, and typically inhabit more offshore habitats (Clarke & Borges, 2005; Ellis et al., 2005; Gerritsen et al., 2010). The sensitivity of eDNA methods is one of its main advantages in being able to detect rare species at low abundances, but with this comes the increased risk of making spurious detections which are sometimes difficult to verify. There are several routes that allochthonous DNA can enter into the sampling and analysis steps. Firstly, field contamination can distort results from the transfer of eDNA between sites via sampling gear or clothing. Laboratory contamination from amplicons generated during previous work can frequently be observed in eDNA studies, despite rigorous decontamination controls being employed (Bowers et al., 2021). Here, this prior knowledge allowed us to flag *Galeus melastomus* reads as likely coming from a previous project. Another consideration is taxonomic assignment artefacts and reference database deficiencies (Ardura, 2019; Jackman et al., 2021), which may lead to misidentifications. Despite global initiatives such as the International Barcode of Life (Weigand et al., 2019), reference databases remain incomplete (Schenekar et al., 2020). Biological explanations are also possible. One factor could be tidal transport, where eDNA from adjacent areas is carried to coastal environments. While there is evidence supporting the influence of tides on eDNA transportation (Jeunen et al., 2018), conflicting studies suggest minimal effects (Larson et al., 2022). This may also especially be the case during reproductive activity, where eDNA quantities are elevated due to spawning (Collins et al., 2022). Non-local eDNA may also be brought in via ballast water locally discharged from shipping (Andres et al., 2023). Future eDNA studies must address these issues and develop new strategies to improve end-user confidence in the results (Blancher et al., 2022).

The ability to identify temporal changes in fish community compositions can play a crucial role in developing a better understanding of complex community dynamics. This study highlights the effectiveness of eDNA metabarcoding in detecting and shedding light on these temporal trends, as demonstrated by the observed patterns in *P. flesus* and European eel populations. Statistical analysis using PERMANOVA shows the presence of significant differences in fish community structure across months and seasons in both sampling locations. Various factors influence the abundance of eDNA. For example, marine organisms that undergo spawning events are expected to shed increased quantities of DNA, through the release of gametes (Collins et al., 2022). Studies have demonstrated the composition and structure of fish communities exhibit seasonal differences in estuarine or coastal ecosystems due to the yearly variations in environmental conditions, spawning, migration and fishing activities (Hallam et al., 2021; Li et al., 2022; Zamani et al., 2022; Jiang et al., 2023).

These temporal fluctuations in the presence of certain fish within these two urban infrastructures highlight the need to also consider ecological factors that may be influencing this behaviour (Sigsgaard et al., 2017). Artificial coastal structures can create unsuitable habitats for various species by diminishing natural heterogeneity and fragmenting the environment (Bulleri and Chapman, 2010), especially from anthropogenic activities (Todd et al., 2019; Mael et al., 2024). Such environmental alterations, such as increased water temperatures, can affect the presence of fish that either favour or avoid these conditions (Dugan et al., 2011). Changes in water pH, often driven by pollution from ocean acidification and coastal eutrophication, can also influence resource availability (Colburn et al., 2016). This is known to have a particularly strong impact on mollusc larvae, leading to cascading effects throughout the food web (Colburn et al., 2016). On the other hand, these environmental alterations are not always detrimental; some structures may actually support unique communities, potentially enhancing habitat heterogeneity (Bulleri and Chapman, 2010). Incorporating environmental data alongside eDNA sampling could provide valuable insights into what might be influencing these behaviours (Rourke et al., 2022). This approach could support the development of monitoring efforts and conservation strategies for species that use these infrastructures as refuges, while also enhancing ecological understanding of these under-researched urban areas.

The results showed that the Albert Dock had consistently higher richness of species overall which was maintained across all seasons. There was a notable decline in species richness during winter, followed by an increase in spring, summer, and autumn. These results align with the anticipated seasonal diversity patterns typical of cold-temperate coastal habitats, in which diversity tends to be greater at the end rather than the beginning of summer (Jovanovic et al., 2007). This fluctuation in diversity is often driven by migration and the reproductive cycles of marine organisms (Connor et al., 2019). These findings highlight the dynamic nature of fish communities, with diversity shifts occurring throughout the year as species display specific reproductive behaviours and life-cycle patterns. While eDNA metabarcoding has proven highly effective—often matching or surpassing traditional sampling methods with less effort involved (Jeunen et al., 2019; Sard et al., 2019; Hallam et al., 2021)—these specific study sites have not been extensively surveyed for fish diversity. To strengthen this research, integrating paired sampling with traditional methods would provide a valuable comparison. This approach would not only assess whether eDNA metabarcoding outperforms conventional methods in this context but also offer additional insights to specific species, which may be absent or less prevalent at the control sites. The overall absence of archived fish survey data shows the limited monitoring of fish species in these areas, emphasising the importance of this study despite the lack of comparisons with traditional surveys and control sites. Through eDNA metabarcoding alone, this research has identified key species of conservation concern and revealed intriguing ecological patterns in migratory fish behaviour, demonstrating the need for further surveys whilst also providing a valuable baseline for future studies in these areas.

Non-target, non-fish species were found in both sampling locations. These ‘bonus’ species consist of several mammals, birds, and even a sea star. A range of different bird species were detected including the cormorant *Phalacrocorax carbo*, a piscivorous bird common in the Mersey and Dee estuaries, as well as other well-known urban species, such as Canada goose *Branta canadensis* and common starling *Sturnus vulgaris*. Mariani et al. (2021) highlight the importance of this molecular by-catch of 'bonus' species, showing that eDNA metabarcoding using 12S Tele02 marker is not limited to detecting fish species alone. As the primers used primarily target teleosts, the presence of these 'bonus' species is likely underestimated, indicating the need for specific primers if future studies aim to focus on these additional taxa. The detection of terrestrial mammals such as the Eastern grey squirrel *Sciurus carolinensis* and brown rat *Rattus norvegicus,* as well as the three domestic species, namely pig *Sus*

scrofa, sheep *Ovis aries*, and chicken *Gallus gallus*– all most likely associated with the human food chain – completes the picture of a typical urban water body.

As part of this molecular by-catch, 66% of the total reads were human. Through extensive checks across the control samples, which contained very minimal reads of human, it can be concluded that this human DNA was derived from the environment itself. Both sampling locations experience significant human activity both in and out of the water, which is likely the primary explanation for these results. The amplification of human sequences by eDNA metabarcoding, particularly using the 12S region, has been documented (Kelly et al., 2014; Miya et al., 2015; Lynsey et al., 2019; Zhang et al., 2020; Ragot and Villemur, 2022). Although the Tele02 12S marker is intended to be fish-specific, this study has identified limitations by revealing the amplification of a substantial quantity of human sequencing reads. Comparing the efficiency of other 12S regions, such as MiFish (Miya et al., 2015), in amplifying human DNA would be valuable. Primer performance may vary, even when targeting similar regions (163-185 bp) (Miya et al., 2015). Additionally, many studies may not report the number of human reads, making it challenging to draw accurate conclusions about the relative effectiveness of each marker based on existing literature. While this represents a potential limitation of eDNA metabarcoding in urban coastal environments, advancements in the field—such as redesigning metabarcoding primers, using primers that do not amplify human DNA, employing human DNA-blocking primers, or increasing sequencing depth—could help address this issue (Zhang et al., 2020). In the meantime, while further research is conducted on how different primers amplify human DNA, it would be beneficial to use human DNA-blocking primers to reduce human DNA amplification. It is important however to acknowledge that the inclusion of these primers may slightly decrease the number of detected fish species, so a multi-pronged strategy that combines human-blocking primers with increased sequencing depth could be employed to balance the trade-offs of each approach. Given global urbanisation trends, it is crucial that sampling in urbanised coastal areas continues, but researchers should take off-target amplification into account and identify pre-emptive mitigations, along with the associated costs for future studies.

*2.6 **Conclusion***

These findings underscore the potential of novel, low-impact molecular technologies to provide valuable insights into the diversity of coastal marine organisms that live alongside us

in urban settings, bringing to the forefront the importance of urban infrastructures as multi-purpose study subjects and monitoring platforms. Most of the docks, piers and sea walls in coastal cities tend to be overlooked as areas of ecological importance but are in fact diverse environments that can serve as a refuge for a plethora of fish species, and they provide easy access for non-invasive sampling methods, such as aqueous eDNA.

Through eDNA metabarcoding, this study has compiled a comprehensive list of teleost species present in both the Liverpool Albert Dock and West Kirby's marine lake, which include species of ecological and conservation importance and have unveiled significant changes in fish community composition between habitats and across different seasons, which point to the important source of habitat heterogeneity that is maintained by these human-made structures.

Overall, eDNA metabarcoding in urban habitats offers a promising and versatile approach for evaluating biodiversity in post-industrial coastal environments. Additionally, these results provide valuable insights into species dynamics and seasonal fluctuations, that can be used to inform the public and guide local authorities in future assessments and monitoring efforts within these diverse and resilient coastal refuges.

3. Chapter 3: Multi-marker eDNA analysis of eukaryotic communities as a blueprint for ecosystem monitoring in urban coastal habitats

3.1 Abstract

Coastal regions impacted by urbanisation are exceptionally susceptible to direct anthropogenic pressures, given their intricate connection between terrestrial and marine environments. These dynamic ecosystems harbour a diverse array of eukaryotic communities, ranging from animals to protists. The potential to characterise entire communities through eDNA metabarcoding, including crucial environmental indicators such as diatoms, offers substantial promise for effectively monitoring these habitats. In this study, Cytochrome oxidase I gene fragment (COI) was employed, known for its universality, and the ribulose-1,5-biphosphate carboxylase oxygenase (rbcL) gene, commonly used for diatoms. In May 2021, six heavily urbanised coastal sampling locations were sampled, all within an approximately 20km radius in Merseyside, Northwest England, situated between the final stretch of the River Mersey estuary and the River Dee on the Wirral peninsula. Three sites were enclosed habitats within industrial urban structures (e.g., Albert Dock, Crosby Marine Lake, West Kirby Marine Lake) as well as three open coastal environments (e.g. Speke and Garston coastal reserve, New Brighton beach, Thurstaston beach). The findings show that the COI marker provides a comprehensive overview of eukaryotic diversity, whereas rbcL offers finer resolution, particularly concerning environmental indicators such as Stramenopiles, including diatoms, and the identification of potentially harmful algal species. Forty indicator species between the markers (twenty per marker) were delineated, unveiling unique species distributions among the locations driven by specific markers, thereby highlighting the advantages of employing these markers in conjunction. Additionally, significant diatom species detected by COI were absent from rbcL, again revealing the advantages of using the markers concurrently. Geographical variations were detected, with higher species richness observed in open coastal habitats compared to enclosed ones, alongside distinct clusters of whole-community eukaryotic assemblages in each sampling location, delineating localised communities on a relatively small spatial scale despite their interconnectivity via tidal waterways. This underscores the versatility of the two barcoding regions in providing

valuable insights into both broad-scale patterns and finer-scale community dynamics, which could guide future environmental management efforts.

3.2 Introduction

Coastal habitats are more vulnerable to direct anthropogenic stressors (such as urbanisation, pollution, and hydrological changes) than the open sea, a result of the dynamic interaction between land and sea. Such habitats host essential and diverse eukaryotic communities, ranging from animals to protists. These organisms are vital to many ecosystem functions (Blaxter et al., 2022) and play a key role in marine food webs as primary producers, primary consumers, and detrivores involved in nutrient cycling and carbon fixation (Xue et al., 2022). These organisms and their associated ecological functions are susceptible to alterations from environmental conditions (Harley et al., 2006). Consequently, they can potentially serve as crucial early indicators of pollution, climate change, urbanisation and invasive species (Grossart et al., 2021; Sagova-Mareckova et al., 2021). One such alteration is the construction of urban infrastructure. Urban infrastructure is widespread along the UK coastlines and can heavily impact the biodiversity of coastal habitats and the communities that reside there (Evans et al., 2019). For instance, shipping docks can act as barriers for species (as explored in Chapter 2), making the identification of sensitive species that serve as bioindicators crucial for informing future environmental surveys and protection efforts (Woolridge et al., 1999). Given the vulnerability of the UK's coastline, accurately and efficiently characterising communities is vital.

The increasing popularity of environmental DNA (eDNA) analysis as a research tool has provided invaluable ecological insights into the world's ecosystems (Deiner et al., 2017). eDNA metabarcoding enables the detection of trace DNA left by organisms in a given environment (Pawlowski et al., 2020), offering unprecedented opportunities for both routine and targeted biomonitoring (Pawlowski et al., 2021; Pont et al., 2021). This method can provide profound insights into whole-community eukaryotes, uncovering thousands of species—often using the COI marker—and revealing the vast, previously underestimated diversity of these small and often overlooked taxa (Bik et al., 2012; Bakker et al., 2019; Antil et al., 2023). Using single or multiple assays can enhance biodiversity measurements which go beyond traditional surveys (Bakker et al., 2019; McElroy et al., 2020). Markers such as the chloroplast gene rbcL (large subunit of ribulose 1,5 bisphosphate carboxylase), used to

primarily target plants and more recently applied to diatoms—a diverse group of photosynthetic microalgae crucial for primary production and commonly used as ecological indicators (Desrosiers et al., 2013)—have predominantly been used in freshwater environments (Kutty et al., 2022; Turk Dermastia et al., 2023; Espinosa et al., 2024). These markers can offer greater precision in identifying environmentally significant species such as bioindicators (Apothéloz-Perret-Gentil et al., 2021). By contrast, ribosomal gene 18S typically target whole community eukaryotes diversity (De Vargas et al., 2015; Guardiola et al., 2015), 16S typically prokaryotes and 12S primarily for vertebrates such as fish (Taberlet et al., 2012; Miya et al., 2015). Overall, eDNA can reveal "hidden diversity" without requiring prior knowledge of species composition in the habitat (Gleason et al., 2023).

As mentioned, the COI marker (Leray et al., 2013) is among the most frequently used DNA fragments for exploring whole-community species diversity among marine organisms (Bakker et al., 2019; Collins et al., 2019; Holman et al., 2019). The versatility of using the COI marker is vast and it has been employed in numerous recent studies, including biodiversity surveys, environmental monitoring, and dietary studies, due to its ability to amplify DNA from most mammals and insects (Clarke et al., 2017; Ruppert et al., 2019; Collins et al., 2019; Othman et al., 2021). However, eDNA metabarcoding studies focusing on whole-community marine eukaryote diversity have been limited as outlined by Bakker et al. (2019) (Lacoursière-Roussel et al., 2018; Castro-Cubillos et al., 2022; Jeunen et al., 2019), with most emphasis on the use of ribosomal markers such as 18S (Jo et al., 2019) and 12S (Miya et al., 2015; Taberlet et al., 2012; Jensen et al., 2022). With each molecular marker having its pitfalls and advantages, selecting the appropriate one is a crucial step in eDNA metabarcoding analysis of eukaryotes. Research into these markers can guide future environmental monitoring efforts, helping to determine the best choice for targeting specific taxa of interest (Wei et al., 2018; Jeunen et al., 2019).

Studies investigating the simultaneous use of additional markers alongside COI to primarily detect whole-community marine eukaryote DNA often examine nuclear ribosomal DNA (18S) in combination. Wangensteen et al. (2018) demonstrated that COI reveals greater species-level diversity compared to the V7 region of the 18S rDNA primer in hard-bottom samples dominated by macroscopic seaweeds and sessile metazoans. Günther and Knebelsberger (2015) showed that COI exhibited higher species clusters and experienced fewer sequencing failures when compared to the ribosomal 18S and 16S markers. Similarly,

Song and Liang (2023) found stronger species specificity and sensitivity in COI compared to 18S when assessing zooplankton community diversity.

On the other hand, plastid genes such as the rbcL marker provide a narrower resolution of eukaryotic diversity (Hamsher et al., 2011; Bailet et al., 2019; Kowalska et al., 2019; Turk Dermastia et al., 2023). The rbcL marker is more commonly used in diatom metabarcoding studies as it is known to better differentiate closely related species (Pérez-Burillo et al., 2022). Using eDNA metabarcoding helps address the challenges associated with traditional methods of identifying diatoms and other microalgae, which have traditionally relied on morphological approaches (Fawley and Fawley, 2020). Due to the diverse and intricate structures of these organisms, traditional identification typically requires skilled experts (Manoylov et al., 2014) and specialised instruments (e.g. scanning election microscopy) (Morales et al., 2001). eDNA metabarcoding has provided additional insights that complement traditional morphological approaches, enriching our understanding of diatom community composition and distribution (Danovaro et al., 2016). Moreover, these methods hold promise for forensic applications, offering compelling evidence to link individuals or objects to specific habitat types or sites (Kutty et al., 2022).

The rbcL marker could aid in focusing on key primary producers within the Stramenopiles, such as diatoms, brown algae, and oomycetes. These important groups of aquatic organisms and plant pathogens, most of which are photosynthetic algae, are poised to be excellent candidates for future eDNA monitoring strategies (Pawlowski et al., 2022; Pérez -Burillo et al., 2022; Mynott et al., 2023). Numerous studies have explored using diatoms for biomonitoring of rivers and streams (Smucker and Vis, 2011; Rimet and Bouchez, 2012; Costa and Schneck, 2022). Their abundance and diverse presence in various aquatic environments, coupled with their widespread distribution, make them valuable indicators. Their environmental sensitivity allows them to reflect changes in ecosystem health, and as primary producers, they provide insights into ecosystem productivity and functioning (Ruppert et al., 2019; Maria et al., 2020; Kulas, 2023). Additionally, their specific habitat associations and potential role in the early detection of harmful algal blooms further underscore their importance for monitoring environmental health (Jacobs-Palmer et al., 2021; Knudsen et al., 2022).

When multiple markers are used alongside rbcL, it is ribosomal markers, in particular 18S that are typically included for comparison. These studies typically have a particular focus in freshwater environments (Apothéloz-Perret-Gentil et al., 2021; Espinosa Prieto et al., 2023; Kezlya et al., 2023) with limited research in marine environments (Esenkulova et al., 2020; Tzafesta et al., 2022). For instance, Apothéloz-Perret-Gentil et al. (2021) compared the rbcL and the V4 region of 18S rRNA in freshwater rivers, discovering that the reference database for rbcL was more complete and confirming the effectiveness of diatom metabarcoding as a tool for assessing river ecological quality. Bailet et al. (2019) tested both rbcL and 18S-V4, finding that the rbcL marker consistently produced molecular datasets of taxa that closely matched those obtained using traditional microscopy. Kelly et al. (2020) used the rbcL marker alone and confirmed its usefulness for environmental monitoring.

Research has also explored the efficacy of employing more than two markers, adopting a multi-marker approach. Guo et al. (2015) compared four different markers: 18S rRNA and ITS, COI and rbcL. In their evaluation, they found that the 18S rRNA gene and rbcL were effective in clustering lower taxa, while the COI marker successfully barcoded species of only certain genera within the Bacillariophyceae, particularly pennates. This led to the conclusion that the COI region is not suitable for clustering analysis of the entire diatom phylum. However, its strengths lie in its potential usefulness for barcoding species of specific genera within the Bacillariophyceae. Exploring a multi-marker approach offers significant benefits for broadly identifying marine eukaryotic communities (Eble et al., 2020; Seymour et al., 2020; Portas et al., 2022; MacNeil et al., 2022), as it can help mitigate issues such as limitations in reference databases and biases toward certain taxonomic groups (Porter and Hajibabaei, 2018; Ficetola and Taberlet, 2023), which can skew species identification toward better-known taxa (Bakker et al., 2019).

Considering this, our study employs both COI (Leray et al., 2013) and rbcL (Bruder and Medlin, 2007; Stoof-Leichsenring et al., 2012) markers simultaneously. While the rbcL marker may fill in gaps associated with COI, which might not achieve clear species-level identification for certain taxa, COI, on the other hand, could mitigate the bias of rbcL towards certain taxa, allowing for both finer resolution and broader taxonomic coverage (Perez-Burillo et al., 2022). Using both markers together could offer comprehensive insights into ecosystem health, leveraging the strengths of each to improve environmental monitoring. The aim of this study is to investigate the merits and limitations of each molecular marker, both

individually and in combination, focusing on taxonomic resolution, ecological inferences, and the detection of potentially harmful species. Additionally, community dynamics and species richness will be explored across the six heavily urbanised coastal sampling locations all within <20km of each other and assess the ecological applications of this approach. By using two distinct barcoding regions, this study will seek to understand the eukaryotic community structure in a highly heterogeneous coastal zone shaped by centuries of urbanisation.

3.3 Material and Methods

3.3.1 Field collection

Sampling occurred in May 2021 at six coastal locations in Merseyside, in the northwest of England (United Kingdom). Merseyside comprises the final stretch of the river Mersey estuary, in Liverpool, and the Eastern bank of the River Dee, on the Wirral peninsula (Figure 3.1). The sites in the Mersey included: 1) Speke and Garston coastal reserve, at the dynamic boundary between freshwater and marine influence; 2) Albert Dock, at the heart of the city of Liverpool; 3) New Brighton tidal beach, situated right at the mouth of the river Mersey 4) Crosby marine lake. The sites in the Dee were: 5) Thurstaston and 6) West Kirby marine lake.

Three sites are classified as enclosed habitats because they are located within industrial urban structures, meaning they are not as exposed to tidal changes as more open coastal locations. These sites are 2) Albert Dock, established in 1846 and not only the historic centre of Liverpool's maritime industries but also a prominent tourist destination with high levels of human activity. The docks are connected to the Mersey estuary via a sluice gate which only opens when needed, for example when boats are moving into or out of the area. 4) Crosby marine lake lacks a sluice gate and is more isolated from the sea. Only exceptionally high tides mix with the lake. 6) West Kirby marine lake, built in 1899, is known for recreational activities such as sailing and wind-surfing. It features a sluice gate regulating tidal water flow, leading to frequent hydrological changes (see Chapter 2, section 2.3.1 for further details).

The remaining three sites are considered more open coastal habitats, influenced by tidal changes rather than constrained within an urban infrastructure dependent on sluice gates. 1)

Speke and Garston coastal reserve, more inland within the River Mersey estuary, experiencing human activity from nearby facilities like business parks and an airport. 3) New Brighton beach also in the Mersey estuary, sits near busy shipping routes (see Chapter 4) and 5) Thurstaston Beach is situated near the mouth of the Dee estuary and is known for recreational activities.

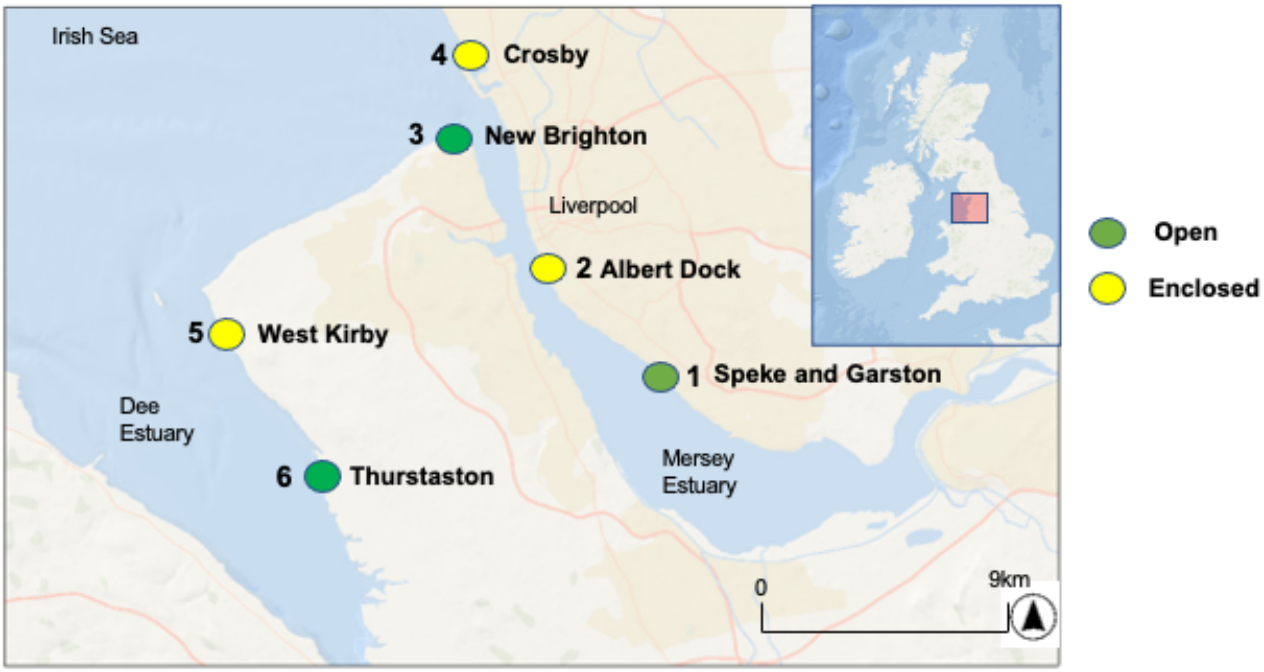

Figure 3.1 Map showing six sampling locations within the Merseyside.
This figure shows 1) Speke and Garston coastal reserve: 2) Albert Dock 3) New Brighton; 4) Crosby marine lake, all situated along or in the outflow of the Mersey Estuary. The locations in the Dee were: 5) Thurstaston and 6) West Kirby marine lake. The legend green "open" and yellow "enclosed" signifies the habitat type.

Three litres of water were collected at each site, with one litre pushed through one 0.22 µm filter using an EZ-Stream filter pump, resulting in three biological replicates from each location. Sampling was completed on the same day and within four hours either side of high tide. Prior to sampling, all field equipment was sterilised with 10% bleach followed by 70% ethanol. Sterile gloves were worn and regularly changed after filtering each sample. Bottles of water collected were stored on ice for approximately 2-8 hours before filtering in the laboratory. To monitor contamination at each site, a blank was taken in which purified water was pushed through the EZ-Stream filter, being treated identically to other samples throughout the collection and extraction process. Each filter was placed inside two sterile bags, and immediately stored on ice. All samples were stored at -20°C in the lab until further processing.

3.3.2 DNA extraction

DNA extraction from the filters was based on the water mu-DNA protocol by Sellers et al. (2018). Please see section 2.3.2 in Chapter 2 for further details.

3.3.3 PCR Amplification

3.3.3.1 COI

PCR amplification was performed in triplicate for each of the eighteen samples, using the COI Leray primer, which targets a ~313 bp fragment of the COI rRNA mitochondrial region (COI, mICOIintF: GGWACWGGWTGAACWGTWTAYCCYCC; Leray et al., 2013, matched to jgHCO2198: TAIACYTCIGGRTGICCRAARAAYCA; Geller et al., 2013), Positive controls were put in place for each PCR batch. Extracts of iridescent shark (*Pangasianodon hypophthalmus*) DNA at 0.05 ng/µl were used, choosing this organism as it is a tropical freshwater fish, unrelated to fish species in UK waters. Primer pairs were uniquely indexed to enable demultiplexing for downstream bioinformatic analysis. For each DNA sample, PCR amplification was performed in triplicate. The reaction mix included:

10µL Mifi DNA polymerase mastermix (2x), molecular grade water 5.84µL, BSA 0.16µL, 1µL of each primer (10 µM) and 2 µL of DNA with a thermocycling profile denaturation step at 95 °C for 15 min followed by a total of 30 cycles of 95 °c for 45 s (denaturation), 55 °C for 45 s (annealing), and 72 °C for 45 s (final extension) (Leray et al., 2013).

3.3.3.2 rbcL

The same eighteen DNA extracts were used to perform PCR amplification, also in triplicate, targeting a 312bp fragment of the rbcL plastid the primer pair Diat_rbcL_708F (Stoof-leichsenring et al., 2012)- AGGTGAAGTAAAAGGTTCWTACTTAAA- and R3 (Bruder and Medlin 2007) - CCTTCTAATTTACCWACWACTG- using uniquely indexed primers. The reaction mix included: 10µL Mifi DNA polymerase mastermix (2x), molecular grade water 5.84µL, BSA 0.16µL, 1µL of each primer (10 µM) and 2 µL of DNA with a thermocycling profile of 95 °C for 15 min followed by a total of 30 cycles of 95 °c for 45 s (denaturation), 55 °C for 45 s (annealing), and 72 °C for 45 s (final extension) following the PCR conditions from Vasselon et al. (2017).

3.3.4 Library preparation

PCR products were run on 2% agarose gels stained with SYBR safe. PCR replicates were pooled and samples were purified with a 1:1 30µL Mag-Bind® TotalPure NGS magnetic beads and 30µL of pooled PCR product. The concentration of each purified PCR was quantified using Qubit 4 Fluorometer dsDNA HS assay kits and pooled in equimolar amounts. The pooled PCR product was quantified using Tapestation 4200 using the high sensitivity D1000 assay. A further 1x bead clean-up was performed on the pooled PCR product. Illumina libraries were prepared using the Perkin-Elmer NEXTFLEX® Rapid DNA-Seq Kit 2.0, using 1 µg as starting concentration of the pooled PCR product following the manufacturers guidelines with library amplification. The library and PhiX control were quantified using qPCR using the NEBNext® Library Quant Kit for Illumina®. The final library and PhiX control were diluted to 85pM (with PhiX 20% of the run) and loaded onto an Illumina® MiSeq™ 100 Reagent v2 (2 x 300 cycles).

3.3.5 Bioinformatics and downstream analysis

The bioinformatic process was based on the OBITOOLS pipeline (Boyer et al., 2016). FASTQC was used to assess the quality scores of the fastq files. ILLUMINAPAIREDEND was then used to merge the paired-end reads and remove alignments with low (<40) quality scores. NGSFILTER

was used to de-multiplex samples. To remove sequences that were not in the target base pair range, the sequence lengths were filtered to 313bp by using OBIGREP. The samples were dereplicated using OBIUNIQ. Chimeras were then removed using the *uchime-denovo* chimera search function in VSEARCH (Rognes et al., 2016). Molecular operational taxonomic unit (MOTU) clustering was implemented using SWARM with "-d 13" (Mahé et al., 2014). Taxonomic assignment per sample was carried out using ECOTAG which was constructed using 'ecoPCR' in silico against the EMBL database (Release version r143 October 2023). The taxonomic assignments included seven levels of biological classification along with a percentage indicating the likelihood of accurate classification.

All downstream analyses were conducted using R version 4.2.2. The package DECONTAM in R was used on the raw MOTU output. MOTUs were filtered by retaining assignments with >95% identity match and removing MOTUs that did not reach this criterion. MOTUs that were non-native and unexpected taxa were cross-checked by manual BLAST against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide). Prior to downstream analysis, data was transformed using Hellinger, which converts species abundances from absolute to relative values followed by square-rooting for standardisation.

The transformed reads were visualised in a heatmap using RESHAPE2. Another heatmap was generated using binary presence/absence data also using RESHAPE2. The *facetwrap* function was used to categorise data by sampling locations and molecular markers.

Species richness and Shannon diversity were calculated using the R package PHYLOSEQ which performs standard alpha diversity estimates (McMurdie and Holmes, 2013). To statistically test for alpha diversity differences, Analysis of Variance (ANOVA) was used to compare species richness for each sampling location and habitat type (open and enclosed) for each independent molecular marker (COI and rbcL). Species richness was compared across sampling locations and habitats using both molecular markers together, again employing ANOVA. Finally, species richness was tested between the molecular markers regardless of location. The species richness, split by marker (COI vs. rbcL), sampling locations, and habitat, was visualised using boxplots in the R package GGPLOT2.

To highlight indicator species, the indicator value species analysis and multilevel pattern analysis were conducted using the *multipatt* function with 999 permutations for the six

sampling locations and molecular markers. The results were visualised with violin plots using the GGPLOT2 and INDISPECIES R packages (De Cáceres and Legendre 2009).

To visualise spatial (among the six locations) and marker-based (COI vs rbcL) differences, nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distance was conducted. Differences between locations and seasons using permutational multivariate analysis of variance (PERMANOVA, 999 permutations) were tested on pairwise distance matrices using the function *adonis* in VEGAN (Oksanen et al., 2013).

3.4 Results

Samples were sequenced across one Illumina Miseq run alongside an unrelated eDNA project, the samples from this study made up 20.7% of the overall sequencing run. There were forty-four samples altogether: 36 eDNA filters, 2 field blanks, 2 extraction blanks and 2 PCR blanks, 2 positive controls. Before the filtering there were 498,515 reads over 18 samples for COI and 565,286 reads over 18 samples for rbcL. After the first filtering step (>95% identity match), the 18 samples amplified by the COI gene, these yielded 201,333 number of reads. For the 18 samples amplified by the rbcL gene, these yielded 487,604 number of reads. No contamination was found in the negative controls after filtering <10 reads, meaning no taxa had to be removed for downstream analysis. One sample, amplified by the COI gene, from the Albert Dock yielded no sequencing reads and therefore was removed from downstream analysis.

In the eighteen eDNA filters amplified by rbcL, a total of nine different Classes were detected. *Coscinodiscophyceae* (centric diatoms) had the highest relative proportion for four of the locations: Speke and Garston, Crosby, West Kirby and Albert Docks. *Bacillariophyceae* had the highest proportion in New Brighton and Thurstaston (both based proximity to the Dee estuary) (Figure 3.2). There were five different kingdoms found in COI (Animalia, Bacteria, Chromista, Fungi and Plantae), whereas only one was found using rbcL: Chromista. Six different classes were shared between the two molecular markers highlighted in figure 2 B (*Phaeophyceae, Fragolariophyceae, Eustigmatophyceae, Coscinodiscophyceae, Chrysophyceae* and *Bacillariophycaeae*). There were seven distinct classes found within Chromista detected by COI marker only and three classes (*Xanthophyceae, Raphidophyceae* and *Bolidophyceae*) by the rbcL marker only.

The highest proportion of classes found with the rbcL gene were *Coscinodiscophycae* and *Bacillariophceae*, where *Coscinodiscophycae* had a higher proportion in five of the six locations, with least proportion in New Brighton. By contrast, the COI marker in that class, Speke and Garston and New Brighton contained the higher proportions. The highest proportion of *Bacillariophyceae* detected by the rbcL marker was in New Brighton and Thursdaston, with Albert Dock containing no detections. A similar pattern occurs in the COI marker where Speke and Garston, New Brighton and Crosby contain the higher proportions whereas Albert Dock, West Kirby and Thurstaston contain low to no proportionate reads. In COI marker, *Prymnesiophyceae* had the highest proportion within Chromista which is located in West Kirby. This is not detected by the rbcL marker (Figure 3.2).

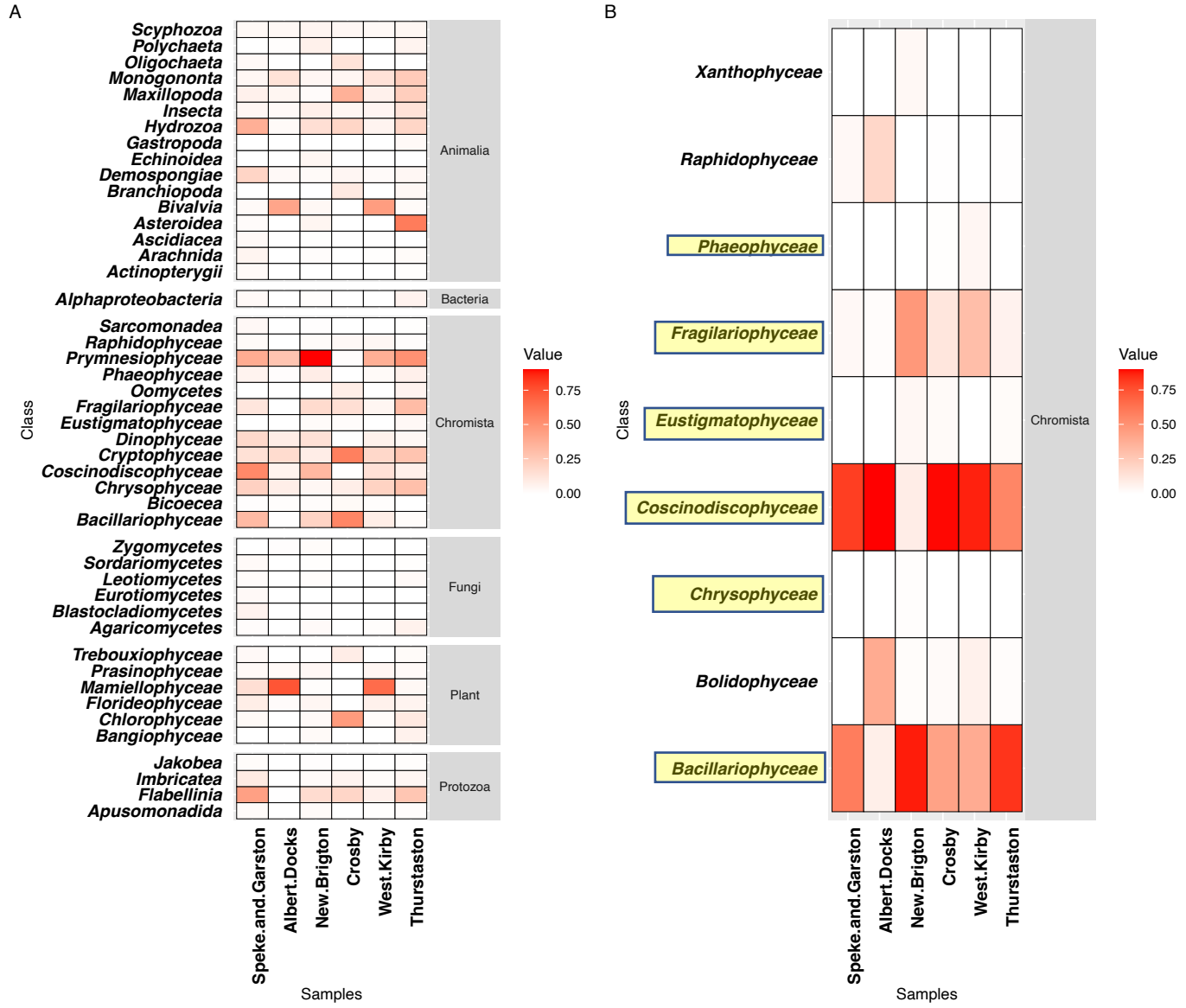

Figure 3.2 Heatmap of classes (A) COI marker (B) rbcL marker based on Hellinger transformed data.

Heatmaps depicting species relative proportion based on hellinger transformed data among the six sampling locations and (A) COI marker and (B) rbcL marker. Colours on the heatmap range from white, indicating low abundance, to red, signifying high abundance. The highlighted groups in the (B) panel indicate the classes shared between both COI and rbcL marker.

The COI marker showed the highest richness in New Brighton (open) and the lowest in West Kirby (enclosed), whereas for the rbcL marker, Thurstaston (open) exhibited the highest richness, and Albert Dock (enclosed) had the lowest (see Figure 3.3).

Regarding species richness detected solely by the COI marker across the six sampling locations (ANOVA F= 2.467; P< 0.0985) or by habitat (ANOVA F= 0.094; P< 0.764), neither was found to be significant. Despite this lack of significance, the open sites showed higher richness in the COI marker, with mean values of 18, 25, and 20, while the enclosed sites exhibited lower diversity, with mean values of 13, 14, and 7.

For the analysis of species richness solely using the rbcL marker, significance was found for the six sampling locations (ANOVA F= 3.82; P< 0.008), but not for habitat (ANOVA F= 0.815; P< 0.38).

When examining species richness across sampling locations using both molecular markers simultaneously, significance was observed for the sampling locations (ANOVA F= 5.863; P< 0.000734) and habitats (ANOVA F= 7.887; P< 0.000473). Additionally, the comparison of species richness between the two molecular markers (COI and rbcL) yielded significant results (ANOVA F= 7.887; P< 0.000473).

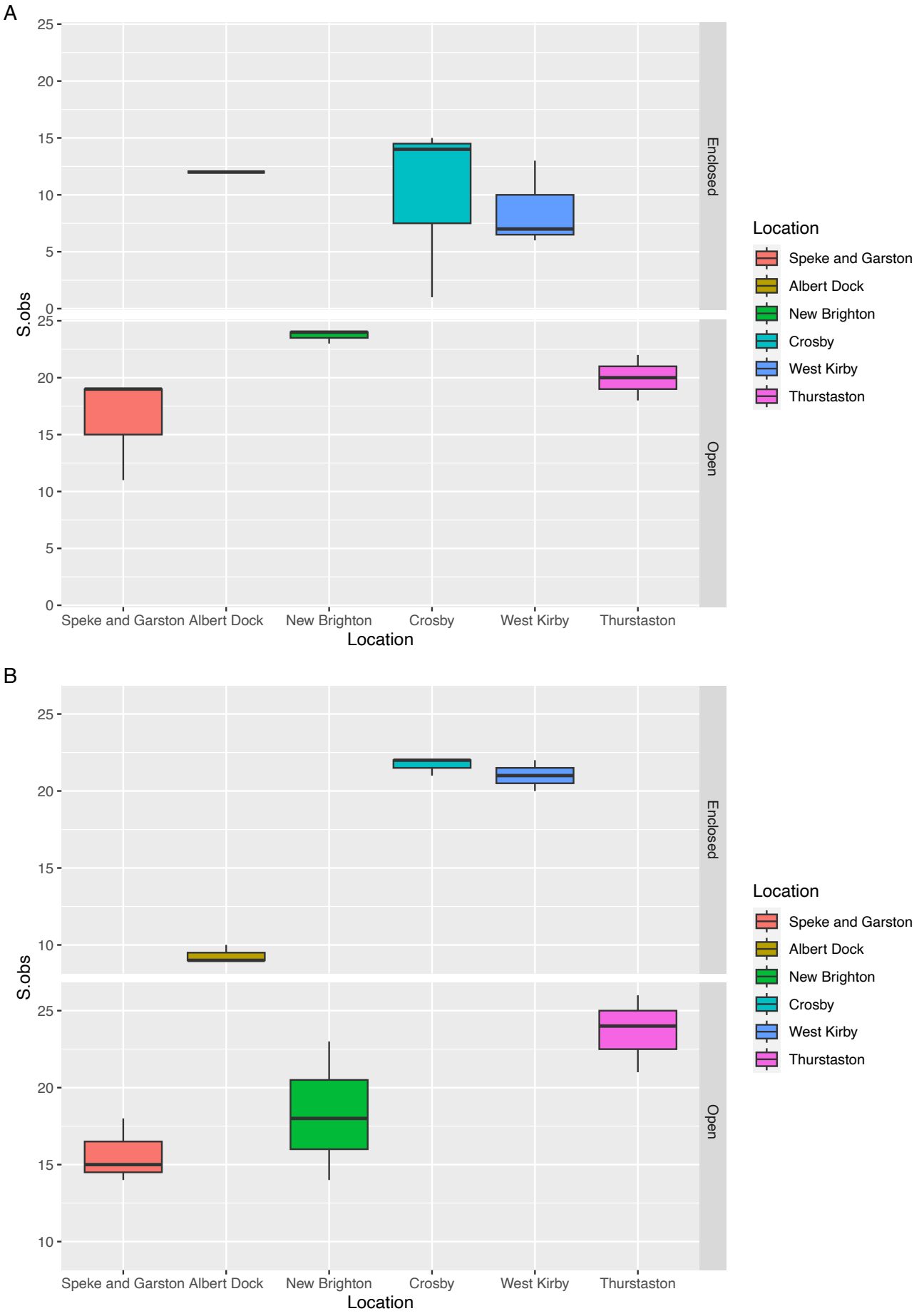
A
B
S.obs
Location
Enclosed
Open
Speke and Garston
Albert Dock
New Brighton
Crosby
West Kirby
Thurstaston

Figure 3.3 Box-whisker plots of species between (A) COI marker and (B) rbcL richness across the sampling location.

Box-whisker plots showing species richness among sampling locations categorised by "open" and "enclosed" habitats and (A) COI marker and (B) rbcL.

To delve deeper into the species-level factors contributing to variations among sampling locations, an indicator species analysis was carried out across the six sampling locations (Figure 3.4). Forty species were sampling site indicators. See Supplementary Table S3.1 and S3.2 for the statistics for each of these indicator species. Figure 3.4 (D) Crosby marine lake has the lowest number of significant species, with *Ankylochrysis lutea* a haptophyte alga, a primary producer which is detected by the rbcL molecular marker only. Whereas (C) New Brighton contains the highest number of indicator species (16) of which consist of four different groups (diatoms, fishes, marine invertebrates and seaweed). Among all six sampling locations, a total of twenty were detected by COI marker, and twenty were detected by rbcL.

Across all six sampling locations, four significant indicator species of diatoms—*Thalassiosira pseudonana, Rhizosolenia setigera, Cyclostephanos sp. WTC16*, and *Cyclostephanos dubius*—were identified using the COI marker. These species were found exclusively at the Speke and Garston sites, comprising four out of the five significant diatom species detected there. The fifth significant species, identified with the rbcL marker, was *Skeletonema potamos.* A remaining seventeen other diatom species were found to be significant among all six locations, which were all detected by the rbcL marker. The category of potential "harmful algae" is explored further in the discussion.

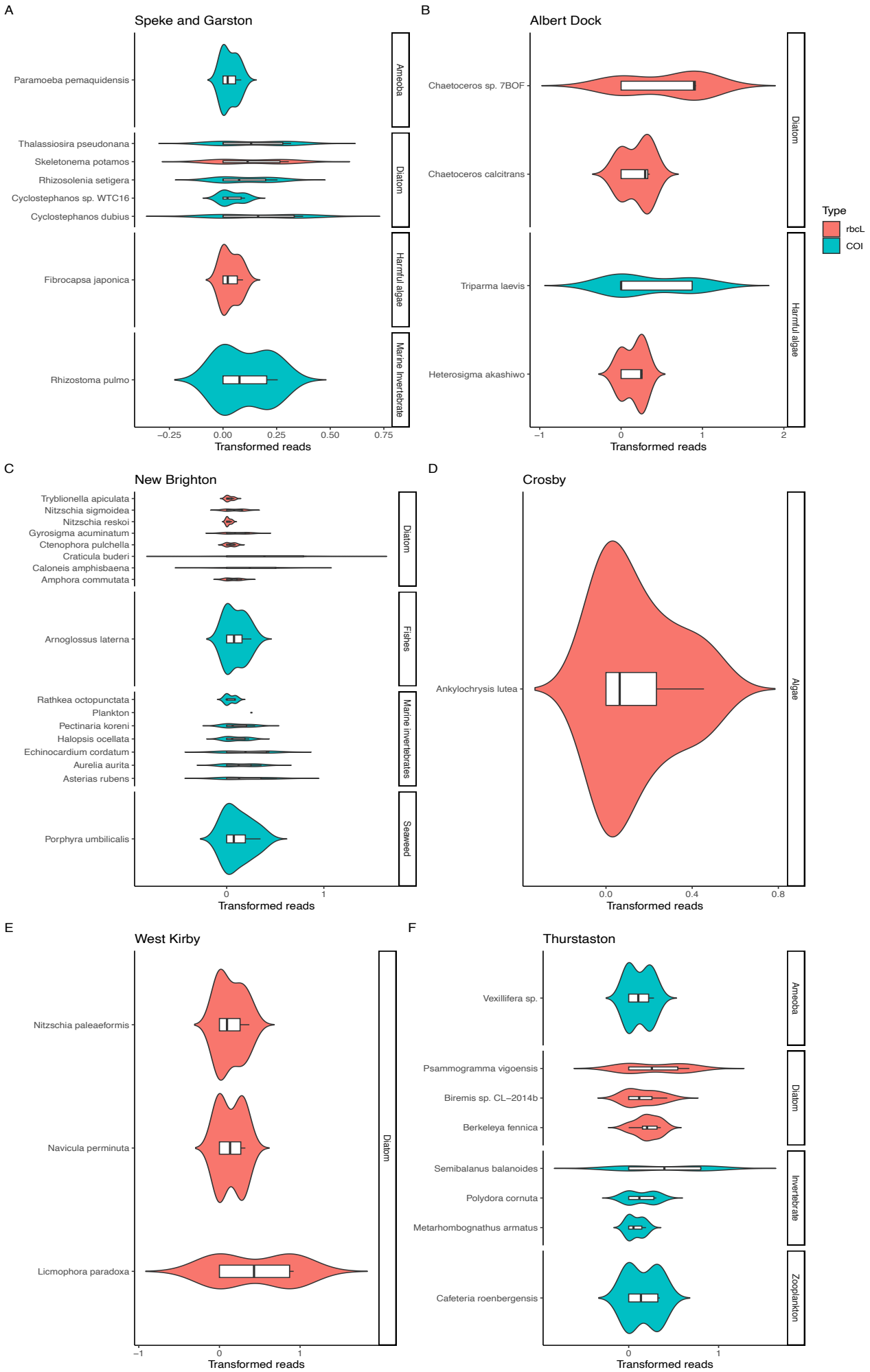
A
Speke and Garston
Paramoeba pemaquidensis
Thalassiosira pseudonana
Skeletonema potamos
Rhizosolenia setigera
Cyclostephanos sp. WTC16
Cyclostephanos dubius
Fibrocapsa japonica
Rhizostoma pulmo
Amoeba
Diatom
Harmful algae
Marine Invertebrate
−0.25
0.00
0.25
0.50
0.75
Transformed reads
B
Albert Dock
Chaetoceros sp. 7BOF
Chaetoceros calcitrans
Triparma laevis
Heterosigma akashiwo
Diatom
Harmful algae
−1
0
1
2
Transformed reads
Type
rbcL
COI
C
New Brighton
Tryblionella apiculata
Nitzschia sigmoidea
Nitzschia reskoi
Gyrosigma acuminatum
Ctenophora pulchella
Craticula buderi
Caloneis amphisbaena
Amphora commutata
Arnoglossus laterna
Rathkea octopunctata
Plankton
Pectinaria koreni
Halopsis ocellata
Echinocardium cordatum
Aurelia aurita
Asterias rubens
Porphyra umbilicalis
Diatom
Fishes
Marine invertebrates
Seaweed
0
1
Transformed reads
D
Crosby
Ankylochrysis lutea
Algae
0.0
0.4
0.8
Transformed reads
E
West Kirby
Nitzschia paleaeformis
Navicula perminuta
Licmophora paradoxa
Diatom
−1
0
1
Transformed reads
F
Thurstaston
Vexillifera sp.
Psammogramma vigoensis
Biremis sp. CL−2014b
Berkeleya fennica
Semibalanus balanoides
Polydora cornuta
Metarhombognathus armatus
Cafeteria roenbergensis
Amoeba
Diatom
Invertebrate
Zooplankton
0
1
Transformed reads

Figure 3.4: Violin plots of indicator species from each sampling location.
Diagram shows violin plots of Hellinger transformed data. Highlighting the identified indicator species splits among the different sampling locations (A) Speke and Garston, (B) Albert Dock, (C) New Brighton, (D) Crosby, (E) West Kirby and (F) Thurstaston. Colours depict the molecular markers.

To further investigate the species-level distinctions between the markers, it is important to now turn the attention to an important subgroup of Stramenopiles, which also harbours several significant species including diatoms. A total of sixty-five different species of Stramenopiles were detected using both COI and rbcL markers: 13 species (20%) were detected only by COI, 51 species (78%) only by rbcL, and 1 species (2%) was shared between the markers. Overall, Crosby had the highest number of species (37), while samples from the Albert Docks had the lowest number of species (15) (Figure 5). Among the Stramenopiles, two harmful algae species, *Heterosigma akashiw*o and *Fibrocapsa japonica*, were detected only with the rbcL marker. Additionally, 45 different species of diatoms were identified using both markers, including the invasive *Didymosphenia geminata* and the potentially harmful *Pseudo-nitzschia sp.* (Figure 3.5).

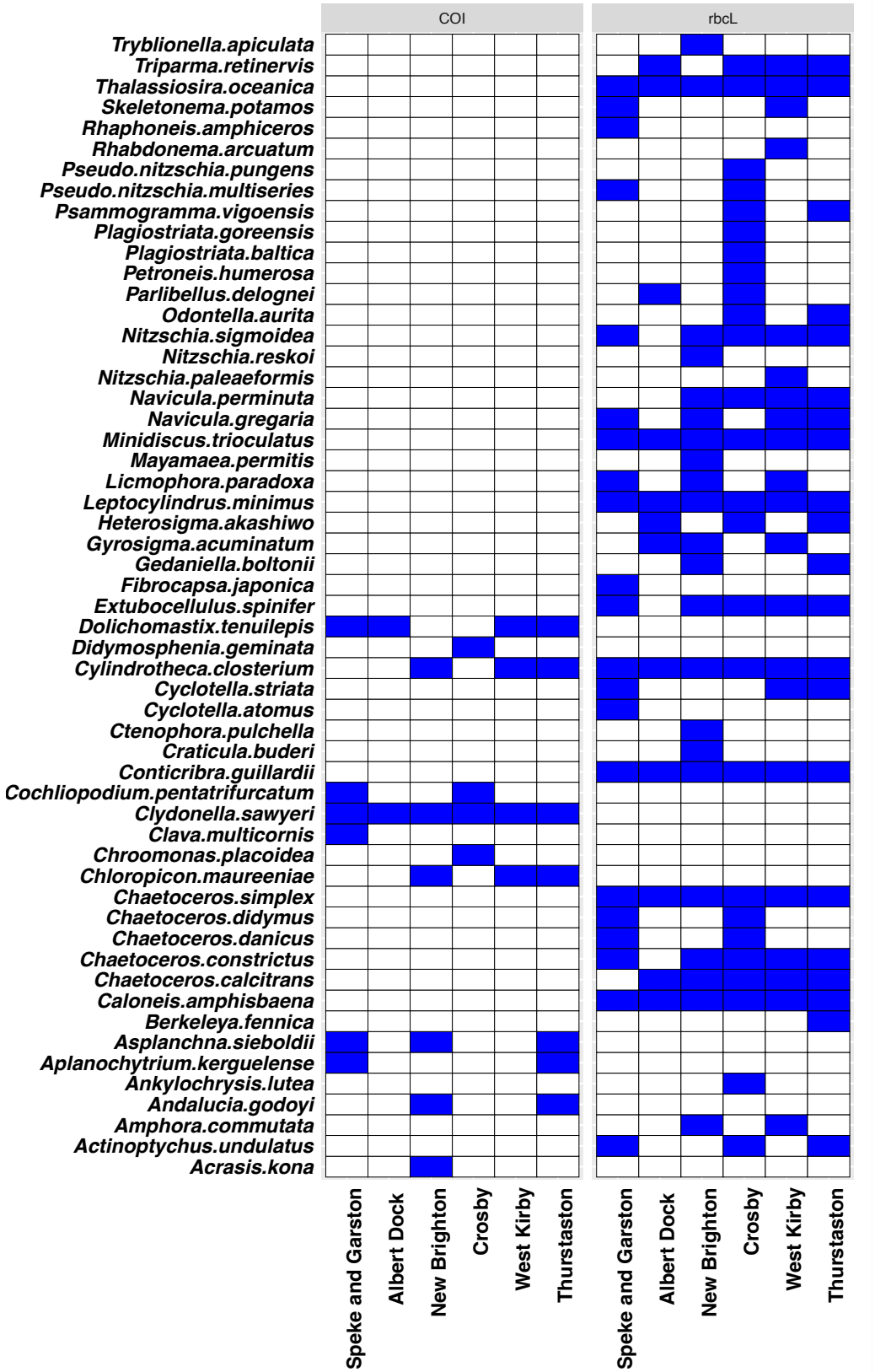

Figure 3.5 Presence absence binary heatmap of Stramenopiles.

Figure displays a heatmap showing presence (blue) and absence (white) of species within the Stramenopiles.

When broader eukaryotic community structure was contrasted across the six different locations, there was a significant difference found (PERMANOVA pseudo-F=2.9932 P= 0.001). Differences in eukaryotic community structure were also significant between the two different barcodes (PERMANOVA pseudo-F=7.3091 P= 0.001). The interaction between location and molecular marker was also significant (PERMANOVA pseudo-F=8.3333 P= 0.001). (Figure 3.6).

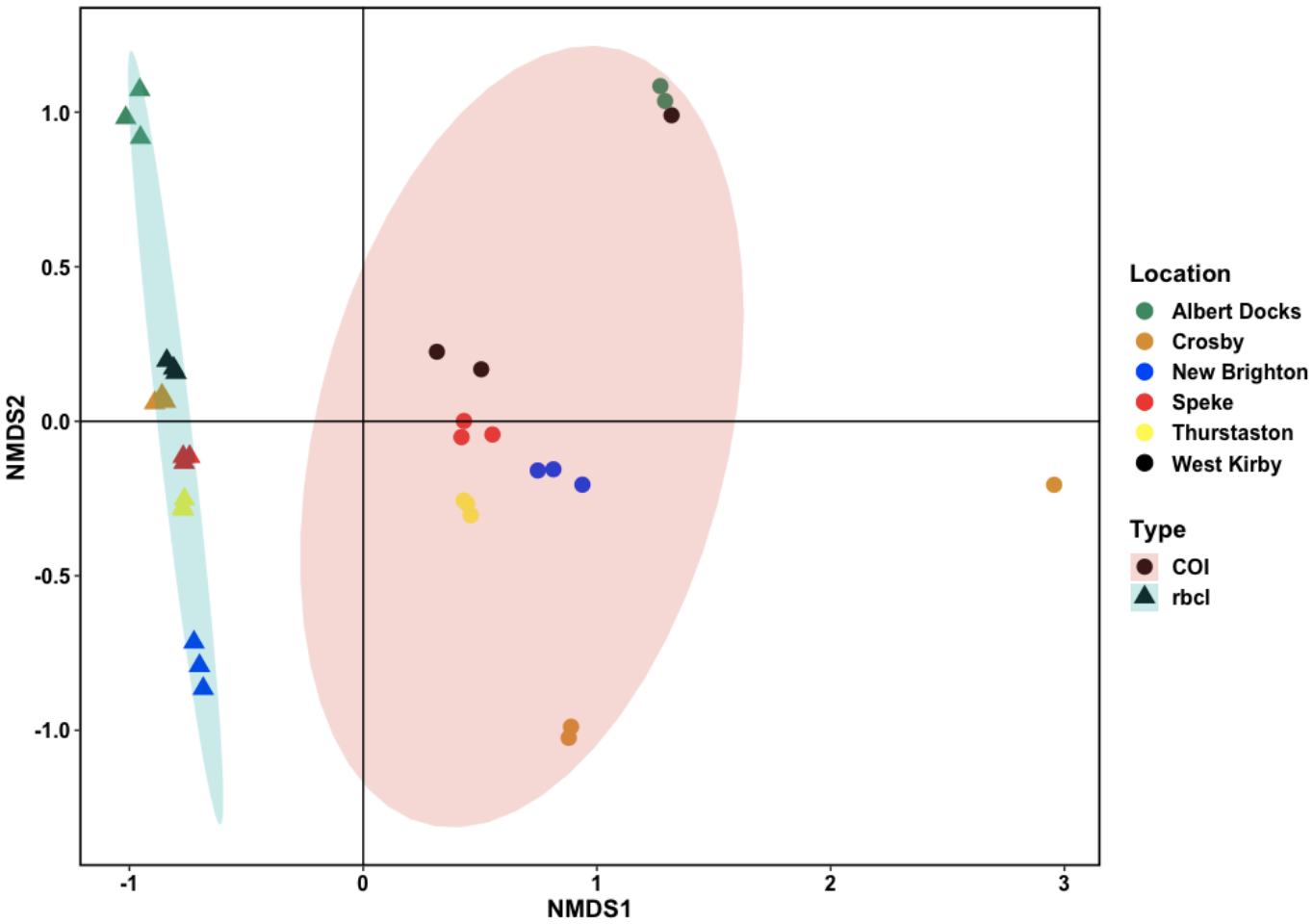

Figure 3.6: Non-metric multi-dimensional scaling showing whole eukaryotic community structure.

Figure shows a non-metric multi-dimensional scaling (NMDS) showing the whole eukaryotic community structure for all samples using Bray-Curtis dissimilarity on Hellinger transformed data. This is for all six locations (depicted by colour) and the two different molecular markers (depicted by shape). The ellipses indicate a 95% confidence level.

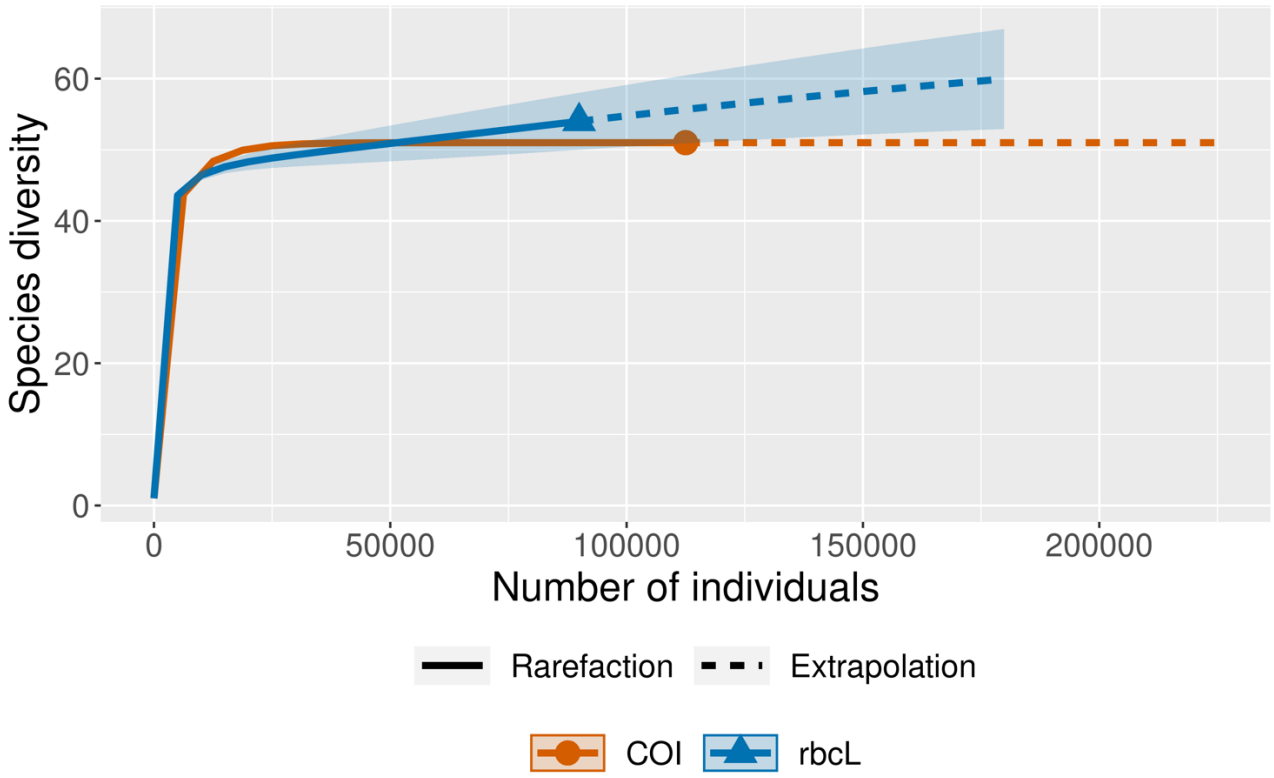

Figure 3.7: Rarefaction curve between the two molecular markers: COI and rbcL.

The dashed line represents the extrapolated estimate calculated with R package iNEXT (Hsieh et al., 2016) and the shaded section shows the 95% confidence interval for the estimation.

3.5 Discussion

This study explores using two molecular markers—the cytochrome c oxidase subunit I gene (COI) and the ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit gene (rbcL)—for characterising eukaryotes in urban coastal environments. Our aim was to assess and monitor eukaryotic communities across different sampling sites within a heavily urbanised coastal area. When examining different taxa, COI unsurprisingly proved better at detecting a wide range of eukaryotes, spanning from marine invertebrates such as jellyfish to diatoms. In contrast, the rbcL marker, while targeting the extensive kingdom Chromista, had a narrower detection scope, identifying mostly Stramenopiles. This finding, though anticipated, reveals the importance of exploring each marker's merits to provide valuable information for environmental managers. COI and rbcL markers were selected to fill research gaps and evaluate whether using these markers together, rather than the commonly used ribosomal markers, could enhance the detection range of whole community eukaryotes and offer finer

resolution for key bioindicator species. While extensive research has been conducted in freshwater environments, limited knowledge exists regarding the use of these markers for eDNA metabarcoding in coastal habitats, especially in heavily human-impacted areas (Ruppert et al., 2019). Although the 18S marker is valuable, its resolution is generally lower compared to COI and rbcL (Hamsher et al., 2011; Dermastia et al., 2023), highlighting how the choice of marker is crucial. In this study, 48 distinct classes and 167 distinct species were revealed across both COI and rbcL markers through eDNA metabarcoding, showcasing the wide biodiversity within six localised coastal urban sites across Merseyside.

3.5.1 Taxonomic resolution of molecular markers: opportunities for environmental managers

The rbcL marker chosen is primarily used for diatom studies, mainly in freshwater environments, and its use for eDNA metabarcoding in coastal environments is not yet fully established (Kezlya et al., 2023). Seventeen of the significant species identified through indicator species analysis using rbcL were diatoms, demonstrating its specificity for diatoms (Figure 3.4). Some of these species are potentially harmful, such as *Chaetoceros calcitrans* found in Albert Dock, which can irritate the gills of fish when cells are in high abundance (Klein et al., 2009). The enclosed habitat of this site coupled with the high human activity means that this detection could provide useful information for environmental managers.

Other indicator species can also serve as species of environmental importance, such as *Nitzschia reskoi* detected in New Brighton. Földi et al (2018) have previously shown that the increasing abundance of *Nitzschia reskoi* was a signal of the decreasing soluble salts in a saline wetland. *Skeletonema potamos,* also found in New Brighton, typically inhabits low salinity areas, therefore indicating the habitat type (Duleba et al., 2014; Heudre et al., 2021). To date, research involving eDNA metabarcoding to detect diatoms in coastal environments has been limited (Gallego et al., 2020; Perez-Burillo et al., 2022), but our study demonstrates the potential advantages of employing the rbcL marker in this regard. Further research expanding on this will be valuable for environmental managers seeking to identify bioindicators for specific locations. I encourage future research to further explore this opportunity.

The rbcL marker is anticipated to offer enhanced resolution for bioindicators, especially in the detection of potentially harmful algae (Bailet et al., 2019; Apothéloz-Perret-Gentil et al., 2021; Kulas, 2023). At the species level within Stramenopiles, several microalgae considered potentially harmful to humans and wildlife were exclusively identified using the rbcL marker. *Heterosigma akashiwo* was detected in Thurstaston beach and Crosby marine lake, with it being an indicator species in the Albert Dock (strongly associated). *Heterosigma akashiwo* is known for inducing harmful algal blooms in coastal and estuarine environments (Zhang et al., 2006; Martínez et al., 2010), which can lead to fish mortality (Allaf, 2023). In the Albert Dock, harmful algal species were detected. Given the quantity of anthropogenic activity that takes place in this habitat, the detection of these harmful species holds significance for environmental managers. It not only has the potential to affect humans from a public health perspective, but also raises concerns as potential transportation or introduction of harmful species to or from other locations can be facilitated by shipping docks and ballast water exchange (Butrón and Orive, 2011; Hamer et al., 2011; Shaw et al., 2019). Leveraging eDNA metabarcoding as a simple sampling method for monitoring urban shipping docks could offer significant advantages for environmental monitoring. Furthermore, eDNA metabarcoding has the potential to aid molecular mapping, thereby supporting global initiatives aimed at controlling the spread of harmful algae worldwide; this information can inform policies and guide environmental agencies in their efforts (Shaw et al., 2019).

The potentially harmful species *Fibrocapsa japonica,* often associated with fish mortality, was detected in the Speke and Garston coastal reserve (Pezzolesi et al., 2010). This reserve is located along the same Mersey estuary as the Albert Dock (within approximately 9km). Both *Fibrocapsa japonica* and *H. akashiwo* are classified as toxic and harmful by the Environment Agency (Environment Agency, 2009), underscoring the significance of effective detection methods for potentially hazardous algae. This is particularly crucial given the need to prevent their impact on human and wildlife communities. *Leptocylindrus minimus*, reported to have harmful effects on aquaculture and natural fisheries (Buschmann et al., 2006), was also found in all six sampling sites exclusively through the rbcL marker. The presence of these species was exclusively revealed by the rbcL marker, suggesting its resolution in detecting harmful algal species. These results demonstrate that without the use of the rbcL marker, certain algal species would have been overlooked, highlighting its importance for environmental monitoring. The identification of these species in urban environments reveals the significant

association between harmful algae and urban settings, indicating their widespread distribution across diverse environments.

Although the rbcL marker seems to provide finer resolution for harmful algae, our findings show that, despite the broader taxonomic coverage of the COI marker, COI identified four Chromista classes and three significant diatom species in Speke and Garston that rbcL did not detect (Figure 3.2). This included *Skeletonema potamos*, typically found in warm, photophilic environments with broad salinity tolerance, indicating the habitat of the location (Duleba et al., 2014). This suggests that even though rbcL has a narrower taxonomic range, it may overlook certain ecologically significant species. Additionally, the invasive benthic diatom *Didymosphenia geminata* (Lyngbye), known for forming dense macroscopic growths (West et al., 2020), with potentially severe impacts on food webs (Clancy et al., 2021), was exclusively identified in Crosby marine lake using the COI marker. When considering indicator species, the COI marker helped identify sites with higher diversity of other eukaryotes, such as New Brighton (fishes, marine invertebrates, and seaweed) and Thurstaston, which correlates with both sites being categorised as open coastal systems located toward or on the mouth of the estuary of the Mersey and Dee, known to have higher biodiversity (Vasconcelos et al., 2015).This is supported by the COI marker detecting higher species richness in New Brighton than rbcL.

The use of both molecular markers detected distinctive communities within each of the six studied locations (Figure 3.6). This observation shows the variation found present in the eukaryotic assemblages across these locations, revealing significant spatial dynamics. The rarefaction curve (Figure 3.7) suggests that the sampling effort for the COI marker has sufficiently captured the overall diversity within the environment. However, the rbcL marker shows signs of slight under-sampling (albeit it is still close to reaching the asymptote), indicating that additional sampling effort might uncover more species. These differences are notable given that the sampling effort was the same for both markers, indicating that the variation could be marker specific. The rbcL marker may require more effort than COI, which has been shown to more accurately represent relative diversity compared to 18S (Casey et al., 2021). Despite this, the combined use of both markers has successfully captured a comprehensive representation of species diversity. This suggests that while the dual-marker approach is successful, further sampling with the rbcL marker may be necessary for a more complete biodiversity assessment. Further refinement and optimisation might also be required

to account for the specific nuances of different markers to achieve optimal results (Furlan et al., 2016). Furthermore, a significant distinction emerged when comparing the community assemblages driven by the two primer regions. This divergence highlights the unique perspectives offered by each primer, showcasing how specific markers can uncover distinct differences in the eukaryotic communities present. While there are differences in the communities associated with the two barcodes, distinct community clusters for each location are evident in both barcodes. The COI marker offers a valuable whole-community perspective, detecting a swath of organisms such as jellyfish, fish, barnacles, and many others. This can be crucial for assessing community dynamics, helping to identify changes in community structure influenced by biodiversity shifts. However, it is important to note that false negatives are likely, and using multiple markers is known to improve detection probability, enhancing the assessment of eukaryotic diversity from eDNA samples (Günther et al., 2018; Stat et al., 2017; Ruppert et al., 2019; Beng and Corlett, 2020; Seymour et al., 2020). A deeper exploration into the eukaryotic species unveiled by COI is warranted and leveraging these markers in combination suggests an advantage. The COI marker offers a more comprehensive comprehension of the entire community, which can be highly valuable for detecting shifts in community structure.

3.5.2 Geographical findings, considerations, and implications

The community distinctiveness of assemblages at each location, despite the interconnected waterways, found with both markers can give us important information. These findings are intriguing because connected tidal environments could potentially facilitate the transport of eDNA over considerable distances (Hinz et al., 2022), which might have resulted in some degree of overlap of communities between the sites. Our study demonstrates the effectiveness of eDNA metabarcoding in discerning localised communities across six coastal habitats within a relatively small spatial scale (<20 km). This suggests limited dispersal of eDNA among these habitats, which has also been observed on an even smaller spatial scale (<5 km) using three barcoding markers (Jeunen et al., 2019). This is particularly interesting when considering the proximity from Speke and Garston to the Albert Docks along the Mersey estuary (approximately 9 km), continuing to New Brighton (approximately 5 km), all interconnected by the Mersey estuary. Revealing such pronounced spatial variations via eDNA metabarcoding holds promise for investigating the transport of eDNA via tides in coastal environments, a topic that is still undergoing exploration. Jeunen et al. (2018)

demonstrated that the dispersal of eDNA among coastal habitats was found to be restricted, with individual taxa exhibiting localised eDNA signals aligned with their specific habitat preferences. Our study supports these findings; nevertheless, I strongly advocate for further research to delve deeper into this topic. Moreover, from a practical standpoint, eDNA metabarcoding can facilitate environmental assessments in interconnected waterways, where conventional sampling methods such as sediment collection may pose challenges or be inaccessible. Additionally, this method presents opportunities for forensic applications, such as linking a suspect to a specific site through diatom identification, an aspect that remains largely unexplored in eDNA metabarcoding (Young and Linacre, 2021). However, further research is necessary to evaluate the viability of this approach in forensic science, though it represents a promising avenue for future exploration.

When comparing species richness among the six locations, it was observed that the COI marker alone did not reveal significant differences, whereas the rbcL marker did (see Figure 3.2). The COI marker did reveal a higher species richness, albeit insignificant, in the open habitats than the enclosed (Figure 3.3). These differences might suggest that the COI marker, owing to its wide range of eukaryote detection, captures a baseline level of biodiversity that is common across all sampled locations (Casey et al., 2021). Alternatively, it could indicate that the COI marker lacks the precision to detect subtle variations in species richness between locations (Zhan et al., 2014). In contrast, the significant differences detected by the rbcL marker may be attributed to its specificity for diatoms; indeed, diatoms are also strong bioindicators relative to other taxonomic groups, such as fish (Desrosiers et al., 2013). This specificity implies that these particular groups exhibit more variability between locations, potentially highlighting subtle environmental differences not evident in the broader eukaryotic community detected by COI. These variations could directly impact taxa such as diatoms more than the broader eukaryotic groups. Furthermore, when comparing species richness between enclosed and open habitats, the significant variations detected solely by the rbcL marker reinforce this pattern. The indicator species identified by rbcL suggest a lack of diversity in enclosed habitats due to the number of species, indicating that these areas are ecologically distinct from open coastal systems. However, while this could indicate marker sensitivity, it could also be attributed to marker bias; rbcL is a chloroplast gene, meaning its use might result in algae and plants being more abundant or diverse at the sampling locations (Turk Dermastia et al., 2023). As discussed in Chapter 2, these enclosed, human-made infrastructures may be driving changes in biodiversity, necessitating further investigation.

There is a need for more exploration regarding the environmental drivers of these changes between the two markers among the sampling locations, incorporating environmental data such as pH and salinity levels alongside eDNA sampling (Stewart, 2019; Ruppert et al., 2019). A comprehensive understanding of these dynamics could inform future environmental decisions and management strategies.

3.5.3 Limitations

Urban coastal environments often introduce pollutants, nutrient runoff, and changes in water quality, creating favourable conditions for the proliferation of harmful algal species (Gilbert, 2020). Recognition of harmful algae through studies emphasises the necessity of monitoring and management strategies in urban water bodies (Berdalet et al., 2016). Detecting harmful algal species facilitates the establishment of preventative measures, such as avoiding the consumption of potentially harmful shellfish (Brown et al., 2020). It is important to note that due to the predominantly presence/absence nature of eDNA metabarcoding results, accurate quantification and assessment of the toxicity of these cells remain challenging (Jacobs-Palmer et al., 2021). However, eDNA metabarcoding of single-celled microeukaryotes can use proportionate read abundance (after data transformation) as an approximation of relative abundance, which should be applied with its limitations in mind (Beng and Corlett, 2020). The benefits of eDNA metabarcoding are further underscored by its fewer limitations compared to common techniques currently used to identify harmful algae (Jacobs-Palmer et al., 2021). Genetic sequences acquired from eDNA in various studies demonstrate the capability to identify taxa that can't be easily distinguished morphologically (cryptic species) through DNA sequences (Thomsen and Willerslev, 2015; Ruppert et al., 2019). this method provides consistency by relying on DNA sequences instead of morphological taxonomic assignments, which require highly skilled individuals and can even be subjective (Jacobs-Palmer et al., 2021). The application of eDNA can function as an early warning system for potentially harmful algae, enhancing comprehension of the conditions and species associated with the proliferation of these organisms in evolving marine environments (Suarez-Menendez et al., 2020; Jacobs-Palmer et al., 2021).

Another consideration of this technique is species masking, where the detection of rare species might be missed due to the dominance of abundant species in PCR amplification, as described by Skelton et al. (2022). However, this concept could reinforce that a high

proportion of harmful algae in a sample likely reflects a genuine high prevalence of that species, allowing for confidence in its abundance. Although eDNA cannot measure toxicity directly, the application that this method could serve as a useful baseline for environmental managers to quickly identify, prioritise areas that contain a higher abundance of harmful species and address urgent issues. Therefore, detecting these species is vital for monitoring and can complement existing practices in water quality management.

One significant limitation is the reference databases' incompleteness and the critical role of taxonomic expertise in eDNA studies (Beng and Corlett, 2020). In this study, 201,333 reads obtained from the COI marker and 65,900 reads from the rbcL marker were unidentified. Without taxonomists' contributions to specimen identification, linking DNA fragments found in water to specific organisms becomes challenging (Manoylov, 2014; Rees et al, 2014), as evidenced by our number of unidentified sequencing reads especially by COI (Taberlet et al., 2012; Stat et al., 2017; Bakker et al., 2019; Ruppert et al., 2019). Our study focused on species from Stramenopiles, including diatoms, due to their ecological significance and the rbcL marker's high species-level detection rate. This focus is partly attributed to the database's extensive collection of Stramenopiles sequences (Xie et al., 2018; Antich et al., 2021). However, database completeness alone isn't sufficient; the actual abundance and distribution of Stramenopiles in the environment enable their detection (Mathieu et al., 2020) and despite database limitations, eDNA metabarcoding still helps reveal hidden diversity (Couton et al., 2022). Since further refinement and optimisation of methods is needed, investigating the use of ASVs as could aid species resolution as OTUs may not fully capture the complexities of eukaryotic metabarcoding (Ruppert et al., 2019). While both MOTUs and ASV algorithms tend to produce similar results for species with high relative abundance, ASVs may provide more biologically realistic outputs (Antich et al., 2022). This indicates that it presents a promising avenue for further exploration. (He et al., 2022).

This study demonstrates significant spatial community trends in coastal habitats, highlighting differences between barcoding regions. The rbcL marker exhibited a greater species richness compared to COI, thereby improving the detection of important species when used together (see Figure 3.5). However, despite these observed findings, it is essential to acknowledge the limitations of this figure, as discrepancies between the markers may be contributing to the observed differences rather than reflecting true biological variations. In future studies, combining this novel molecular approach with the more established technique of taxonomic

identification could offer a more comprehensive comparison and a clearer understanding of the communities across different sites. While the findings are important and provide a valuable baseline for future studies, comparing them with traditional morphological surveys is essential to assess whether eDNA metabarcoding can serve as a reliable tool for ecological managers. Although this has been shown to match and even surpass traditional morphological methods in freshwater environments (Wang et al., 2024), there is still a shortage of research in dynamic coastal environments. Expanding this could help accelerate its adoption in ecological surveys, as has been seen more frequently in freshwater contexts (Apothéloz-Perret-Gentil et al., 2021; Kutty et al., 2022). Nonetheless, the inclusion of this figure is still valuable, provided its limitations are recognised. It underscores the importance of developing reference databases and conducting method comparisons, both of which are crucial for advancing eDNA metabarcoding in coastal environments. The disparity in the number of unidentified reads also reveals differences between the taxa availability in databases. COI, capturing a wider array of taxa, is more prone to encountering unidentified reads, which may be a concern for environmental managers. Furthermore, when considering a multi-marker approach, it is crucial to assess the cost-benefit trade-offs: employing multiple markers increases costs, thus future research should aim to assist managers in making informed decisions regarding the advantages and disadvantages of different markers.

3.6 Conclusion

Using eDNA metabarcoding, comprehensive and diverse list of eukaryote species were compiled across six heavily urbanised areas within a small <20km scale in Merseyside, all connected through tidal waterways. These species included those of ecological and conservation importance, including potentially harmful algae. Significant changes in whole-eukaryote community composition between molecular markers and across localised spatial scales were observed. Species richness and diversity were lower in enclosed habitats, highlighting the importance of potential habitat heterogeneity maintained by these human-made structures. The exploration of molecular markers showed COI region, with its broader scope, is advantageous for detecting a diverse array of eukaryotic organisms across a wide range of taxa. In contrast, the rbcL barcode is particularly beneficial for species-level investigations of microalgae, especially diatoms within the Stramenopiles, capable of identifying potentially harmful species. Although eDNA cannot directly indicate the toxicity of these species, their detection is crucial for informing environmental management decisions

and monitoring these valuable and vulnerable habitats. Despite the limitations of existing reference databases, eDNA metabarcoding provides important insights, and ongoing research and analysis will help address these limitations.

The significant divergence observed in eukaryotic communities across the six different locations demonstrates the utility of either barcode in identifying localised spatial trends. This underscores the versatility of the two barcoding regions in offering valuable insights into both broad-scale trends and finer-scale community dynamics, contributing to a comprehensive understanding of the studied ecosystems. Overall, these insights can guide future environmental management efforts aimed at effectively monitoring these intricate eukaryotic communities within vital urban coastal habitats.

4. Chapter 4: Exploring intertidal sea anemones (Actinia equina) as natural eDNA samplers for coastal biodiversity assessment

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Note: Please see the "Contribution to Data Chapters" section for more information. As described, sample collection, wet and dry lab processes, bioinformatics, data analysis and writing of the manuscript was conducted by Alice Cunnington. Co-authors reviewed the manuscript.

4.1 Abstract

Biodiversity in coastal marine environments is under unprecedented threat from anthropogenic disturbances. To better evaluate such impacts, it is imperative to develop accurate, cost-effective and expedient methods of assessing biodiversity. The analysis of environmental DNA (eDNA) – typically obtained through artificially filtered water samples – can paint a detailed picture of fish diversity in marine coastal environments. More recently, the analysis of natural sampler DNA (nsDNA) has emerged as a potential alternative to water filtering. Natural samplers are usually filter-feeding invertebrates that naturally trap eDNA in their tissues. This study investigated the widely distributed beadlet anemone (*Actinia equina*) as a potential natural eDNA sampler. Anemone samples were collected from two coastal locations in the United Kingdom: Rhosneigr – Anglesey, North Wales, and New Brighton – Wirral, North-western England. Sampling took place over two different months, and samples were compared to concomitantly sampled water. DNA metabarcoding via 12S Tele02 fish-specific primers revealed successful detection of a range of fish and other vertebrate species. Differences in species detected between conventional eDNA and sea anemone nsDNA samples were observed, as well as a significant difference in seasonality detected through nsDNA. Our results indicate that the beadlet anemone can be a successful natural eDNA sampler, but that its value is more likely to reside in its complementarity alongside established eDNA methods.

Keywords: Metabarcoding, intertidal environments, Environmental DNA, Natural sampler DNA, beadlet anemone, coastal fishes, Irish Sea

4.2 Introduction

The escalating severity of the global biodiversity crisis affecting terrestrial and aquatic life should be at the forefront of conservation biology (Sutherland et al. 2023). Coastal environments are vibrant hotspots of biodiversity that play a pivotal role in securing a range of important ecosystem services (Jones et al. 2020). These habitats are being increasingly threatened by a variety of stressors - including urbanisation, pollution and climate change, underscoring the urgent need to enhance and refine methods of monitoring and measuring biodiversity changes and their impacts on ecosystem functions and services.

In coastal ecosystems, observational methods, such as underwater visual surveys or baited remote underwater videos, and capture-based netting and trapping are used widely to measure fish biodiversity (Jovanovic et al. 2007). These techniques can be time consuming, expensive, often inaccurate, and sometimes destructive (Baker et al. 2016). Recent advances in high throughput, non-invasive molecular methods, particularly environmental DNA (eDNA) metabarcoding, hold substantial promise for enhancing the precision and scope of biodiversity surveys (Aglieri et al. 2021). At present, aquatic eDNA is typically obtained through passing water through an artificial filter with the aid of a range of manual or automated water pumping devices (Aglieri et al. 2021). As the field of eDNA metabarcoding advances, more cost-effective and accessible sampling methods are emerging; for example, passive sampling techniques (Bessey et al. 2021), which utilise various artificial materials and objects to trap and accumulate eDNA from the surrounding environment.

Natural eDNA samplers present a compelling alternative to artificial filters, offering a different avenue to the retrieval of eDNA fragments. The scope of natural eDNA samplers is extensive, spanning from natural substrates such as cobbles (Shum et al. 2019) and spider-webs (Gregorič et al., 2022) to the gut contents of aquatic generalist feeders (Siegenthaler et al. 2019, Shum et al. 2023), and extending to sea sponges (Phylum: Porifera), the taxon that best epitomises aquatic filter-feeding (Mariani et al. 2019). These organisms prompted further exploration of eDNA sampling properties in other aquatic invertebrates, such as mussels (Weber at al. 2022).

This paper explores whether sea anemones are viable natural eDNA samplers for detection of fish biodiversity in benthic intertidal habitats. Anemones are abundant and distributed in both deep oceans and coastal zones globally (Steinberg et al. 2020). This research focuses on the common and widespread suspension feeding beadlet anemone (*Actinia equina*), a common species distributed along the coasts of the United Kingdom, Western Europe and much of the East Atlantic (Davenport et al. 2011). In order to conduct this research, a small number of anemones were sacrificed. However, this organism is abundant, and not of conservation concern (Kipson et al. 2015). Further, the scientific use of a small number of these organisms is of minimal impact when compared to the habitat damage and animal fatalities associated with established marine surveying techniques, such as the use of nets, traps, dredges and grabs. It should be emphasised that this is a proof-of-concept study aimed at gaining an initial understanding of whether these organisms can enhance biodiversity studies beyond what is achieved through eDNA collected from non-invasive water filters. Future research could instead focus on using opportunistic methods, for example using anemones already obtained, rather than collecting them directly from the environment. This is observed with sea sponges, where initial research focused on exploring the potential scope of nsDNA, while more recent studies have shifted towards opportunistic sampling, such as obtaining samples from museum collections (Neave et al., 2024). With this in mind, the prevalence of beadlet anemones in intertidal zones at the water line makes them readily accessible for eDNA metabarcoding analysis. *A. equina* are generalist feeders and opportunistic omnivores feeding predominantly on arthropods and bivalves (Davenport et al., 2011). As sedentary organisms (although capable of moving slowly), species detections through nsDNA should reflect the sampling environment.

To test whether *A. equina* can be an effective natural eDNA sampler, fish specific primer pair was used. To broaden understanding of taxa detected by the anemones, a conventional water eDNA approach was used as a reference point. DNA was extracted from the whole body of the anemone (including the gut), using a fish specific primer enabled us to focus on taxa that are unlikely to be primary targets of the anemone's diet, making the nsDNA detections most comparable to aqueous eDNA data for vertebrate biodiversity monitoring. In this context, it is important to consider the digestion time of the anemones and the influence this might have on eDNA degradation. While no study has so far attempted to measure eDNA degradation time in sea anemones, this is likely to be in the same order of magnitude as the known degradation time of eDNA in seawater, which is between 24 and 72 hours (Collins et al. 2018): Kruger &

Griffiths (1997) report a gut retention time in *A. equina* between 12 and 23 hours, when feeding on planktonic crustaceans, with longer digestion times for of 40-60 hours in the case of shelled prey (Shick et al. 1991). These digestion times indicate that aqueous eDNA and anemone nsDNA approaches are comparable, allowing us to consider the merits of these candidate natural eDNA samplers in the context of coastal biodiversity assessments.

4.3 Materials and Methods

4.3.1 Field Collection

In an initial experiment, six beadlet anemones were collected in May 2022 from rockpools at New Brighton, Wirral (Figure 1B). Subsequently, ten *Actinia equina* and 3L of water samples were collected in October 2022 from both New Brighton and Rhosneigr, Anglesey, North Wales (Figure 1C). Rhosneigr is an exposed rocky coastal site, whilst the rockpools at New Brighton are situated under man-made concrete groynes (Figure 4.1) on a sandy beach.

Figure 4.1 Map showing sampling locations (A) New Brighton and (B) Rhosneigr. *Sampling locations (A). Concrete groynes at New Brighton, Wirral (B) Rocky coastline at Rhosneigr, Anglesey (C).*

Prior to sampling, all field equipment was sterilised with 10% bleach followed by 70% ethanol. Sterile gloves were worn and regularly changed after handling each sample. To monitor contamination at each site, purified water was filtered and used as a field blank, which were treated identically to other samples throughout the collection and extraction process. Anemones were collected and stored separately in 100% ethanol and placed in a cooler on ice in the field. The three 1-litre water samples (collected from the same rockpool as the sampled anemones) were pushed through 0.22 µm Sterivex filters, with each filter placed inside two sterile bags, and immediately stored on ice. All samples were stored at -20°C in the lab until further processing.

4.3.2 Laboratory procedures

4.3.2.1 eDNA Sterivex filters

DNA extraction from Sterivex filters followed the mu-DNA protocol for water (Sellers et al. 2018) which is outlined in Chapter 2, section 2.3.2.

4.3.2.2 Anemone nsDNA

Extractions were based on the mu-DNA extraction protocol by Sellers et al. (2018) and DNA isolation from marine sponges (Harper et al., 2023). Anemones were preserved in 100% ethanol and stored at -20°C prior to DNA extraction. Each anemone was cut into small ~5mm pieces (including stomach, tissue, and tentacles) with residual ethanol removed using Whatman blotting paper. Based on Harper et al (2023), 500mg of tissue was used per extraction with the remaining sample stored at -20°C. 730µL Lysis solution, 230µL tissue lysis additive, 40µL Proteinase K per sample were combined to make the lysis master mix. 1ml of this was added to each sample. This was placed on a thermomixer for 12 hours at 55°C at 650rpm. Samples were then centrifuged at 10,000 x g for 1 minute at room temperature. The supernatant was transferred to new tubes and 0.3x volume flocculant solution per sample was added and this was placed for 10-30 minutes on ice. Samples were then centrifuged 10,000 x g for 1 minute and the supernatant transferred to 2ml Eppendorf tubes. 2x volume of the tissue binding buffer was added and vortexed. The sample was then transferred to the spin columns and centrifuged. This step was repeated until the entire volume had passed through the filter. 500µL of wash solution was added to each sample and centrifuged. This was repeated twice. 100µL of elution buffer was added directly to the spin column, centrifuged and the supernatant was retained.

PCR amplification was performed in triplicate for each sample, using the Tele02 fish-specific primers, which target a ~167 bp fragment of the 12S rRNA mitochondrial region (Taberlet et al. 2018). Primer pairs were uniquely indexed to enable demultiplexing for downstream bioinformatic analysis. Positive controls were put in place for each PCR batch. We used 0.05 ng/µl DNA extracts of iridescent shark catfish (*Pangasianodon hypophthalmus*), a tropical freshwater fish absent from UK coastal waters). PCR amplicons were pooled in a single library and sequenced on an Illumina iSeq100 using v2 150x2 chemistry. Further details on PCR conditions and library preparation can be found in Chapter 2 section 2.3.3.

4.3.3 Bioinformatics and Downstream Analysis

Bioinformatic analysis followed the OBITOOLS pipeline (Boyer et al. 2016). FASTQC was used to assess the quality scores of the fastq files, ILLUMINAPAIREDEND was used to align reads, and NGSFILTER was used to de-multiplex samples. The sequence lengths were filtered to 120-200bp in order to remove sequences that were not in the target base pair range by using OBIGREP. Chimeras were then removed using VSEARCH (Rognes et al., 2016). Molecular operational taxonomic unit (MOTU) clustering was implemented using SWARM with d=3 (Mahé et al., 2014). Taxonomic assignment per sample was carried out using ECOTAG with a 12S reference database which was constructed using 'ecoPCR' in silico against the EMBL database (Release version r143).

Taxonomic assignments were validated by cross-checking non-native and unexpected taxa by manual BLAST against the nucleotide GenBank database. The package DECONTAM in R was used on the raw MOTU output. MOTUs were filtered by retaining assignments with >97% identity match and removing MOTUs that did not reach this criterion.

A Venn diagram was used to visualise fish MOTUs in nsDNA samples from May and October to portray seasonal variation. Samples were grouped per site and nsDNA/eDNA capture method for October only, and then visualised using a stacked bar chart of proportion percentage per class (fish and birds). To visualise the species proportions, a bubble plot was used for fish species only. Proportions were calculated using the number of sequencing reads of each species/MOTU per sample divided by the total sample reads; this represents the proportional read count per species and serves as a proxy for relative abundance.

To visualise temporal differences (May vs. October) between anemone nsDNA samples from New Brighton, nonmetric multidimensional scaling (NMDS) based on Jaccard distances using presence/absence data were used. These differences were tested using permutational multivariate analysis of variance (PERMANOVA, 999 permutations) on binary pairwise distance matrices using the function *adonis* in VEGAN (Oksanen et al. 2013). PERMANOVA was also used to test for differences between the two sampling sites in October. Finally, to identify whether there were any species significantly associated with certain months, indicator species analysis in R using the INDISPECIES package (Cáceres et al. 2016) was used, after Hellinger-transforming read count data.

4.4 ***Results***

Twenty-eight samples from October (Supplementary Table S1) made up one third of an iSeq100 run, yielding 602,127 reads, which after the first filtering step (>97% identity match) were reduced to 535,263 (88.9%). Although no contamination was found in the controls, two anemone samples were removed as they contained no sequencing reads. The eight samples from May (Supplementary Table S2) yielded 379,994 reads from an iSeq100 run, of which 374,432 (98.5%) were retained for downstream analyses. In this batch, one anemone sample was removed as it contained no sequencing reads. See Supplementary Material S1.5.
17 fish species were identified across anemone nsDNA and water eDNA from October 2022 samples (Figure 4.2). Although the DNA marker used is primarily designed to detect vertebrates, *Actinia equina* was also detected, albeit with only 115 reads across all samples, as were five distinct bird species: Spotted sandpiper *Actitis macularius*, Ruddy turnstone *Arenaria interpres*, Rock dove *Columba livia*, European herring gull *Larus argentatus*, and Common redshank *Tringa totanus*.

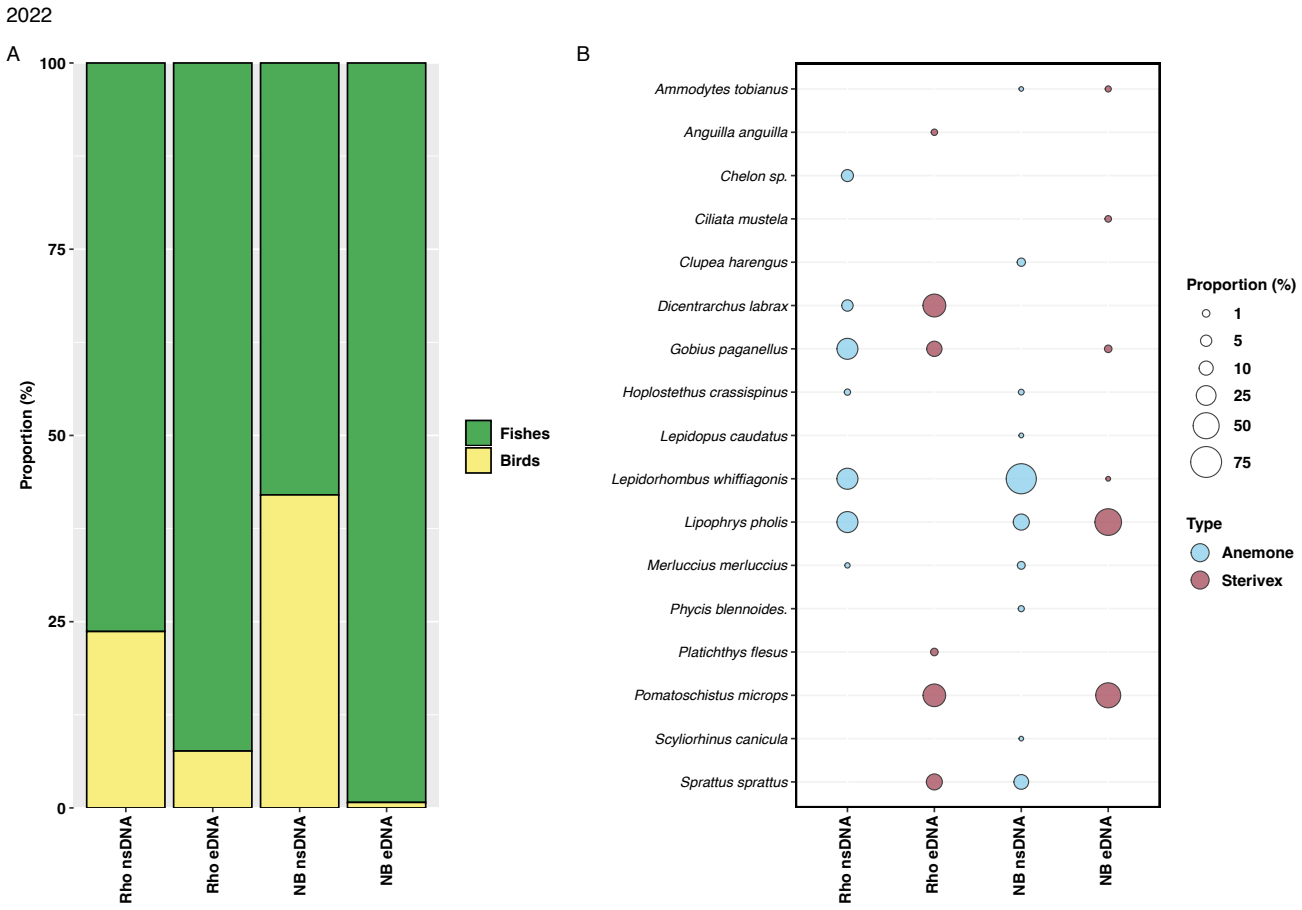

Figure 4.2: (A) Stacked bar chart and (B) bubble plot showing percent proportions of species between nsDNA and eDNA.

A stacked bar chart showing percent proportions for fish and birds between nsDNA and eDNA between the two sites in October only. Bubble plot showing the percent proportions per species per sampling site between nsDNA and eDNA for October only.

The bubble plot offers an approximation of species proportional representation, as derived from the relative number of sequencing reads obtained per species (Figure 4.2). This shows a higher diversity of fish was detected in New Brighton samples however, no significant difference in community composition was detected between the two sites (PERMANOVA pseudo-F=1.0165, df= 1.0, p= 0.393). There was no significant difference detected in community composition between nsDNA and eDNA in both locations in October (PERMANOVA pseudo-F=0.5629, df= 1.0, p=0.19) (supplementary figure S4.3). Indicator species analysis showed that three vertebrate species were statistically more abundant in eDNA samples: common goby *Pomatoschistus microps* (p=0.0063), European eel *Anguilla anguilla* (p=0.0167) and common redshank *Tringa totanus* (p=0.0110).

When New Brighton samples from May and October were contrasted (Figure 4.3), only three taxa (12.5%) were shared between the two months. Six MOTUs (25%) were detected only in May, while 15 (62.5%) were found only in October. This results in a strong separation of these temporal samples (Figure 4.3A), which is also supported statistically (PERMANOVA F= 5.8614, df= 1.0 p= 0.001). Indicator species analysis shows significant abundances in May for Shanny *Lipophrys pholis* (stat = 0.733, p=0.004), and Common sole *Solea solea* (stat = 0.382, p=0.0358) and significantly higher abundance in October for Megrim *Lepidorhombus wiffiagonis* (stat =0.676, p=0.0056), Common goby (stat = 0.573, p=0.0434), and ruddy turnstone (stat = 0.676, p= 0.0056),

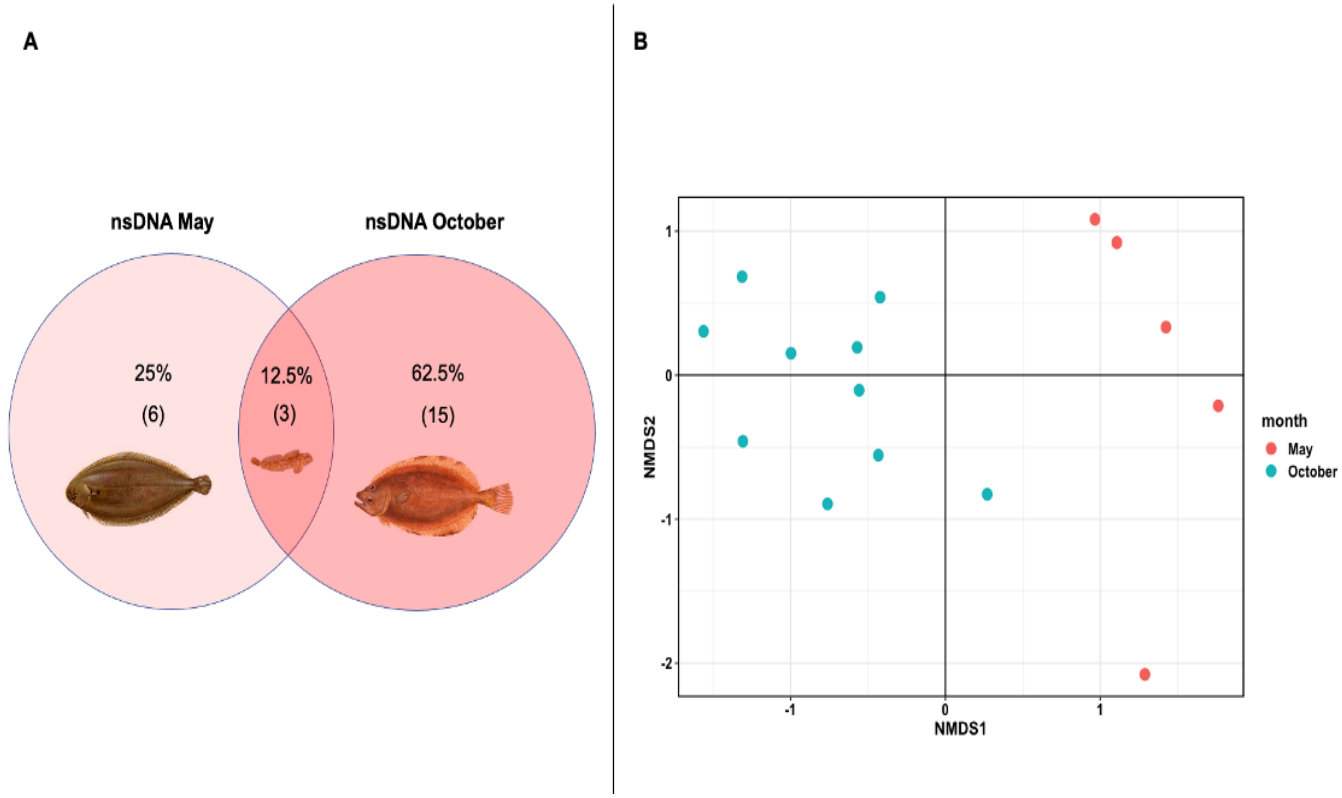

Figure 4.3: (A) Venn Diagram showing nsDNA OTUs and (B) non-metric multidimensional scaling of nsDNA samples.

Venn diagram representing the degree of overlap, in terms of nsDNA Operational Taxonomic Units (OTUs), between May and October samples from New Brighton only (A). Pictures show species of fish with the highest read count: May (Solea solea), shared (Lipophrys pholis) and October (Lepidorhombus whiffiagonis). NMDS showing nsDNA samples collected from New Brighton in May and October based on Jaccard distances using binary presence/absence data (B).

4.5 Discussion

Easily accessible, sessile, filter-feeding invertebrates make ideal candidates for coastal nsDNA applications, especially if, as in the case of the beadlet anemone, they exhibit remarkable resilience to fluctuations in temperature and salinity (Maskrey et al. 2020), which results in a wide distribution range. With this first attempt to evaluate the role of sea anemones as natural eDNA samplers to aid fish biodiversity assessments, it begins to understand the extent and circumstances in which such an approach may be beneficial. Wells et al. (2021) amplified DNA extracts of gut content from the giant plumose anemone, *Metridium farcimen*, targeting the mitochondrial COI region to investigate the diet of the organism. While the use of the COI primer pair enabled successful identification of the organisms on which the anemone fed, it also allowed detection of several fish species, indicating that anemones may have potential as successful natural eDNA samplers assessing biodiversity beyond the organisms that they select for consumption.

Our study demonstrates the effective use of *A. equina* nsDNA in detecting fish species representative of their environment. There was no significant difference found in species detected between nsDNA and eDNA, suggesting their similar efficiency; however, this could be due to the relatively small sample size in the number of samples collected. As the results clearly confirmed that anemone nsDNA successfully detects fish species within its environment, larger scale initiatives should be encouraged in the future to investigate further whether differences in species detected between intertidal nsDNA and eDNA conform to a consistent, ecologically relevant pattern that could be generalised. However, expanding the sample size is essential to accurately evaluate the effectiveness of anemone nsDNA. The next steps should prioritise understanding its capacity as a biodiversity tool and the scope for its use in future ecological surveys. Larger-scale initiatives are necessary, but these efforts should focus on using opportunistic samples, such as anemones collected from other surveys, or even archived samples, as seen in studies involving marine sponges (Neave et al., 2023), to minimise the impact. Although these initial findings are promising and warrant further research, if it is determined that anemone nsDNA provides comparable results without offering additional benefits over water eDNA, environmental agencies might prefer the non-invasive approach to minimise environmental impact.

It is found that while nsDNA can identify a subset of vertebrate species that are also detected by conventional eDNA capture methods, it uniquely identifies vertebrate species that have a minimal presence in aqueous eDNA samples. This was particularly evident in the detection of birds such as Ruddy Turnstone (*Arenaria interpres*), Spotted sandpiper (*Actitis macularius*) and the European herring gull (*Larus argentatus*). These species are closely associated with intertidal zones, with *A. interpres* known to predominantly look for molluscs, crustaceans, and small invertebrates (Kendall et al. 2004). It could be speculated that beadlet anemones are either predated on and/or exposed to some shoreline birds (or their guano), especially when the tide goes down, resulting in a higher proportion of reads of these species in the anemone nsDNA. This would indicate that in some circumstances this approach could be more effective than conventional eDNA filtration at monitoring rare and endangered coastal wading birds. The inclusion of non-target species enriches this study and suggests that forthcoming conservation research could employ bird-specific primers to specifically examine and delve deeper into these intriguing findings.This study therefore underscores the importance of utilising both nsDNA and eDNA techniques in biodiversity assessments. Relying solely on either method would have resulted in several vertebrate species going undetected. Given their relatively early stages of development, both standard eDNA and anemone nsDNA methodologies stand to benefit greatly from further refinement and advancements. There may be considerable value in using these two methods in tandem, potentially maximising our ability to reconstruct a comprehensive picture of the vertebrate community of a given environment.

Monitoring seasonal changes in fish assemblages in coastal environments via traditional methods is challenging, but eDNA has been shown to serve this purpose (Sigsgaard et al. 2017). Here, a strong temporal signal was found in anemone nsDNA between May and October, which indicates its sensitivity to detect seasonal variations in vertebrate communities. In cold-temperate intertidal habitats, there is a greater diversity at the end of summer rather than the spring (Jovanovic et al. 2007), with fluctuations in diversity driven by migration and spawning (Connor et al. 2019). Anemone nsDNA between these two months demonstrated strongly divergent patterns of read proportions in some species. In May, shanny (*L. pholis*) and common sole (*S. solea*) contained a higher percentage of reads than in October. This potentially reflects spawning behaviour, as shanny are known to typically spawn between the months of April to August (Shackley et al. 1977). The spawning season of

common sole is known to peak in the Irish Sea between mid-April to Late May (Armstrong et al. 2001), which correlates with its higher proportion found in May. This reinforces the argument that anemone nsDNA can detect environmental seasonal changes suggesting that in future, analysis of anemone nsDNA could be used to characterise seasonal patterns in certain coastal communities. However, the rarefaction curve (supplementary S4.6) illustrates that a more comprehensive representation of diversity is likely to be achieved through a larger number of samples rather than simply increasing sequencing depth. While it is crucial to acknowledge that under sampling has occurred, this study serves as an initial proof of concept. The next steps should involve expanding the sampling effort—ideally using already collected specimens—to enhance future research, as the current potential of anemone nsDNA may still be unknown.

It must be emphasised that this study is an initial step in validating *A. equina* as a natural eDNA sampler to aid biodiversity assessment. While these findings offer promising insights, further research is necessary to explore certain aspects in greater depth. This includes investigating the potential correlation between the feeding habits of sea anemones and the outcomes of eDNA metabarcoding, which could significantly influence our interpretation of biodiversity in these environments. Despite the informative nsDNA data garnered from this study, future studies may focus on optimising laboratory methods, exploring variation in nsDNA collection between anemone species, and the influence of feeding behaviour on nsDNA. To minimise the impact of sampling, further research could also investigate the efficacy of non or less invasive techniques for obtaining eDNA, for example biopsies or swabs. Overall, this is a compelling introduction of a new phylum to the field of eDNA and nsDNA analysis and reinforces the potential of this technique, at least in conjunction with conventional eDNA methods, to obtain a more comprehensive picture of species diversity in intertidal environments, thereby enhancing biodiversity monitoring.

5. Chapter 5: Mariculture facilities as eDNA monitoring stations: a Scottish sea loch case study

5.1 Abstract

Coastal habitats are ecosystems that provide an essential permanent or temporary home to a range of fish species. These habitats must adapt to considerable environmental variation, including stressors anthropogenic in nature. Scottish sea lochs are complex, sheltered fjordic inlets that have become optimal habitats for mariculture, especially traditional rope-grown mussel farming. Although aquaculture activities can have mixed impacts on the ecological equilibria of these habitats, they offer potential as platforms for ecological monitoring. In this study eDNA samples were collected in proximity of mussel farming ropes with two main objectives: i) to test whether eDNA could detect seasonal changes in the fish assemblages of the loch, ii) to test the natural eDNA sampling potential of farmed mussels. By using the fish-specific 12S Tele02 metabarcoding marker, 34 species across six classes were detected, from water samples, over two sampling events, in July and November. Analysis of DNA extracted from the tissue of blue mussels yielded no fish sequencing reads, suggesting there may be some limitations to the use of natural sampler DNA in this context. There was a notable seasonal variance in migratory species, including some of conservation importance, such as the European eel (*Anguilla anguilla*) and thornback ray (*Raja clavata*). The substantial molecular 'bycatch' of non-target species recovered offers important insights into the nature of land-sea interaction in these coastal habitats and confirms the vast reach and potential of eDNA monitoring for conservation and ecological applications.

5.2 Introduction

Coastal habitats across the globe are currently experiencing adverse effects from various stressors, including pollution, the impacts of climate change, and habitat destruction (Figueiredo et al., 2019). These fragile yet highly dynamic ecosystems play a crucial role for a wide array of organisms (Sheaves, 2009) and form an integral part of migratory routes for aquatic animals, including serving as vital locations for spawning and nurseries (Seitz et al., 2014).

In Scotland, sea lochs are fjordic inlets that have been predominantly formed through glacial processes. These settings are characterised by the interplay between marine and freshwater, resulting in a diversity of ecological conditions due to variations in salinity levels (Gillibrand et al., 1996; Angus, 2017). Various species rely on these environments for essential activities such as spawning, feeding, and seeking shelter (Gordon, 2003; Haggan & Pitcher, 2005), as happens in most coastal habitats (Seitz et al., 2014). Their sheltered nature also makes them important habitats for different types of aquaculture (Callaway et al., 2012). Sea lochs, especially those located in the Western Isles, serve as suitable environments for rope-grown mussel farming (Dias et al., 2009). This approach is recognised for its sustainability, as it exerts minimal influence on the nearby ecosystem (Dias et al., 2011). The ropes used in this activity offer supplementary surfaces for the attachment of various marine species, thereby generating artificial habitats that contribute to the enhancement of biodiversity (Suplicy, 2020), and providing a larger biomass of associated invertebrates and fish compared to similar areas without farms (Shumway et al., 2003).

Due to the critical significance of coastal regions, the precise and efficient identification of fish species holds great importance. However, conventional survey methods such as gillnets, trawling and electrofishing can inflict harm (Stage and Archipelago, 2016). Visual techniques – such as diving, snorkelling or camera deployment – may be less damaging, but their accuracy may be diminished by meteorological constraints and the reliance on taxonomic expertise (Harvey et al., 2012; Holmes et al., 2013; Bessey et al., 2020). Environmental DNA (eDNA) metabarcoding, which uses fragments of DNA shed by organisms, is becoming an increasingly popular method of identifying fish communities in a range of habitats (Miya, 2022). Studies have shown that eDNA metabarcoding can comprehensively identify trends of fish distribution (Keck et al., 2022), for example distinguishing seasonal trends of fish

communities in river systems (Milhau et al., 2019). Even though eDNA has been successfully integrated in aquatic monitoring to identify patterns of community composition across various ecological and temporal scales (Thomsen et al., 2015; Port et al., 2016), there remains uncertainty around ways to establish regular coastal eDNA monitoring observatories.

With the massive development of mariculture in Norwegian fjords and Scottish sea lochs, the eDNA metabarcoding approach had been identified as a valuable tool to assess the impact of fish farming on the surrounding ecosystem through the screening of bacterial and protist communities (Pawlowski et al., 2016; Dully et al., 2021). Given the threat to biodiversity in these important coastal habitats, heightened by the prevalence of salmon aquaculture, sea lochs offer a suitable natural scenario to test the applicability of eDNA metabarcoding (Pawlowski et al., 2016; Dully et al., 2021) for monitoring other community components of ecological importance.

The use of natural filter-feeding organisms as natural eDNA filterers adds an exciting branch to the realm of eDNA metabarcoding. This approach typically (but not exclusively) capitalises on the natural processes of filter-feeders to retrieve eDNA fragments, further reducing sampling effort. Previous studies have investigated fish diversity by examining the gut contents of shrimp (Siegenthaler et al., 2019) and sea anemones (see Chapter 4). Among the most extensively researched natural eDNA samplers are sea sponges, which have demonstrated success in detecting fish and other vertebrate species (Mariani et al., 2019; Turon et al., 2020). The development of this innovative approach is still in its infancy and warrants further exploration, particularly regarding the influence of various factors on eDNA degradation rates and transport within sponge tissues (Cai et al., 2022; Harper et al., 2023).

As the field of natural eDNA sampling undergoes rapid and continuous expansion, the widely distributed blue mussel *Mytilus edulis* emerges as a promising potential natural eDNA sampler. Mussels present a unique advantage due to their abundant presence and accessibility in both coastal and man-made structures (Bulleri and Chapman, 2010), as well as their ubiquitous filter feeding capacities (Van Colen et al., 2021) making them ideal candidates for natural eDNA sampling. Additionally, mussel farms offer a unique opportunity for convenient sample collection, as samples can be easily obtained year-round. Moreover, when used in conjunction with pelagic eDNA filters, mussel farms have the potential to provide a more comprehensive perspective, as mussels are suspended deeper in the water on ropes

which can be easily retrieved (Hartstein et al., 2005). This is particularly noteworthy as other natural eDNA samplers may be limited by sampling requirements, such as the need for scuba diving or taxonomic expertise for retrieval.

This study leverages the routine operations of a mussel farm in Loch Spelve on the Isle of Mull, to investigate fish assemblages sampled in summer and autumn 2021. Surface water and benthic mussel samples were collected with ease using mussel farming vessels. Through the application of eDNA metabarcoding, this study aims to provide insights into the seasonal patterns of the species inhabiting this ecosystem. Well-established water filtering methods were employed as a standard eDNA procedure, while the mussels growing on the ropes were also investigated as potential natural environmental DNA samplers (Mariani et al 2019; Junk et al 2023).

5.3 Methods and Materials

5.3.1 Field Collection

Sampling was conducted in July and November 2021 in proximity of, and with the assistance of, the rope-farmed mussel company Inverlussa, situated in Loch Spelve, a sea loch in Craignure on the Isle of Mull, Scotland, UK. The loch basin has a maximum depth of 58 metres and a relatively narrow opening (~350m wide) into the Firth of Lorn. Freshwater inlets from the Lussa River flow into the loch, which is surrounded primarily by rural land.

Sampling took place on board a mussel collection vessel from which three sites were identified (56.406490, -5.724396; 56.399342, -5.723838; 56.391623, -5.723967). Site 1 was located at a rope closest to the shore and Site 3 was at a rope located furthest away (Figure 5.1). Three mussels were collected at each site, extracted from a rope at approximately 2-3 m depth, which had been pulled up from the vessel. Three litres of water were collected from the surface of the loch, at the same location as where the mussels had been collected. Altogether, 18 eDNA filters, 18 mussels, 6 field blanks, 2 extraction blanks, 2 PCR blanks and 2 positive controls were used.

Figure 5.1 Map showing sampling sites in Loch Spelve, Isle of Mull.

Map showing the three different sites in Loch Spelve, Isle of Mull created using qGIS. The photos depict the ropes in Loch Spelve, the working mussel boat equipped with the device used to hoist the ropes, and the organisms attached to the mussel ropes.

Prior to sampling, all field equipment was sterilised with 10% bleach followed by 70% ethanol. Sterile gloves were worn and regularly changed after filtering each sample. To monitor contamination at each site, a field blank was taken in which purified water was pushed through a Sterivex filter with blanks treated identically to other samples throughout the collection and extraction process. The 3-litre water samples were pushed through 0.22 µm Sterivex filters, with each filter placed inside two sterile bags, and immediately stored on ice. The mussels were immediately placed in 100% ethanol in a sterile 5ml tube and then stored on ice. All samples were stored at -20°C in the lab until further processing.

5.3.2 DNA extraction

The DNA extraction of the Sterivex filters was based on the mu-DNA water protocol by Sellers et al. (2018). Firstly, the eDNA filters were removed using pincers and the filter paper removed using sterile dissecting scissors and forceps. Filters were cut up into small, ~10mm^2 pieces and half of the filter was used in DNA extraction with the other half stored in a -20°C freezer. Further outlined in Chapter 2, section 2.3.2.

Mussel DNA extraction was based on the mu-DNA tissue protocol by Sellers et al (2018) and adapted from sponge eDNA isolation (Harper et al., 2023). The whole of the inside of the mussel (including gut, gills etc.) was cut up into small, ~2mm pieces and blotted dry. 500mg of dry weight was used for DNA extraction. Next steps are further outlined in the mu-DNA tissue protocol for anemone nsDNA in Chapter 4, section 4.3.2.

5.3.3 Library preparation

PCR amplification was performed in triplicate for each sample, using the Tele02 fish specific primers, which target a ~167 bp fragment of the 12S rRNA mitochondrial region (Taberlet et al., 2018). Positive controls were put in place for each PCR batch. Extracts of iridescent shark catfish (*Pangasianodon hypophthalmus*) DNA were used at a concentration of 0.05 ng/µl, choosing this organism as it is a tropical freshwater fish, unrelated to fish species in UK waters. Primer pairs were uniquely indexed to enable demultiplexing for downstream bioinformatic analysis.

Please see Chapter 2, section 2.3.3 for the library preparation outlined.

The library and PhiX control were quantified using qPCR using the NEBNext® Library Quant Kit for Illumina®. The final library and PhiX control were diluted to 85pM (with PhiX 20% of the run) and loaded onto an Illumina® iSeq™ 100 Reagent v2 (300-cycle).

5.3.4 Bioinformatics and downstream analysis

The bioinformatic pipeline used was based on the OBITOOLS software (Boyer et al., 2016). FASTQC was used to assess the quality scores of the fastq files. ILLUMINAPAIREDEND was then used to align reads and alignments with low (<40) quality scores were removed. NGSFILTER was used to de-multiplex samples. In order to remove sequences that were not in the target base pair range, the sequence lengths were filtered to 120-200bp by using OBIGREP. The samples were dereplicated using OBINUNIQ. Chimeras were then removed using VSEARCH (Rognes et al., 2016). Molecular operational taxonomic unit (MOTU) clustering was implemented using SWARM with d=3 (Mahé et al., 2014). Taxonomic assignment per sample was carried out using ECOTAG with a 12S reference database which was constructed using 'ecoPCR' in silico against the EMBL database (Release version r143). The taxonomic assignments included levels of biological classification (such as superkingdom and family) along with a percentage indicating the likelihood of accurate classification. After taxonomic assignment, non-native and unexpected taxa were cross-checked by manual BLAST against the NCBI reference database. MOTUs that could not be assigned to genus or species level were also cross-checked manually against the NCBI reference database.

All downstream analyses were conducted using R version 4.2.2. The package DECONTAM in R was used on the raw MOTU table output. MOTUs were filtered by retaining assignments with >97% identity match for each biological classification (ie. family, genus, species). Alpha diversity indices (richness and Shannon) were calculated using PHYLOSEQ and VEGAN package, visualised using ggplot2 and patchwork R packages. Prior to downstream analysis, to standardise data, the number of reads were transformed into relative abundances by applying the Hellinger data transformation (Legendre & Gallagher, 2001) using the *decostand* function in vegan package (Oksanen et al., 2020). The Hellinger transformation corrects for biases produced by high read values and reveals an improved performance with eDNA metabarcoding data sets (Laporte et al., 2021; Legendre & Legendre, 2012). Alpha diversity indices (richness and Shannon) were calculated using the PHYLOSEQ and VEGAN packages (Mcmurdie and Holmes, 2013; Oksanen et al., 2013). ANOVA was used to test for

significant differences (distribution and abundance) in Shannon diversity among sites and months.

To visualise spatial (sites) and temporal (July and November) differences in fish communities among eDNA samples, nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distance were used. Differences between sites and seasons were tested using permutational multivariate analysis of variance (PERMANOVA, 999 permutations) on pairwise distance matrices using the function *adonis* in VEGAN.

5.4 Results

Samples were sequenced across one Illumina iSeq100 run alongside an unrelated eDNA project. The samples from this study made up 38% of the overall sequencing run: these included 18 eDNA filters, 18 mussels, 6 field blanks, 2 extraction blanks, 2 PCR blanks and 2 positive controls (N=48). After the first filtering step (>97% identity match) and removing human reads, 16 eDNA filters yielded 250,672 reads, with an average of 17,905 reads per sample. Three eDNA filters from each site from November yielded no reads and were removed from downstream analysis. The 24 mussel samples yielded no results apart from one mussel containing 1403 sequencing reads from Red Deer (*Cervus elaphus*). As the mussels yielded no results, these were removed from downstream analysis.

Across all eDNA filters six different groups were detected: amphibians, bacteria, birds, fishes, mammals and starfishes. Across all three sites in July, mammals accounted for 57.4% of mammal reads, while fish accounted for 37.6%. Across all three sites in November, mammals and fishes represented respectively 13.6% and 12.6% of sequencing reads. Across all sites and months, a total of 20 fish, 5 mammals, 3 birds, 1 amphibian and 1 starfish were detected (Figure 5.2).

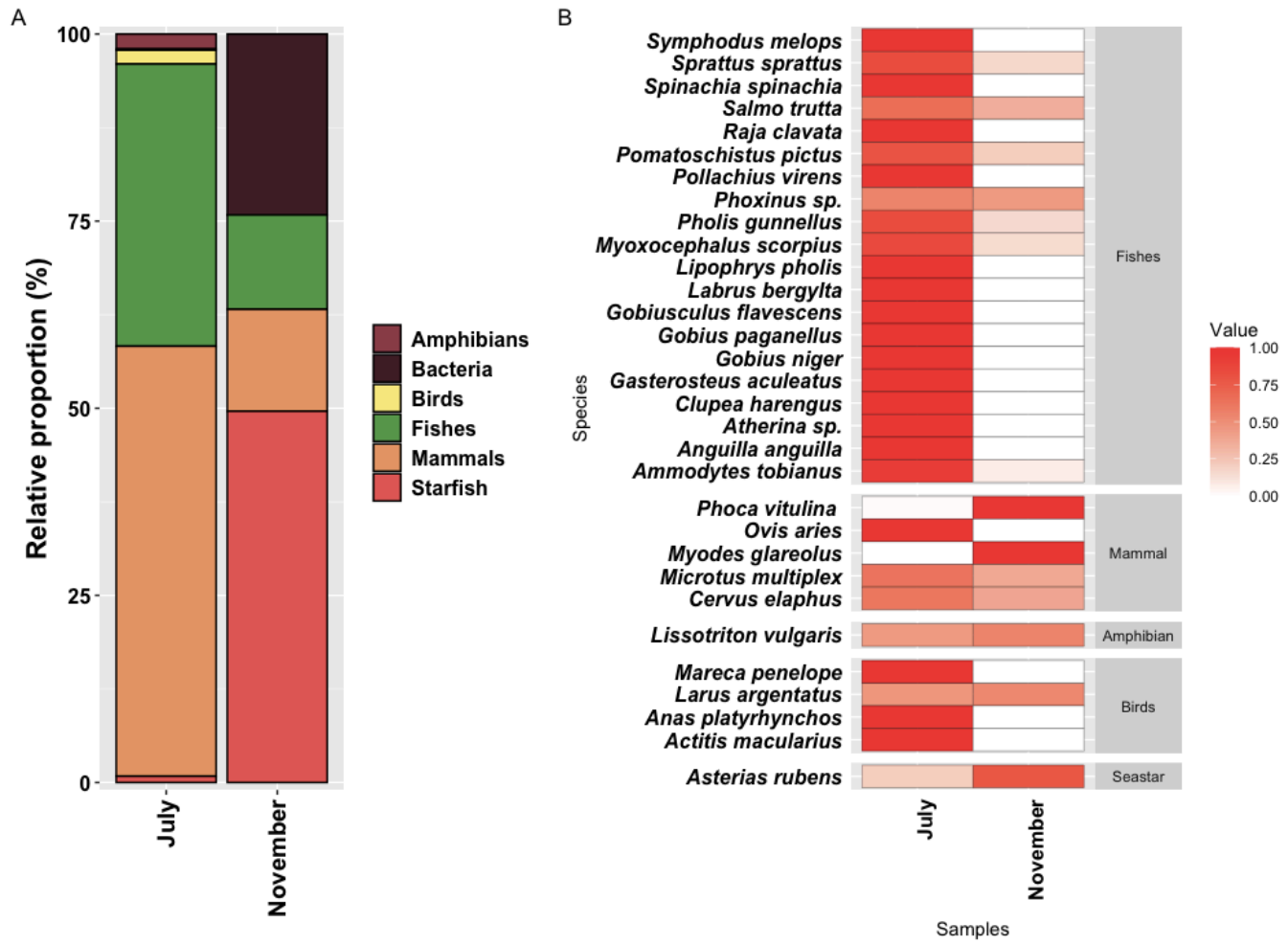

Figure 5.2: (A) Stacked bar chart showing proportional counts of classes and (B) heatmap showing proportional counts of species.

Figure shows (A) Proportional transformed read counts split into six different groups: Amphibians, bacteria, birds, fishes, mammals and starfish. (B) A heatmap depicting proportion of species based on transformed data, illustrates the distribution of species across sites and between the two months. Colours on the heatmap range from white, indicating low abundance, to red, signifying high abundance.

Species richness and Shannon diversity was higher in July than November overall (when all sites are combined). Species richness for July was between 17 and 24 OTUs and for November, species richness was between 1 to 9 OTUs. Site 2, in July, contained the highest species richness out of all sites and months with species richness between 5 and 24 OTUs (Figure 5.3). Even though species richness was higher in July it was not significant (ANOVA $F= 0.502$; $P< 0.49$), or by site (ANOVA $F= 0.887$; $P< 0.436$). The Rarefaction curve (Figure 5.4) shows that species diversity was not reached during sampling.

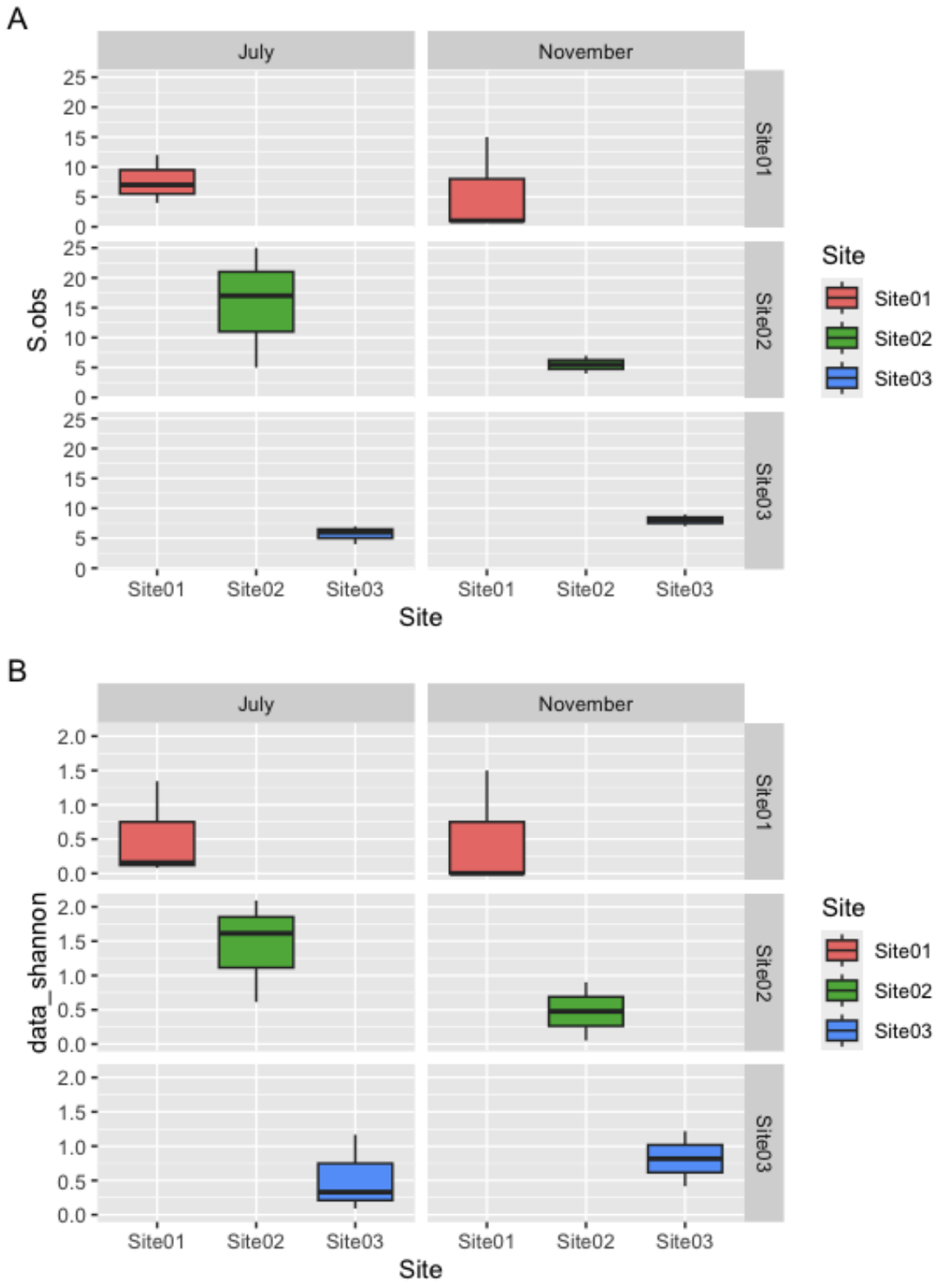

Figure 5.3: Box-whisker plots showing (A) species richness and (B) shannon diversity. *Box-whisker plots showing (A) species richness and (B) Shannon index alpha diversity between July and November for all three sites.*

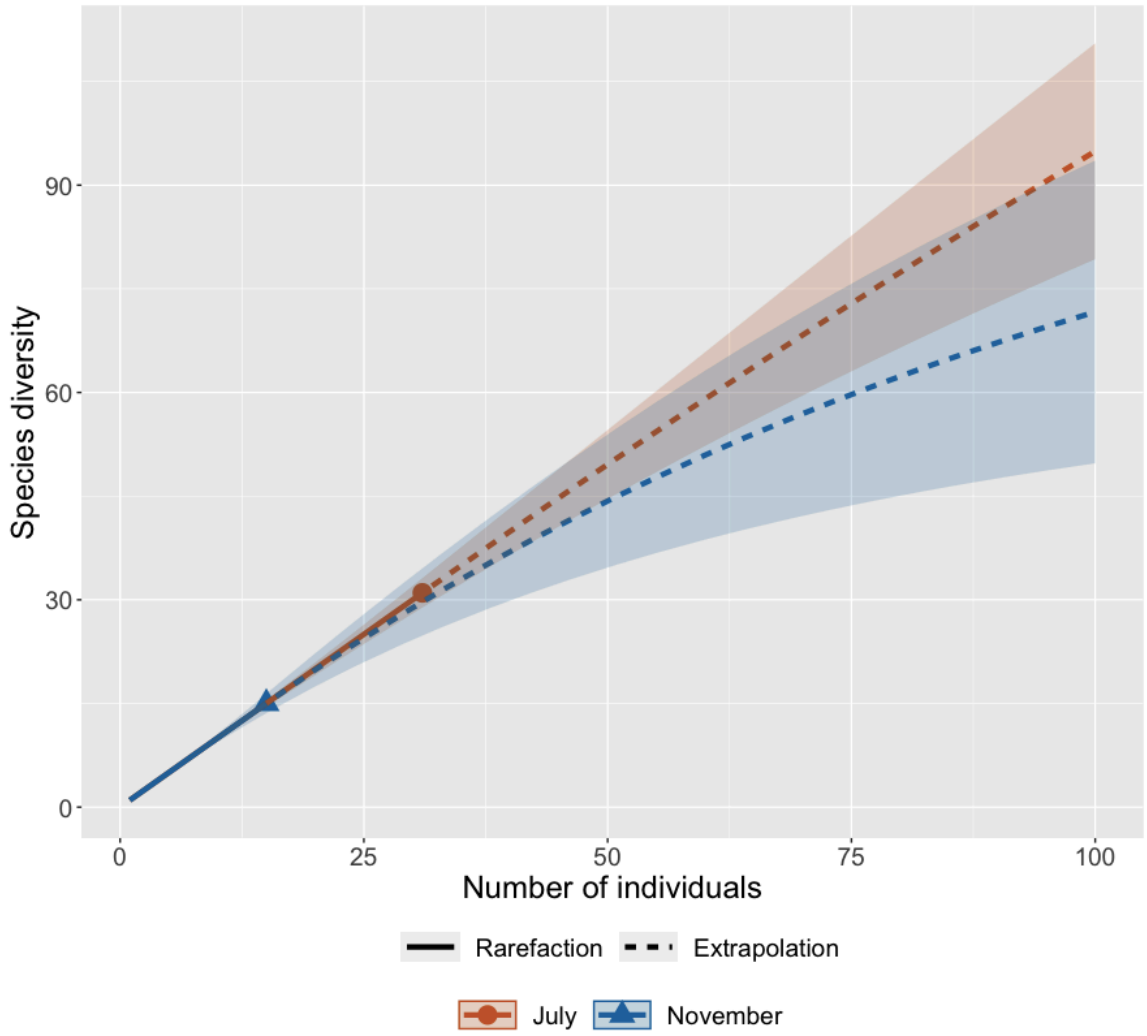

Figure 5.4 Rarefaction curve between July and November.

Rarefaction curve between July and November. The dashed line represents the extrapolated estimate calculated with R package iNEXT (Hsieh et al., 2016) and the shaded section shows the 95% confidence interval for the estimation.

When fish community structure was compared across the two months (July and November), a significant temporal (seasonal) difference was found (PERMANOVA F=2.3074 P= 0.023), but no differences among sites (PERMANOVA F=1.3111 P= 0.111). However, the interaction between season and sites (temporal and spatial) was found to be significant (PERMANOVA F=1.7381 P=0.004) (Figure 5.5).

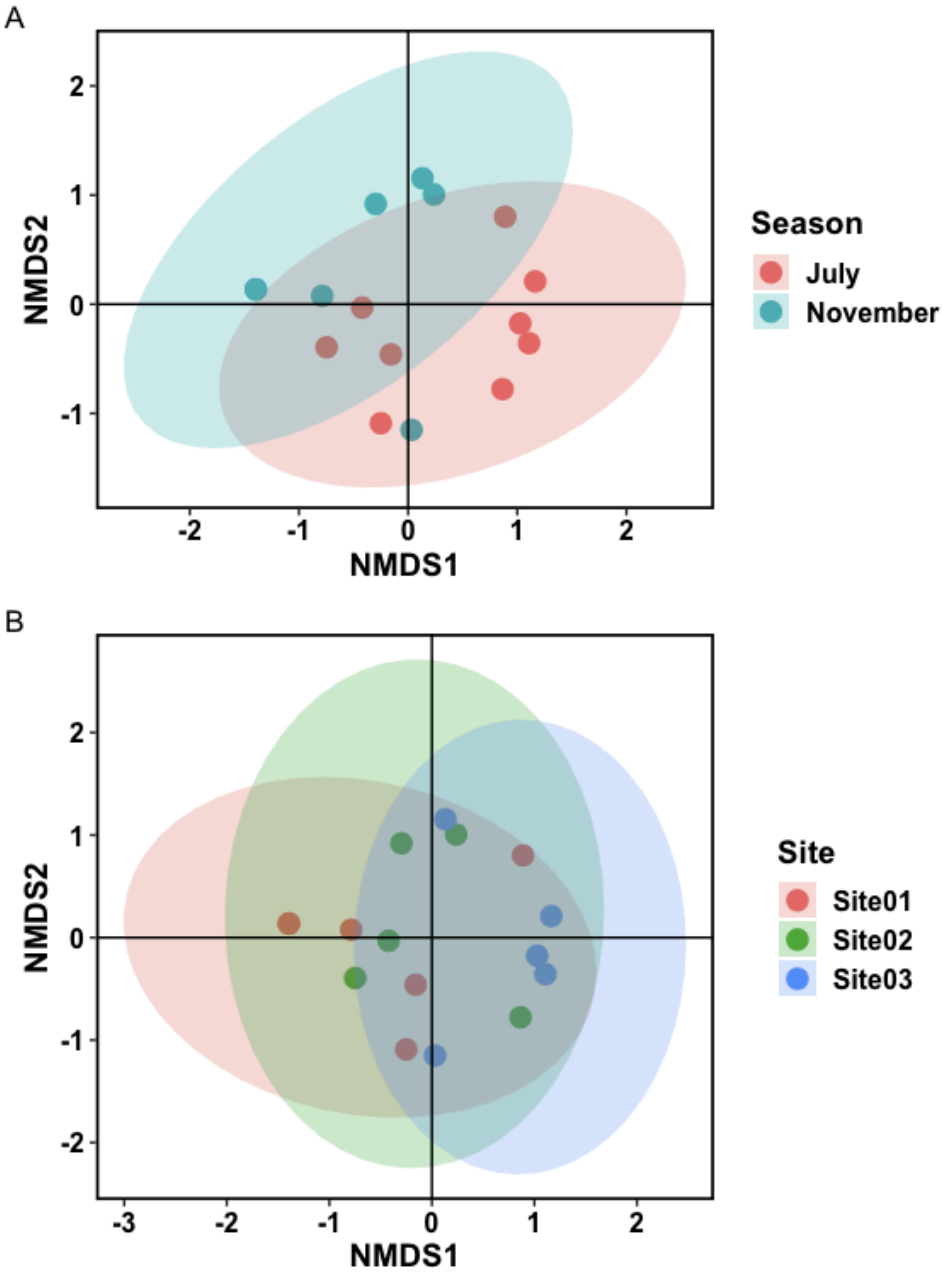

Figure 5.5 Non-metric multidimensional scaling of fish species composition between (A) months and (B) sites.

NMDS plot showing (A) fish species composition between July and November and on and (B) between sites using bray-curtis dissimilarity on Hellinger transformed data. The ellipses indicate a 95% confidence level.

5.5 Discussion

Using eDNA metabarcoding, a total 30 distinct species were detected belonging to 6 different classes across all eDNA Sterivex filter samples collected during both months. These species

were consistent with what would typically be expected in the sampled habitat. the observed results do not demonstrate statistically higher Shannon diversity in July when compared to November, this is almost certainly due to the small sample size. Figure 5.4 suggests that the sampling effort is insufficient to capture the full diversity of species present. It is important to highlight, as it could impact the communities observed. This indicates that if more samples were collected, additional taxa would likely be detected, suggesting the presence of false negatives in the dataset. Issues related to under-sampling can be potentially addressed by using more than one barcoding region; targeting different genomic regions can help increase diversity of DNA fragments amplified (Jeunen et al., 2019). Additionally, increasing sequencing depth could also address this, however if sampling effort is the issue sequencing depth will not help (Ruppert et al., 2019).

When examining fish species composition, significant differences between the two months were identified, revealing temporal variation. Scottish sea lochs, being coastal habitats, typically harbour numerous migratory fish species, which tend to aggregate in these areas for feeding and spawning during the spring and summer seasons before migrating to deeper or warmer waters in winter, leaving behind the year-round residents (Pemberton, 1976; Tuck et al., 1998; Middlemas et al., 2009). In November, seven distinct fish species were present across all three sites, with four of these being recognised as year-round residents: Sculpin *Myoxocephalus Scorpius*, Rock gunnel *Pholis gunnellus*, Painted goby *Pomatoschistus pictus* and, *Phoxinus sp*. Interestingly, there was a low abundance of migratory species such as the Atlantic herring *Clupea harengus* and Brown trout *Salmo trutta* in November. The differences in species composition between the two months highlight the dynamic nature of fish communities, with shifts occurring throughout the year due to species-specific reproductive behaviours and life-cycle patterns. However, these findings should be interpreted with caution, as under sampling occurred. Future sampling efforts should be refined and optimised to gain a more comprehensive understanding of community dynamics.

In July, the migratory sand smelt *Atherina* is observed. Two species of *Atherina* are known to inhabit the UK: the sand smelt *Atherina presbyter* and the Big-scale sand smelt *Atherina boyeri*. *A. presbyter* is commonly found along the UK coast (Palmer, 1983), whereas *A. boyeri* primarily inhabits specific populations in southern regions (Bowers and Naylor., 1964). Since only one lineage is detected, unlike in Chapter 2, it is more likely to be *A. presbyter* due to its common occurrence in the UK and its location on a sea loch rather than a

heavily human-influenced area. However, due to the database limitations, as discussed in Chapter 2, achieving absolute certainty remains challenging, and therefore, it is still referred to as *Atherina sp.* rather than specifying the species from the NCBI GenBank database. These historical misidentifications have raised doubts about the accuracy of reference databases for the *Atherina* sp. Further investigations are required to develop a reliable method for distinguishing between these two *Atherina* species in the UK. Once the current uncertainties surrounding reference sequence data are resolved, environmental DNA analysis could be particularly effective for monitoring the distribution of sand smelts, given the difficulties encountered in identifying them to the species level in the field.

As in previous Chapters (notably Chapter 2), the presence of the European eel is interesting, and it is notable that high abundances are observed across all three sites in July. This is particularly of note given its critically endangered status, as indicated on the IUCN Red List of threatened species (IUCN, 2024). Even though it is not unusual to find endangered European eels in the UK, it is important to identify areas of potential refuge for these animals. This identification will in turn enable us to better understand which environments require protection and conservation. Our findings suggest that sea lochs may hold significance for eels, possibly due to the vital connection they provide between freshwater rivers and the Atlantic Ocean, when *A. anguilla* migrate to their spawning grounds in the Sargasso Sea (Kettle et al., 2006). Using eDNA metabarcoding, these appearances and disappearances correspond with periods of migration to the sea or the potential arrival of juveniles, thereby enhancing temporal resolution and enabling the identification of monitoring peaks. Furthermore, the Thornback ray *Raja clavata*, detected in July, is a notable species. These rays are classified as near threatened on the IUCN Red List (IUCN, 2024) and are predominantly found in coastal waters (Hunter et al., 2006). The identification of threatened species, even in cases where the sampling scale is limited, underscores the value of eDNA metabarcoding in these areas.

Although the communities are not significantly different across the three sites, and the dataset is limited by under sampling, there are still notable observations of differences between the communities. In Site 1, which is situated closest to land and the furthest site from the open coastal environment, we noted detections of fish species typically found in areas of lower salinity, such as the Three-spined stickleback *Gasterosteus aculeatus* and the Sea stickleback *Spinachia spinachia*. However, the Common minnow *Phoxinus phoxinus* and Brown trout

Salmo trutta, which are primarily freshwater species, are detected in all three sites. A reason for the similarities between sites could be that sea lochs, with their steep sides and multiple inlets, receive freshwater inputs at various locations which suggest the homogenous nature of fjordic sea lochs. The presence of these species underscores the intricate interplay between freshwater and marine influences and emphasises the relationship and flow between freshwater and the fjordic inlets.

As increasingly documented, fish-targeting eDNA surveys typically also capture significant 'molecular bycatch' of other vertebrates (Mariani et al., 2021; Ritter et al., 2022; Zhang et al., 2023). Several mammals species were detected in this study, including both terrestrial and aquatic species, with harbour seal *Phoca vitulina* representing one distinct aquatic molecular bycatch. Among the terrestrial species detected were Red deer *Cervus elaphus*, Short-tailed vole *Microtus agrestis*, Bank vole *Myodes glareolus* and sheep *Ovis aries*. four bird species were also detected, European wigeon *Mareca penelope*, European Herring gull *Larus argentatus*, Mallard Anas platyrhynchos and Spotted sandpiper *Actitis macularius*. The mixture of these terrestrial and aquatic mammals and birds in our findings again reinforces the connection of land to sea through water runoff and freshwater rivers flowing into the loch. Within this unexpected "bonus" category of non-target species, the detection of the Smooth newt *Lissotriton vulgaris* is of note, as it is typically associated with ponds, and visually distinguishing them from the palmate newt *Lissotriton helveticus* can be challenging (Parry et el., 2015). Given the substantial abundance of non-targeted teleosts in our study and throughout chapters 2 and 3, it reveals the potential of eDNA analysis to identify a wide range of vertebrates, offering valuable insights for future ecological studies.

Another interesting insight garnered from our study is from our analysis of mussels. We emphasise that this is a novel attempt to use *Mytilus edulis* as natural eDNA samplers to aid in fish biodiversity assessments, extracting DNA from the whole organism. An initial attempt by Jeunen et al. (2022) revealed similar results, however only extracted DNA from the gills. Mussels hold promise as natural eDNA samplers, given their global distribution and their daily filtration of substantial water volumes (Bologna et al., 2005). Previous studies have explored the use of the COI barcoding region to identify metazoan taxa, however they predominately yielded mussel reads (Krehenwinkel et al., 2017) and mostly informed on the organisms that mussels typically eat (Junk et al., 2023). Weber et al. (2022) employed primers that effectively minimised mussel amplification, revealing valuable eukaryote dietary

data. However, they noted a significantly higher richness in extractions from concomitantly taken water samples, suggesting selectivity in mussel filtration (Baker & Levinton, 2003) and proposed that the mussels have a selective retention of particle sizes larger than 5µm in Dreissena mussels (Sprung & Rose, 1988). This is reinforced by Strohmeier et al. (2012) who reported that M. edulis effectively retained particles as small as approximately 7µm, with a maximum retention size of 35µm. Considering that Sterivex water eDNA filterers have pore sizes of 0.22µm, it can be hypothesised that some of the smaller particles typically detected using aqueous eDNA analysis may not be retained by mussels. This could be a viable explanation to why no 12S reads were yielded from the mussels in our study.

Given the infancy of natural eDNA samplers this study based the extraction methods on an optimised DNA extraction method for sponge nsDNA (Harper et al., 2023), which determined 500mg as the maximum required dry weight. Given the absence of a definitive standard method for natural samplers, this research embarked on using techniques previously employed with marine sponges. The effectiveness of the DNA extraction process was confirmed by successfully amplifying the same DNA extracts using the COI molecular marker (Leray et al., 2013). For a summary of the results, please refer to the supplementary section Supplementary S5 in the appendix. While future investigations into modifying extraction techniques should be encouraged, such as targeting specific organismal parts such as the gills or stomach, the emphasise should also consider the advantages of natural eDNA samplers. The practicality of stomach removal (or any dissections) must be weighed against the scientific value of the results obtained; stomach isolation could elongate sampling time and necessitate technical proficiency, potentially complicating eDNA retrieval. Additionally, exploring alternative mussel species is warranted to ascertain if they may offer better performance. As evidenced in marine sponges (Cai et al., 2022), it is plausible that different mussel species may exhibit varying effectiveness, necessitating further inquiry.

5.6 Conclusion

This research highlights the clear benefits of using eDNA metabarcoding to evaluate vertebrate diversity in Scottish sea lochs, leveraging the accessibility of mussel farming vessels for water sample collection to effectively inform both the public and conservation management. Through this method, various teleost species inhabiting Loch Spelve were detected during both July and November 2021, including species of significant conservation

importance such as the European eel and Thornback ray. Although under sampling occurred in the dataset, an important caveat when interpreting ecological inferences, the results unveil notable seasonal trends. In July, mammals comprised over 50% of proportional reads, uncovering intriguing additional terrestrial and aquatic organisms. The incidental molecular by-catch which was gained without any extra sampling effort, suggests that eDNA metabarcoding holds potential for future ecological investigations targeting avian or mammalian species. This study presents a potential avenue that provides more user-friendly protocols that coastal stakeholders (e.g., aquaculturists, fishermen, recreational operators) could readily support by facilitating the establishment of regular or coordinated "observatories" for eDNA collection.

Ideally – and in the original spirit of this research effort – no bespoke water filtering equipment would be even required if mussels had proved effective as natural eDNA samplers, as a handful of them would periodically deliver the biodiversity information needed. Yet, even with *M. edulis* not meeting the standard of broad spectrum nsDNA (Neave et al., 2023; Reisgo et al., 2024), COI analysis of this species' intake can still provide valuable insights into the surrounding ecological dynamics (Junk et al. 2023). Irrespective of natural eDNA samplers, eDNA metabarcoding is emerging as a promising, non-invasive method for characterising temporal trends within fish communities. These artificial structures present future opportunities for different sampling methods, such as attaching passive eDNA samplers such as metaprobes to mussel ropes (Bessey et al., 2021; Maiello et al., 2022), which may be a novel and logical avenue to explore in the near future.

6. Chapter 6: General Discussion

6.1 Main Findings

This thesis focussed on eDNA metabarcoding, looking at both the more ubiquitous aquatic eDNA collection, as well as more innovative techniques, such as anemone nsDNA. These approaches are intended to aid the monitoring of coastal locations heavily impacted by human activities. In locations such as the Albert Dock in the heart of a bustling city or West Kirby marine lake (Chapters 2 and 3), environmental monitoring not only helps identify to aid conservation efforts (especially fish of conservation importance) but also addresses potential public health concerns where people are readily using the area for recreational areas. eDNA metabarcoding has proven to be a powerful tool with universal applications for assessing biodiversity across various habitats, from characterising whole communities of macro-organisms to identifying specific potentially harmful algae. This thesis has shown that these complex urban systems can be monitored spatially and temporally through eDNA metabarcoding, which can detect distinct trends in fish communities and changes consistent with ecological behaviours (such as spawning) (Chapters 2, 4, and 5), as well as distinct spatial whole-community eukaryote trends on a localised scale (Chapter 3). eDNA and nsDNA enhance the environmental assessment toolkit, offering insights that can guide future research and management decisions, and bringing attention to often overlooked aspects, places, and processes.

This research has effectively demonstrated that both water eDNA and anemone nsDNA metabarcoding analysis can successfully identify significant temporal trends in fish communities. A recurring theme across chapters 2, 4, and 5 is the seasonal variations observed, typically with higher species richness during summer and autumn, followed by a decline in the colder winter months, which aligns with species-specific behaviour and life-histories (Orio et al., 2017). For instance, the European flounder migrates to warmer waters in winter before returning to coastal areas to spawn in spring, while Atlantic herring spawns in autumn in the Irish Sea (Chapter 2). Interestingly, these seasonal trends observed in the more open coastal location of West Kirby were not evident in the Albert Dock, which maintained higher richness throughout the year (Orio et al., 2017). This suggests that factors such as location and infrastructure may influence fish species presence and disappearance throughout the year, deviating from typical temporal patterns. When considering the dispersal of eDNA, seasonal variation may impact genetic material differently (Barnes et al., 2021): warmer

temperatures may accelerate eDNA detection (Tsuji et al., 2017; Jo et al., 2019; Saito and Doi, 2021), while colder water temperatures and reduced fish activity during winter may lead to less genetic material being shed, thus influencing detection rates (Troth et al., 2021; Rourke et al., 2022). In urban coastal habitats, human impacts could accelerate fluctuations in water temperatures (Cloern et al., 2016), in turn potentially affecting the dispersal of eDNA (Jeunen et al., 2018). Another question to consider is whether coastal infrastructure, such as docks, given their typically closed system, could influence eDNA dispersal. This aspect warrants future research attention, as eDNA dispersal has been investigated in freshwater, sea, estuaries, and coasts, but not in coastal infrastructures. While this factor should be acknowledged when conducting eDNA sampling in these areas, it should not dissuade future sampling endeavours. Even if docks, as closed systems, could potentially lead to eDNA accumulation, the likelihood of false positives is minimised due to reduced water flow and eDNA degradation While it is challenging to determine the exact dispersal model for each new harbour studied, the knowledge that there is more of a rapid degradation of eDNA in inshore environments helps ensure the accuracy of species detection (Collins et al., 2018).

Chapter 3 emphasises how eDNA dispersal in coastal habitats does not prevent the maintenance of strong spatial clustering among eukaryote communities. Notably, a significant distinction emerged when comparing whole- community assemblages identified by two primer regions (COI and rbcL), revealing the distinctiveness of assemblages at each location despite interconnected tides and waterways. This is of note because connected tidal environments could potentially facilitate eDNA transport over considerable distances (Hinz et al., 2022), leading to ecological homogenisation. Chapter 3 demonstrates the effectiveness of eDNA metabarcoding in discerning localised communities across six coastal habitats within a relatively small spatial scale (<20 km), reflecting limited dispersal of eDNA among these habitats, as observed on an even smaller spatial scale (<5 km) using three barcoding markers (Jeunen et al., 2019), or indicating that, despite some dispersal, the ecological microhabitat features determine sufficiently distinctive biodiversity assemblages. This not only provides a comprehensive view of diversity across different sites but also aids in pinpointing specific areas of concern, prioritising monitoring efforts, and implementing mitigation measures, particularly for bioindicators or harmful/invasive species. The ability to detect spatial disparities in communities with ease will be crucial for future biodiversity monitoring.

Several species of conservation importance were detected through eDNA metabarcoding, with the European eel being particularly noteworthy and detected in West Kirby, Albert Dock, Rhosneigr, and Loch Spelve (Chapters 2, 4, and 5). Once abundant in UK waters, its population has sharply declined due to factors such as habitat loss, pollution, and overfishing. Given its critically endangered status, monitoring its presence and distribution is crucial for conservation efforts in the UK, and the abundance of detections in these chapters is an exciting and encouraging sign. Recent efforts have included eDNA analysis to assess the occurrence and abundance of European eels in UK habitats, extending beyond academic research to mainstream news articles (Hillsdon, 2023; Horston, 2023; Rodriguez, 2023). The presence of this species in various locations is both promising and, in certain areas, puzzling, particularly the year-round detection of eels in the Albert Dock. While the discovery of an essential refuge for such a renowned endangered species amidst a bustling city is exciting, concerns arise regarding the design and operation of the Albert Dock, raising the possibility that these catadromous animals may become ensnared within its structures and unable to complete their lengthy reproductive journey (Verhelst et al., 2018). Demonstrating that these species can be identified and monitored via eDNA metabarcoding shows its potential significance for future monitoring efforts, as it facilitates the easy identification of areas where species are present.

This study also showed how eDNA can help illuminate currently uncertain taxonomic and ecological questions relating to certain poorly understood coastal fishes. In the UK, two species of *Atherina* are distinguished: the Sand smelt *Atherina presbyter* and the Big-scale sand smelt *Atherina boyeri*. *A. presbyter* is widespread along the UK coast (Palmer, 1983), whereas *A. boyeri* primarily occupies specific populations in southern regions (Bowers et al., 1964). Chapter 2 identified two distinct lineages of *Atherina sp*., whereas only one lineage was found in Loch Spelve in Chapter 5. Due to historical misidentification and uncertainties surrounding this species (Ardura, 2019), as evidenced by DNA reference databases such as NCBI, assigning the two divergent sequences identified in this study to either *A. presbyter* or *A. boyeri* is currently impossible. Despite the absence of a definitive distinction, it is noteworthy that *Atherina sp. 2* was exclusively observed in the Albert Dock. Additionally, the discovery of only one lineage in Loch Spelve (Chapter 5) further informs this finding. When examining the haplotypes, *Atherina sp. 1* in the Albert Dock matched the haplotype of *Atherina* sp. found in Loch Spelve. This suggests that the less common *Atherina* species (A. boyeri) may indeed be *Atherina sp. 2* and tends to inhabit specific coastal urban refuges. This

discovery underscores how eDNA monitoring can serve to pinpoint focal area for further research and epitomises how little is still known about this fairly common but ecologically important fish species around our coasts, as the adaptations of sand smelts may potentially hold value in the context of environmental monitoring.

In Chapters 2 and 4, this research reported detection of deep-sea species, including some unexpected ones, particularly in Chapter 2, where sampling sites around Liverpool revealed species such as *Galeus melastomus, Leucoraja circularis, Lepidorhombus whiffiagonis*, and *Phycis blennoides.* It is important to address the rationale behind the removal of certain deep-sea species from the analysis in chapter 2, while no such exclusions were made in Chapter 4. It should be noted that while eDNA methods offer high sensitivity in detecting rare species at low abundances, this can lead to the risk of spurious detections that are challenging to verify. Prior knowledge allowed identification of *Galeus melastomus* reads as likely originating from a previous project. To maintain consistency, the other deep-sea species were discussed, considering the possibility of their genuine presence due to tidal transport of eDNA or increased eDNA levels during spawning events (Jeunen et al., 2018; Collins et al., 2022). In contrast, the deep-sea species identified in Chapter 4 (*Lepidorhombus whiffiagonis*, *Merluccius merluccius*, *Phycis blennoides* and *Platichthys flesus*) there was no prior knowledge of potential contamination in the lab process. Since they are not commonly associated with coastal habitats, it was crucial to cross-reference their known behaviours and spawning patterns to assess the plausibility of their detections. It was concluded that these detections may indeed be genuine based on their known behaviours. However, it is essential for future eDNA studies to address these challenges and develop new strategies to enhance confidence in the results among end-users (Blancher et al., 2022).

This thesis reinforces the knowledge that eDNA metabarcoding using the 12S Tele02 marker in coastal habitats can reveal the presence of species that are typically found in terrestrial landscapes, highlighting the role of coastal environments as effective collectors of eDNA transported through catchment drainage at the edge of the land mass. This is particularly pronounced in the sea loch, where steep sides likely facilitate substantial drainage, emphasising the close connection between land and water. These findings are significant additions to traditional fish surveys, as terrestrial species may go unrecorded by conventional methods, thus enhancing the overall value of the survey and its potential applications in conservation and biomonitoring. The mostly mammal and bird taxa obtained serve as

valuable records of organism presence, establishing baselines for future surveys without requiring additional investment. It is essential to acknowledge that the molecular bycatch detected represents an underestimation of actual diversity within the study areas, resulting in false negatives. Targeted primers designed to detect birds (Ushio et al., 2018) or mammals (Sales et al., 2020) could provide further insights into this aspect. Notably, anemone nsDNA revealed an intriguing bycatch, indicating the presence of wading birds that were absent in water eDNA samples. Despite the likelihood of false negatives, this observation suggests potential interactions between wading birds and anemones or offers opportunities for future wading bird detections in these areas. While false negatives will occur, the additional taxa identified are valuable, providing extra, cost-free information that could aid environmental managers in the future.

6.2 Exploring optimisation of laboratory processes for natural eDNA samplers

This thesis has drawn attention to potential advancements that may be achieved through further natural eDNA sampler applications, particularly concerning anemone nsDNA (as discussed in Chapter 4). While acknowledging that this chapter primarily serves as a proof-of-concept study, and much exploration must still be undertaken, this Chapter serves as an initial step in demonstrating the potential of anemone nsDNA to contribute to vertebrate biodiversity monitoring. As stated within Chapter 4, future optimisation of laboratory techniques should be advocated, acknowledging that the efficiency of eDNA extraction from anemone tissue could influence the taxa identified. To address this, various optimisation approaches were investigated, drawing upon methods outlined in Harper et al.'s (2023) study on sponge nsDNA. Gel electrophoresis was used to evaluate the efficacy of PCR amplification and ascertain both the existence and size distribution of amplified DNA fragments associated with the target gene region. Specifically, experimented with a non-destructive swabbing technique of the anemone, yet only minimal amplification compared to tissue samples was shown on the gel, leading to not to pursue this method. Despite the challenges posed by the size of these organisms, it is reasonable to expect that some form of non-destructive techniques may yet be devised to minimise harm.

Additionally, the impact on DNA amplification of blotting the anemone dry versus homogenising the organism in a tissue lyser was investigated. Gel images indicated that

blotting the ethanol dry resulted in more successful amplification compared to homogenisation, aligning with findings from sponge nsDNA (Harper et al., 2023). Moreover, experimentation with different sample weights revealed that a maximum weight of 500mg yielded the best amplification on the electrophoresis gel. However, to gain a comprehensive understanding of the taxa yielded, repeats of this testing is needed. Overall, while this study provides valuable insights, further research is essential to refine and expand upon these findings. Continued optimisation of laboratory techniques, exploration of non-destructive sampling methods, and comprehensive sequencing efforts will be pivotal in harnessing the full potential of anemone nsDNA for vertebrate biodiversity monitoring.

A similar method was used to attempt the isolation of eDNA from blue mussel tissue (Chapter 5). Given the intended ease of obtaining and processing nsDNA, extractions were performed on the entire organism without isolating specific body parts to streamline the DNA extraction process. Subsequently, comparisons were made between homogenisation and blotting methods, with gel electrophoresis of amplifications indicating no discernible difference between the two techniques. Blotting was then chosen for its lower risk of contamination and quicker processing. Additionally, varying sample weights revealed that a maximum weight of 500mg produced optimal amplification on the electrophoresis gel. Although no taxa were detected using the 12S Tele02 metabarcoding region, the very same DNA extracts were amplified with COI primers, resulting in numerous reads, albeit predominantly from the mussel itself (outlined in the supplementary material in S5). This observation demonstrates that the DNA extraction was successful and reinforces that mussel nsDNA may not be suitable for vertebrate eDNA studies. Forthcoming research efforts might be able to develop alternative laboratory methodologies to improve taxa detection. However, it is important to remember the original objectives of using natural eDNA samplers were speed, simplicity and sustainability. Therefore, it remains crucial to assess whether additional, potentially more intricate, steps in the workflow might compromise the intended nsDNA goals.

In summary, this thesis has shown that anemone nsDNA has already showcased its utility in eDNA metabarcoding research, even prior to the optimisation that will be necessary. This highlights its potential for future applications, whether as a supplementary tool to enrich water eDNA studies or as a standalone method, contingent upon the specific sampling

objectives. It is imperative to remember the significance of prioritising essential aspects and striking a balance between enhancing monitoring capabilities and effectively managing costs.

6.3 Exploring limitations and future directions for eDNA metabarcoding in coastal environments.

Given that the majority of this research involved sampling in coastal areas impacted by high levels of human activity (as discussed in Chapters 2, 3, and 4), using the fish-specific vertebrate primer presented challenges. While the incidental amplification of molecular bycatch vertebrate DNA enhanced the studies with bonus taxa, it also resulted in the amplification of human DNA, unsurprisingly yielding the highest sequencing reads in the Albert Dock, with human DNA comprising 66% of the entire study's reads. The negative and positive controls consistently exhibited minimal human reads, indicating that these reads are likely originating from the environment. This is particularly relevant given the high levels of human activity at both locations, especially in the water (from water sports and boats) and in the surrounding areas—especially at Albert Dock, which hosts multiple hospitality and recreational venues. It is important to consider that water runoff could transport human DNA to these sites, and the enclosed nature of these areas may affect the transport and persistence of human DNA (Collins et al., 2018; Harrison et al., 2019). This further highlights the necessity of consistently implementing field and lab controls to determine whether human DNA is a result of environmental factors or contamination during sampling and wet lab processes. It is crucial to stress that researchers should not be dissuaded from using eDNA metabarcoding in coastal areas with high levels of human activity, as these regions particularly require monitoring and attention. However, they should remain aware of this potential limitation and take steps to mitigate it. Furthermore, recent literature, including Whitemore et al. (2023), has drawn attention to potential ethical dilemmas that may arise from the unintended capture of human genomic data, an issue that could become more significant as eDNA metabarcoding becomes increasingly widespread.

Future studies should be encouraged to investigate potential solutions to this issue, such as the use of human-blocking primers or increasing sequencing depth to minimise the impact of human DNA (Beng et al., 2020; Zhang et al., 2020). Additionally, exploring the differences in human DNA amplification between various primers that target similar regions, such as 12S Tele02 and MiFish (Miya et al., 2015), is essential, as this area remains largely unexamined.

Many studies may not report the number of human reads, complicating efforts to draw accurate conclusions. Understanding these differences could enhance future eDNA applications in heavily urbanised environments. While deeper sequencing and the use of human blocking primers may reduce the chances of false negatives, they could also incur higher costs that environmental managers need to consider. Despite these suggested mitigations, the presence of human DNA remains a potential issue. However, this does not diminish the value of the studies, as the detection of fish species remains invaluable. Furthermore, despite the high presence of human DNA reads, clear spatial and temporal community differences were observed, and endangered species were identified. Researchers should bear this in mind when conducting eDNA metabarcoding using the 12S metabarcoding region in urban coastal environments in the future.

This research also identified limitations regarding the identification of certain species, particularly evident with the two species of sand smelt (*Atherina* spp), and the lack of resolution in the case of grey mullet in the genus *Chelon* genus (Chapter 2 and 4). These limitations show the existing gaps in the reference database for certain species, and the occasional inadequacy of the established metabarcoding markers in discriminating closely related species. As eDNA metabarcoding progresses, advancements in reference databases are anticipated to bridge these gaps, including initiatives such as the Tree of Life Gateway, which aims to sequence over 70,000 eukaryotic species (Threlfall and Blaxter, 2021). However, until these advancements happen, exercising caution is advised. This reliance on databases may introduce bias towards well-studied taxa, as evidenced by the high species-level detection rate of Stramenopile species attributed to the rbcL marker's database richness (Chapter 3). However, it is important to note that the actual abundance and distribution of Stramenopiles in the environment indicate that, despite a possible database bias, their detection confirms their presence at the sampling site.

As eDNA metabarcoding continues to expand, especially through large-scale initiatives and global citizen science projects, exploring ASV pipelines may be more appropriate for studies involving multiple sequencing runs and events (Giebner et al., 2020). However, recent evidence suggests that in eDNA-based fish diversity studies, OTU-based pipelines outperform ASV-based ones in terms of species richness, sensitivity, diversity, and reducing false positives (Li et al., 2024). On the other hand, ASVs may offer greater advantages in studies involving eukaryotes and multiple markers (as discussed in Chapter 3), although their

use in these areas has not been fully explored. This gap highlights the need for further research to investigate their potential benefits in future studies. As these bioinformatic tools evolve and datasets become more complex, future research should explore the potential for using a multi-faceted informatics approach that combines the strengths of both OTU and ASV methodologies. As ongoing studies continue to enhance understanding of various pipelines, it is hoped that these efforts will ultimately contribute to the standardisation of methodologies, facilitating the implementation and guidance of eDNA techniques for environmental managers in the future.

Other limitations to eDNA metabarcoding highlighted through this research can be mitigated through careful primer selection. It has been consistently demonstrated that different primers used for metabarcoding yield variable datasets, affecting taxonomic communities and richness (Wangensteen et al., 2018; Piñol et al., 2019). Chapter 3 illustrates the expected differences between the two molecular markers (COI and rbcL) but also reveals unexpected diversity. This information is valuable for ecosystem managers interested in both broad-scale responses of ecological communities and finer resolution, such as the rbcL primer, which identified unique harmful algae not detected by the broader COI marker. The study also underscores the advantages of using two primers simultaneously, capturing a wider range of taxa. This finding is echoed in Chapter 4, where the simultaneous use of water eDNA and anemone nsDNA, albeit with a single primer, resulted in an expanded range of taxa detected, thereby enhancing the study's scope. Research of this nature contributes to informed decision-making for environmental managers, guiding them on the selection of molecular markers or sampling methods. While the idea of using a diverse array of primers may seem enticing to improve monitoring efforts, it is imperative to strike a balance between the ecological question at hand and the most suitable method to address it, as employing multiple primers can escalate costs and time.

6.4 Final Considerations and Pathways to Application

eDNA metabarcoding is not a universal solution for future environmental monitoring; like most methods, it has its limitations. Identifying and outlining these limitations not only guides future researchers in improving these specific areas but also provides valuable information that can help gain acceptance from environmental agencies, facilitating implementation. Coastal areas are under significant anthropogenic pressures. Recently, the health of our coastal waters has garnered substantial mainstream media attention, extending

beyond academia and gaining traction with the public. The condition of these ecosystems is now a major concern for public health and biodiversity. Moreover, eDNA metabarcoding has also featured in mainstream news for the detection of European eels in the UK, demonstrating its reach beyond academia. This public visibility is crucial, as research should not remain confined to academia. Scientific research must continue to extend its reach to the public and environmental agencies, providing accessible knowledge about our coastal habitats and their inhabitants. By bridging the gap between academia and the public, our research becomes more accessible, which can drive meaningful change.

There is a pressing need for further empirical evidence in eDNA metabarcoding to meet the requirements of stakeholders such as environmental agencies and managers and prompt the necessary adjustments to their monitoring and surveying strategies. As research in eDNA metabarcoding progresses, its efficacy in enhancing biodiversity surveys becomes increasingly apparent. Research that both builds upon and delineates the constraints of eDNA metabarcoding remains invaluable, as it equips environmental agencies with crucial insights to inform applications and address these limitations. The accessibility of eDNA metabarcoding is improving, with emerging techniques such as nsDNA and passive eDNA sampling potentially streamlining and reducing costs. For example, nsDNA may alleviate the necessity for expensive filtration equipment such as Sterivex by leveraging naturally occurring organisms or surfaces. These advancements are pivotal for scaling up eDNA applications, particularly given the disparities in traditional and molecular methods among different countries and establishing global frameworks could prove instrumental in guiding management decisions (Ruppert et al., 2019). Citizen science initiatives that foster public involvement in eDNA collection not only broaden research endeavours but also engage and educate the public, showcasing the simplicity of sample collection. As eDNA and nsDNA methodologies evolve and procedures are refined, interdisciplinary collaboration—from human health to environmental science—will be indispensable for the full integration and endorsement of these methods by environmental agencies.

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8. Appendix

8.1 Supplementary material – Chapter 3

S3.1 Supplementary Table of indicator species per sampling site

Group Albert Dock #sps. 4		
		stat **p.value**
Heterosigma.akashiwo	0.741	0.0008 ***
Chaetoceros.sp..7BOF	0.727	0.0009 ***
Chaetoceros.calcitrans	0.678	0.0026 **
Triparma.laevis	0.516	0.0345 *
Group Crosby #sps. 1		
Ankylochrysis.lutea	0.641	0.0153*
Group New Brighton #sps. 16		
Porphyra.umbilicalis	0.666	0.0156 *
Ctenophora.pulchella	0.665	0.0162
Echinocardium.cordatum	0.663	0.0156
Gyrosigma.acuminatum	0.66	0.014
Craticula.buderi	0.659	0.0142
Halopsis.ocellata	0.654	0.0156
Amphora.commutata	0.653	0.014
Arnoglossus.laterna	0.639	0.0156
Caloneis.amphisbaena	0.635	0.0135
Tryblionella.apiculata	0.625	0.0162
Pectinaria.koreni	0.604	0.0156
Nitzschia.reskoi	0.602	0.0162
Nitzschia.sigmoidea	0.591	0.013
Aurelia.aurita	0.573	0.0162
Rathkea.octopunctata	0.519	0.0313
Asterias.rubens	0.5	0.0452

Group Speke #sps. 8		
Cyclostephanos.dubius	0.673	0.0157 *
Skeletonema.potamos	0.664	0.0115
Fibrocapsa.japonica	0.646	0.0157
Cyclostephanos.sp..WTC16	0.635	0.0157
Rhizosolenia.setigera	0.621	0.0103
Thalassiosira.pseudonana	0.61	0.0145
Paramoeba.pemaquidensis	0.597	0.0113
Rhizostoma.pulmo	0.52	0.0404
Group Thurstaston #sps. 8		
Berkeleya.fennica	0.863 0	.0002 *
Polydora.cornuta	0.674	0.0182
Metarhombognathus.arm atus	0.663	0.0182
Cafeteria.roenbergensis	0.642	0.0182
Biremis.sp..CL.2014b	0.615	0.0177
Semibalanus.balanoides	0.600	0.0126
Psammogramma.vigoensis	0.577	0.0213
Vexillifera.sp.	0.566	0.0199
Group West Kirby #sp3		
Licmophora.paradoxa	0.671	0.0135 *
Nitzschia.paleaeformis	0.652	0.0177 *
Navicula.perminuta	0.555	0.0198 *

S3.2 Supplementary Table of indicator species per molecular marker

Group COI #sps. 24			
		stat p.value	
Phaeocystis.globosa 0.	693	0.0001 *	**
Ditylum.brightwellii	0.59	0.0002	***

Rhizostoma.pulmo	0.57	0.0001	***
Cylindrotheca.closterium	0.553	0.0002	***
Gymnodinium.catenatum	0.53	0.0003	***
Bathycoccus.prasinos	0.482	0.0004	***
Micromonas.pusilla	0.454	0.0001	***
Thalassiosira.pseudonana	0.452	0.0001	***
Fucus.spiralis	0.445	0.0044	**
Nannochloropsis.granulata	0.444	0.0051	**
Semibalanus.balanoides	0.425	0.0041	**
Akashiwo.sanguinea	0.423	0.0083	**
Vexillifera.sp.	0.411	0.0097	**
Paramoeba.pemaquidensis	0.399	0.0045	**
Pycnococcus.provasolii	0.399	0.0074	**
Rhizosolenia.setigera	0.384	0.0036	**
Aurelia.aurita	0.38	0.0193	*
Mytilus.edulis	0.368	0.0007	***
Cafeteria.roenbergensis	0.347	0.0173	*
Asterias.rubens	0.34	0.0498	*
Heterocapsa.rotundata	0.336	0.0464	*
Rathkea.octopunctata	0.317	0.047	*
Fistulifera.solaris	0.284	0.0439	*
Actinia.equina	0.262	0.0001	***
Group rbcL #sps. 24			
		stat p.value	
Leptocylindrus.minimus 0.	734	0.0001 *	**
Thalassiosira.sp..16BOF	0.712	0.0001	***
Minidiscus.trioculatus	0.708	0.0001	***
Cylindrotheca.closterium.1	0.689	0.0001	***
Extubocellulus.spinifer	0.655	0.0001	***
Chaetoceros.simplex	0.653	0.0001	***

Conticribra.guillardii	0.635	0.0001	***
Thalassiosira.sp..15BOF	0.598	0.0002	***
Thalassiosira.oceanica	0.529	0.0003	***
Triparma.retinervis	0.49	0.0011	**
Chaetoceros.constrictus	0.483	0.0025	**
Navicula.perminuta	0.47	0.0019	**
Chaetoceros.calcitrans	0.459	0.0002	***
Navicula.gregaria	0.452	0.0003	***
Nitzschia.sigmoidea	0.431	0.0035	**
Actinoptychus.undulatus	0.418	0.0195	*
Caloneis.amphisbaena	0.405	0.0001	***
Gedaniella.boltonii	0.388	0.0422	*
Chaetoceros.sp..7BOF	0.368	0.0004	***
Craticula.buderi	0.355	0.0001	***
Odontella.aurita	0.344	0.0483	*
Cocconeis.cf..mascarenica.SZCZCH283	0.332	0.0457	*
Heterosigma.akashiwo	0.318	0.0489	*
Skeletonema.potamos	0.315	0.0451	*

8.2 Supplementary material – Chapter 4

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NOTE

Exploring intertidal sea anemones *Actinia equina* as natural eDNA samplers for coastal biodiversity assessment

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ABSTRACT: Biodiversity in coastal marine environments is under unprecedented threat from anthropogenic impacts, which highlights a need for cost-effective and expedient survey methods. The analysis of environmental DNA (eDNA) — typically obtained through artificially filtered water samples — can paint a detailed picture of fish diversity in marine coastal environments. More recently, the analysis of natural sampler DNA (nsDNA, based on filter-feeding invertebrates that naturally trap eDNA in their tissues) has emerged as a potential alternative to water filtering. In this study, we investigate the widely distributed beadlet anemone *Actinia equina* as a potential natural eDNA sampler. Anemone samples were collected from 2 coastal locations in the UK: Rhosneigr (Anglesey, North Wales) and New Brighton (Wirral, north-western England). Sampling took place over 2 different months, and samples were compared to concomitantly sampled water. DNA metabarcoding via 12S Tele02 fish-specific primers revealed successful detection of a range of fish and other vertebrate species. We observed differences in species detected between conventional eDNA and sea anemone nsDNA samples, as well as a significant difference in seasonality detected through nsDNA. Our results indicate that the beadlet anemone can be a successful natural eDNA sampler, but that its value is more likely to reside in its complementarity alongside established eDNA methods.

KEY WORDS: Metabarcoding · Intertidal environments · Environmental DNA · Natural sampler DNA · Beadlet anemone · Coastal fishes · Irish Sea

1. INTRODUCTION

The escalating severity of the global biodiversity crisis that is affecting terrestrial and aquatic life should be at the forefront of conservation biology (Sutherland et al. 2023). Coastal environments are vibrant hotspots of biodiversity that play a pivotal role in securing a range of important ecosystem services (Jones et al. 2020). These habitats are being increasingly threatened by a variety of stressors — including urbanisation, pollution and climate change, underscoring the urgent need to enhance and refine methods of monitoring and measuring biodiversity changes along with their impacts on ecosystem functions and services.

In coastal ecosystems, observational methods (such as underwater visual surveys or baited remote underwater videos) and capture-based netting and trapping are used widely to estimate fish biodiversity (Jovanovic et al. 2007). These techniques can be time-consuming and expensive and are often inaccurate and sometimes destructive (Baker et al. 2016). Recent advances in high-throughput, non-invasive molecular methods — particularly environmental DNA (eDNA) metabar-

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coding — hold substantial promise for enhancing the precision and scope of biodiversity surveys (Aglieri et al. 2021). At present, aquatic eDNA is typically obtained by passing water through an artificial filter with the aid of a range of manual or automated water pumping devices. As the field of eDNA metabarcoding advances, more cost-effective and accessible sampling methods are emerging, such as passive sampling techniques (Bessey et al. 2021), which utilise various artificial materials and objects to trap and accumulate eDNA from the surrounding environment.

Natural eDNA samplers present a compelling alternative to artificial filters, offering a different avenue to the retrieval of eDNA fragments. The scope of natural eDNA samplers is extensive, spanning from natural substrates such as cobbles (Shum et al. 2019) and spider webs (Gregorič et al. 2022) to the gut contents of aquatic generalist feeders (Siegenthaler et al. 2019), and extending to sponges (Porifera), the taxon that best epitomises aquatic filter-feeding (Mariani et al. 2019). These organisms prompted further exploration of eDNA sampling properties in other aquatic invertebrates, such as mussels (Weber et al. 2023).

In this paper, we explore whether sea anemones are viable natural eDNA samplers for the detection of fish biodiversity in benthic intertidal habitats. Anemones are abundant and distributed in both deep oceans and coastal zones globally (Steinberg et al. 2020). Here, we focus on the common and widespread suspension-feeding beadlet anemone *Actinia equina*, a common species distributed along the coasts of the UK, Western Europe and much of the East Atlantic (Davenport et al. 2011). In order to conduct this research, a small number of anemones were sacrificed. However, this organism is abundant and not of conservation concern (Kipson et al. 2015). Furthermore, the scientific use of a small number of these organisms is of negligible impact when compared to the habitat damage and animal fatalities associated with established marine surveying techniques, such as the use of nets, traps, dredges and grabs. *A. equina* are generalist feeders and opportunistic omnivores (Davenport et al. 2011), and their prevalence in intertidal zones makes them readily accessible for eDNA metabarcoding analysis. As sedentary organisms (although capable of moving slowly), species detections through natural sampler DNA (nsDNA) should reflect the sampling environment.

To test whether *A. equina* can be an effective natural eDNA sampler, we used a fish-specific primer pair. To broaden our understanding of the taxa detected by the anemones, we used a conventional water eDNA approach as a reference point. As we extracted DNA from the whole body of the anemone (including the gut), using a fish-specific primer enabled us to focus on taxa that are unlikely to be primary targets of the anemone's diet, making the nsDNA detections most comparable to aqueous eDNA data for vertebrate biodiversity monitoring. In this context, it is important to consider the digestion time of the anemones and the influence this might have on eDNA degradation. This is likely to be on the same order of magnitude as the known degradation time of eDNA in seawater, which is between 24 and 72 h (Collins et al. 2018). Kruger & Griffiths (1997) reported a gut retention time in *A. equina* between 12 and 23 h when feeding on planktonic crustaceans, with longer digestion times of 40–60 h reported in the case of shelled prey (Shick 1991). These digestion times indicate that aqueous eDNA and anemone nsDNA approaches are comparable, allowing us to consider the merits of these candidate natural eDNA samplers in the context of coastal biodiversity assessments.

2. MATERIALS AND METHODS

2.1. Field collection

In an initial experiment, 6 beadlet anemones were collected in May 2022 from rockpools at New Brighton, Wirral; subsequently, 10 anemones and 3 l of water samples were collected in October 2022 from both New Brighton and Rhosneigr, Anglesey, North Wales (Fig. 1A). Rhosneigr is an exposed rocky coastal site (Fig. 1C), whilst the rockpools at New Brighton are situated under man-made concrete groynes on a sandy beach (Fig. 1B).

To monitor contamination at each site, purified water was filtered and used as field blanks, which were treated identically to other samples throughout the collection and extraction process. Anemones were collected using sterile gloves, stored separately in 100% ethanol and placed in a cooler on ice in the field. The three 1 l water samples (collected from the same rockpool as the sampled anemones) were pushed through 0.45 µm Sterivex filters, with each filter placed inside 2 sterile bags and immediately stored on ice. All samples were stored at −20°C in the lab until further processing.

2.2. Laboratory procedures

DNA extraction from Sterivex filters followed the modular universal DNA (mu-DNA) protocol for

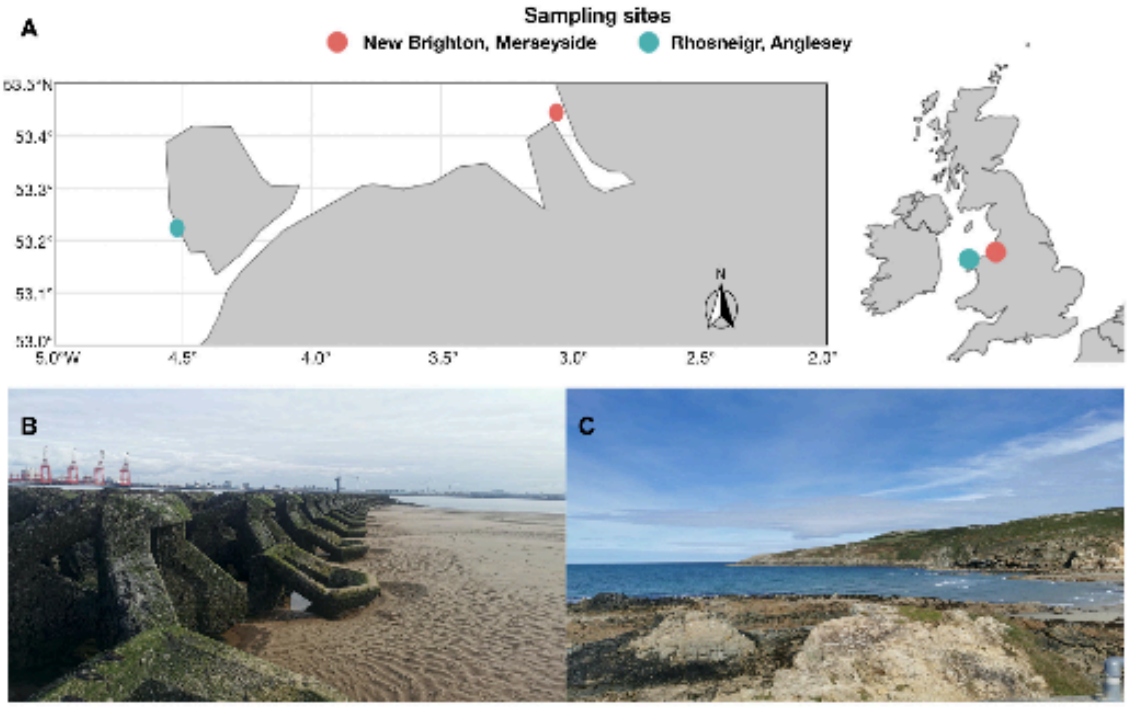

Fig. 1. (A) Sampling sites for sea anemones *Actinia equina*, showing (B) concrete groynes at New Brighton, Wirral, and (C) the rocky coastline at Rhosneigr, Anglesey

water, while anemone DNA extraction followed the mu-DNA extraction protocol for tissue (Sellers et al. 2018). For full procedures see Texts S1.1 & S1.2 in the Supplement at www.int-res.com/articles/suppl/m743p159_supp.pdf. To extract DNA from beadlet anemones, sections from the entire organism (including stomach, tissue and tentacles) were dried by blotting the ethanol used for preservation. The dried material was then cut into small pieces, and 500 mg of the resulting dry weight from the whole organism's tissue was used for DNA extraction.

PCR amplification was performed in triplicate for each sample using the Tele02 fish-specific primers, which target a ~167 bp fragment of the 12S rRNA mitochondrial region (Taberlet et al. 2018). Primer pairs were uniquely indexed to enable demultiplexing for downstream bioinformatic analysis. Iridescent shark catfish *Pangasianodon hypophthalmus* was used as a positive control. PCR amplicons were pooled in a single library and sequenced on an Illumina iSeq100 using v2 150×2 chemistry. Further details on PCR conditions and library preparation can be found in Text S1.3 in the Supplement.

2.3. Bioinformatics and downstream analysis

Bioinformatic analysis followed the OBITOOLS pipeline. For the full protocol, see Text S1.4 in the Supplement. Taxonomic assignments were validated by cross-checking non-native and unexpected taxa by manual BLAST searches against the GenBank nucleotide database. The package 'decontam' in R was used on the raw molecular operational taxonomic unit (MOTU) output (Davis et al. 2018). MOTUs were filtered by removing those that did not reach a sequence identity match of at least 97%.

A Venn diagram was used to visualise fish MOTUs in nsDNA samples from May and October to portray seasonal variation. Samples were grouped per site and nsDNA or eDNA capture method for October only and then visualised using a stacked bar chart of the proportion of fish and birds detected by each method. To visualise the species proportions, a bubble plot was used for fish species only. Proportions were calculated using the number of sequencing reads of each species or MOTU per sample divided by the total sample reads; this represents the

proportional read counts per species and serves as a proxy for relative abundance.

To visualise temporal differences (May vs. October) between anemone nsDNA samples from New Brighton, we used nonmetric multidimensional scaling (NMDS) based on Jaccard distances using presence–absence data. These differences were tested using permutational multivariate analysis of variance (PERMANOVA, 999 permutations) on binary pairwise distance matrices using the function 'adonis' in the R package 'vegan' (Oksanen et al. 2013). PERMANOVA was also used to test for differences between the 2 sampling sites in October. Finally, to identify whether there were any species significantly associated with certain months, we used an indicator species analysis in R using the 'indicspecies' package (De Caceres et al. 2016), after Hellinger-transforming read count data.

3. RESULTS

A total of 28 samples from October (Table S1 in the Supplement) made up one-third of an iSeq100 run, yielding 602 127 reads. After the first filtering step (>97% identity match), this was reduced to 535 263 reads (88.9%). The 8 samples from May (Table S2 in the Supplement) yielded 379 994 reads from an iSeq100 run, of which 374 432 reads (98.5%) were retained for downstream analyses. Although no contamination was found in the controls, 3 anemone samples were removed as they contained no sequencing reads. See Text S1.5 in the Supplement.

We identified 17 fish species across anemone nsDNA and water eDNA samples from October 2022 (Fig. 2). Although the DNA marker used is primarily designed to detect vertebrates, *Actinia equina* was also detected, albeit with only 115 reads across all samples, as were 5 distinct bird species: spotted sandpiper *Actitis macularius*, ruddy turnstone *Arenaria interpres*, rock dove *Columba livia*, European herring gull *Larus argentatus*, and common redshank *Tringa totanus*.

A higher diversity of fish was detected in New Brighton (Fig. 2); however, no significant difference in community composition was detected between the 2 sites (PERMANOVA pseudo-F = 1.0165, df = 1.0, p = 0.393). There was no significant difference detected in community composition between nsDNA and eDNA in both locations in October (PERMANOVA pseudo-F = 0.5629, df = 1.0, p = 0.19) (Fig. S1 in the Supplement). Three vertebrate species were statistically more abundant in eDNA samples: common goby *Pomatoschistus microps* (p = 0.0063), European eel *Anguilla anguilla* (p = 0.0167) and common redshank *Tringa totanus* (p = 0.0110).

Only 3 taxa (12.5%) were shared between May and October samples (Fig. 3). Six MOTUs (25%) were detected only in May, while 15 (62.5%) were found only in October. This results in a strong separation of these temporal samples (Fig. 3A), which is also supported statistically (PERMANOVA F = 5.8614, df = 1.0, p = 0.001). Indicator species analysis showed significant abundances in May for shanny *Lipophrys pholis* (stat = 0.733, p = 0.004) and common sole *Solea solea* (stat = 0.382, p = 0.0358) and significantly higher abundance in October for megrim *Lepidorhombus whiffiagonis* (stat = 0.676, p = 0.0056), common goby (stat = 0.573, p = 0.0434) and ruddy turnstone (stat = 0.676, p = 0.0056).

4. DISCUSSION

Easily accessible, sessile suspension-feeding invertebrates make ideal candidates for coastal nsDNA applications, especially if, as in the case of the beadlet anemone, they are widely distributed. With this first attempt to evaluate the role of sea anemones as natural eDNA samplers to aid fish biodiversity assessments, we begin to understand the extent and circumstances in which such an approach may be beneficial. Wells et al. (2022) amplified DNA extracts of gut content from the giant plumose anemone *Metridium farcimen*, targeting the mitochondrial *COI* region to investigate the diet of the organism. While the use of the *COI* primer pair enabled successful identification of the organisms on which the anemone fed, it also allowed the detection of several fish species, indicating that anemones may have potential as successful natural eDNA samplers, assessing biodiversity beyond the organisms that they select for consumption.

Our study demonstrates the effective use of *Actinia equina* nsDNA in detecting fish species representative of their environment. We found no significant difference in species detected between nsDNA and eDNA, suggesting their similar efficiency; however, this could be due to the relatively small sample size. Therefore, further studies comparing intertidal nsDNA and eDNA should be conducted to establish whether consistent, ecologically relevant differences exist between these capture methods. We found that, while nsDNA can identify a subset of vertebrate species that are also detected by conventional eDNA capture methods, it uniquely identifies vertebrate species that have a minimal presence in aqueous

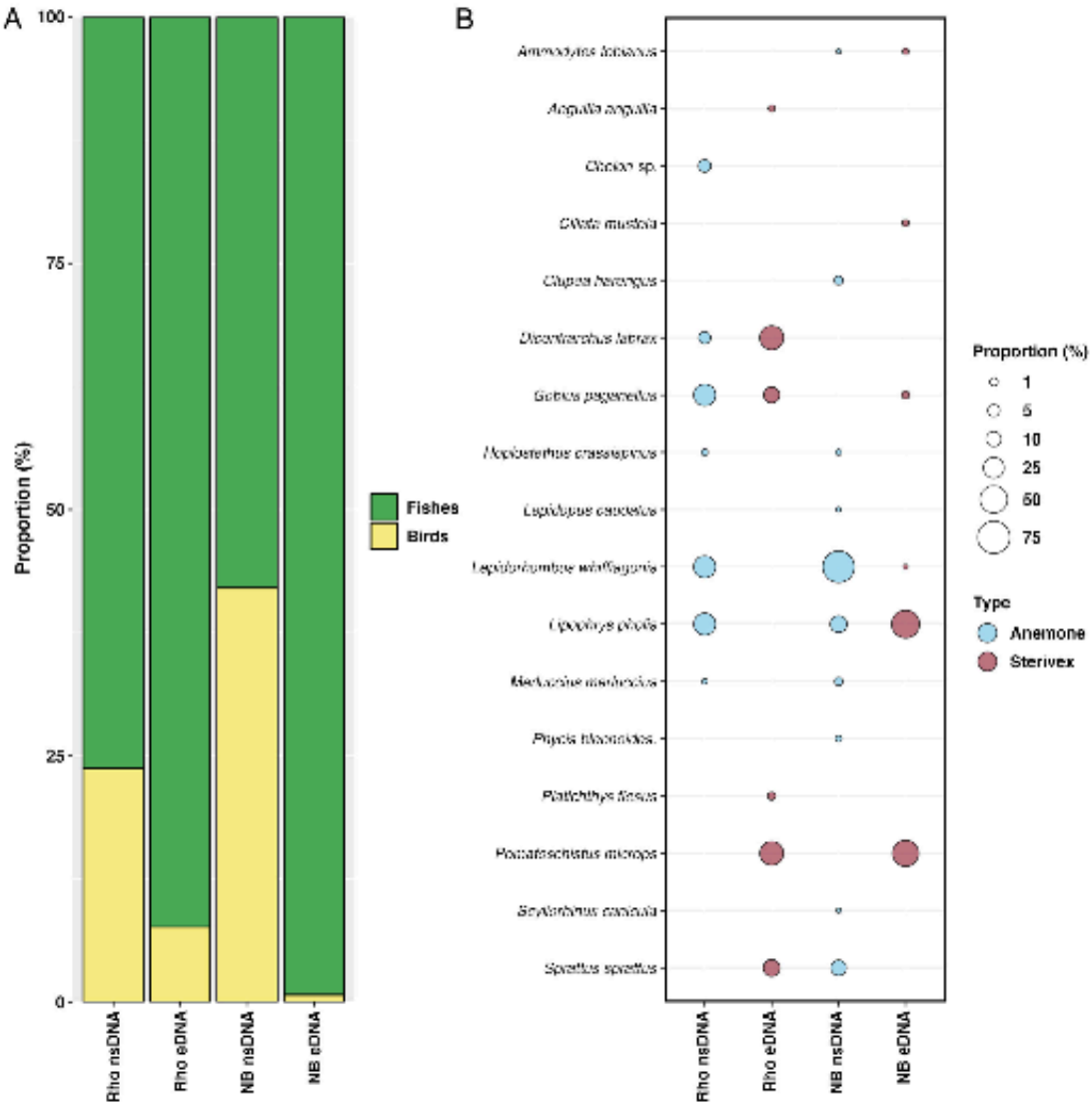

Fig. 2. (A) Proportions of fish and birds detected by natural sampler DNA (nsDNA) and environmental DNA (eDNA) from the 2 sites (Rho: Rhosneigr; NB: New Brighton). (B) Bubble plot showing the proportions of species per sampling site detected by nsDNA and eDNA. Both panels show October 2022 samples only

eDNA samples, such as the shorebirds ruddy turnstone *Arenaria interpres* and spotted sandpiper *Actitis macularius*. These species are closely associated with intertidal zones, with *A. interpres* known to feed on molluscs, crustaceans and small invertebrates (Kendall et al. 2004). We speculate that a high proportion of shorebird reads results from predation attempts on beadlet anemones or their mere exposure to wading birds or their guano at low tide, resulting in the anemone nsDNA. This would indicate that in some circumstances, this approach could be more effective than conventional eDNA filtration at monitoring rare and endangered coastal shorebirds. The inclusion of non-target species enriches this study and suggests that forthcoming conservation research could employ bird-specific primers to strengthen these findings.

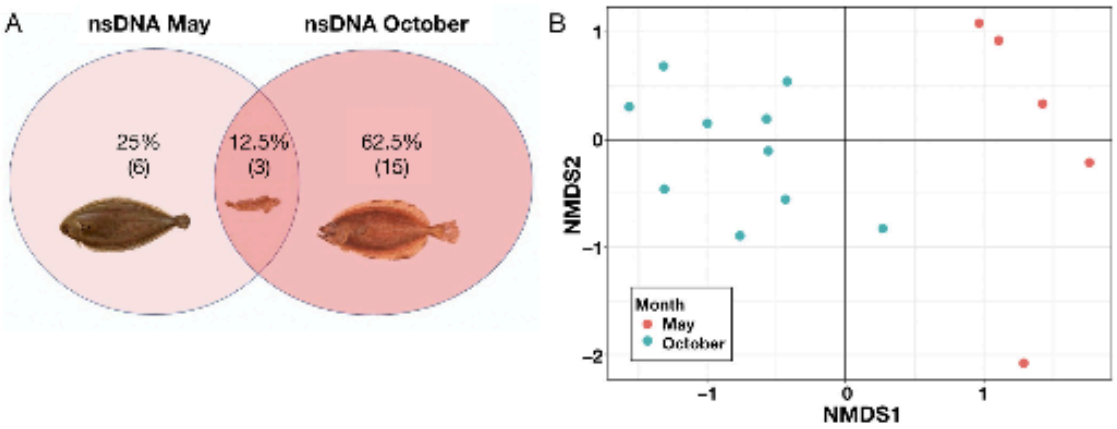

Fig. 3. (A) Venn diagram representing the degree of overlap, in terms of natural sampler DNA (nsDNA) operational taxonomic units, between May and October 2022 samples from New Brighton. Pictures represent species of fish with the highest read counts: May, *Solea solea*; shared, *Lipophrys pholis*; and October, *Lepidorhombus whiffiagonis*. (B) Nonmetric multidimensional scaling plot (NMDS) showing nsDNA samples collected from New Brighton in May and October 2022 based on Jaccard distances using binary presence–absence data

Our study thus underscores the importance of utilising both nsDNA and eDNA techniques in biodiversity assessments. Relying solely on either method would have resulted in several vertebrate species going undetected. Monitoring seasonal changes in fish assemblages in coastal environments via traditional methods is challenging, but eDNA has been shown to serve this purpose (Sigsgaard et al. 2017). Here, we found a strong temporal signal in anemone nsDNA between May and October, which indicates its sensitivity to detect seasonal variations in vertebrate communities. In cold-temperate intertidal habitats, there is a greater diversity at the end of summer than in the spring (Jovanovic et al. 2007), with fluctuations in diversity driven by migration and spawning (Connor et al. 2019). Anemone nsDNA between these 2 months demonstrated strongly divergent patterns of read proportions in some species. In May, there was a higher percentage of shanny *Lipophrys pholis* and common sole *Solea solea* reads than in October. This potentially reflects the spawning time of these species (Shackley & King 1977, Armstrong et al. 2001), thus reinforcing the argument that anemone nsDNA can detect environmental seasonal changes.

With this study being an initial step in exploring anemones as natural eDNA samplers, further research is necessary to optimise laboratory methods, explore variation in nsDNA collection between anemone species and investigate the influence of feeding behaviour on nsDNA recovery. We also encourage further research to investigate less invasive techniques of obtaining eDNA, such as biopsies or swabs to minimise the impact on these invertebrates. This is a compelling introduction of a new phylum to the field of eDNA and nsDNA analysis and reinforces the potential of this technique, at least in conjunction with conventional eDNA methods, to obtain a more comprehensive picture of species diversity in intertidal environments.

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8.2.1 Downstream analysis

To visualise differences between anemone nsDNA and Sterivex eDNA for both locations in October only, we used nonmetric multidimensional scaling (NMDS) based on Jaccard distances using presence/absence data. We tested differences between the two sampling months using permutational multivariate analysis of variance (PERMANOVA, 999 permutations) on pairwise distance matrices using the function *adonis* in VEGAN (Oksanen et al., 2013). Please see supplementary figure S3. Total sum scaling was used to calculate the relative abundance which converts number of reads to appropriately scaled ratios (Alberdi et al., 2019). The rarefaction curve was generated using the *rarefy* function from the package VEGAN to assess whether the sequencing depth was adequate for the anemone nsDNA.

8.2.2 Sequencing and sample information

The samples for this study were pooled alongside unrelated libraries for a different eDNA project. 10 anemone samples and 1 water sample from Rhosneigr, 10 anemone samples and 1 water sample as well as 2 extraction blanks, 2 field blanks and positive control made up the 28 samples from the October library. The six anemone samples from May (plus 1 extraction blank and 1 positive control) made up the 8 samples from the May library. Please see supplementary table S1.

S4.1 Supplementary Table: Sample layout for sequencing runs.

Number of samples for each sample type which make up two separate sequencing runs: May 2022 (8 samples total) and October 2022 (28 samples total).

	New Brighton		**Rhosneigr**
Sample Type	**May 2022**	**October 2022**	**October 2022**
Anemone	6	10	10
Water	0	1	1
Field-blank	0	1	1
Extraction-blank	1	1	1
Positive control	1	1	1

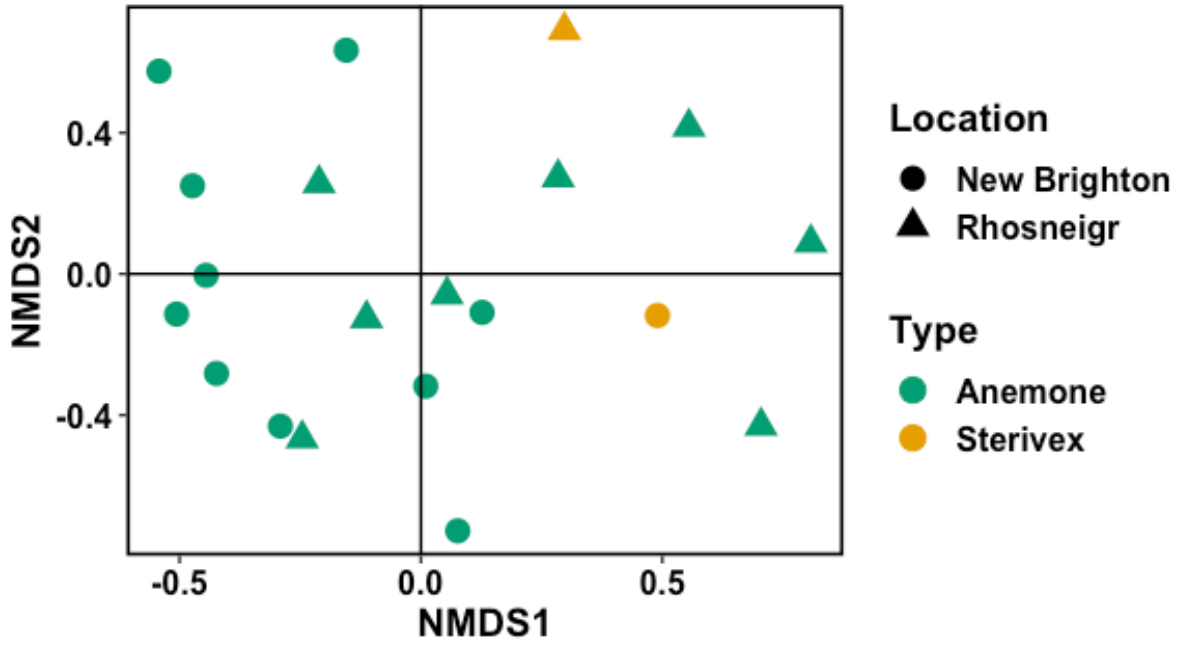
S4.2 Supplementary Table: List of species from nsDNA between May and October 2022.

List of vertebrate species (venn diagram fig. 3A) detected in anemone nsDNA in May 2022, October 2022 or detected in both months. Only at New Brighton.

nsDNA May		**Shared**		**nsDNA October**	
Scientific name	**Common name**	**Scientific name**	**Common name**	**Scientific name**	**Common name**
Solea solea	Common sole	*Lipophrys pholis*	Shanny	*Arenaria interpres*	Ruddy turnstone
Thunnus thynnus	Atlantic Bluefin tuna	*Larus argentatus*	European herring gull	*Lepidorhombus whiffiagonis*	Megrim
Sarda sarda	Atlantic bonito	*Phycis blennoides*	Greater forkbeard	*Pomatoschistus microps*	Common goby
Zeugopterus punctatus	Topknot			*Sprattus sprattus*	European sprat
Helicolenus dactylopterus	Blackbelly rosefish			*Columba livia*	Rock dove
Arnoglossus laterna	Mediterranean scaldfish			*Clupea harengus*	Atlantic herring
				Merluccius merluccius	European hake
				Lepidopus caudatus	Silver scabbardfish
				Scyliorhinus canicula	Small-spotted catshark
				Actitis macularius	Spotted sandpiper
				Chelon auratus	Golden grey mullet
				Ammodytes tobianus	Lesser sand eel
				Gobius paganuellus	Rock goby
				Phoxinus phoxinus	Eurasian minnow
				Salmo trutta	Sea trout

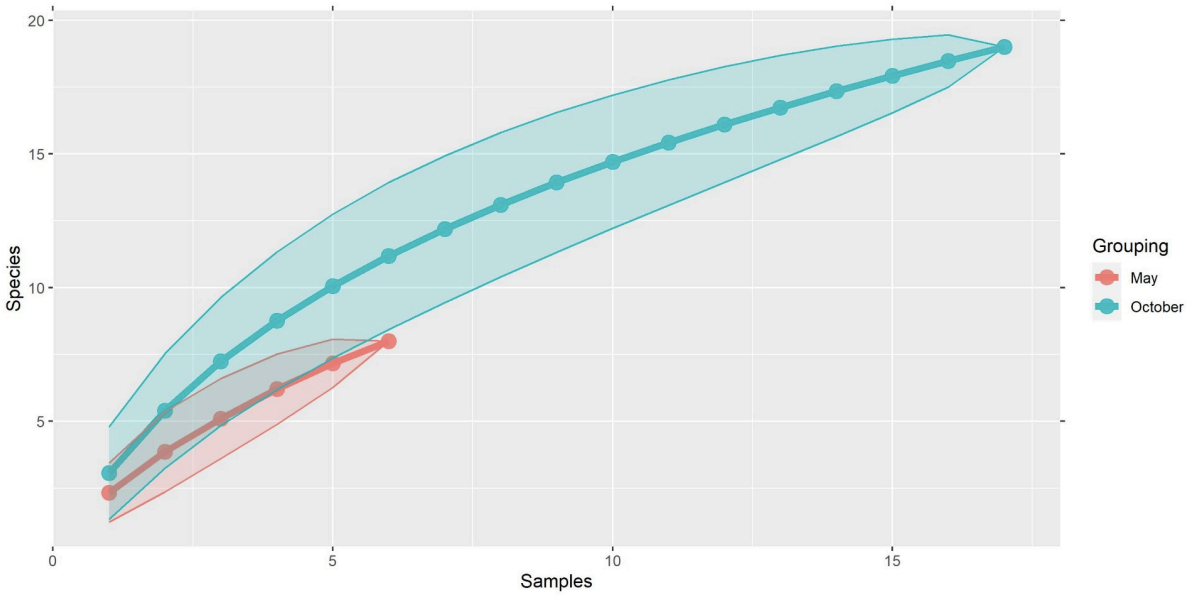
S4.3 Supplementary Figure: Non-metric Multi-Dimensional scaling of nsDNA and eDNA between locations.

Figure shows nsDNA and eDNA samples collected from New Brighton and Rhosneigr in October only based on Jaccard distances using binary presence/absence data.



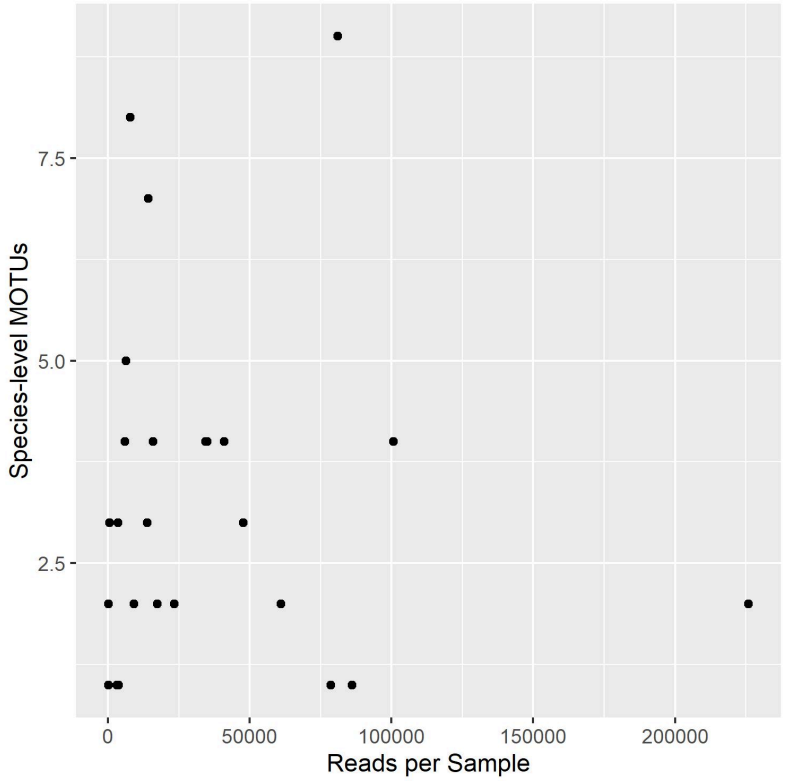
S4.4 Supplementary Figure: Species accumulation curve of anemone nsDNA.

Species accumulation curve showing number of species detected by anemone nsDNA only between May and October.



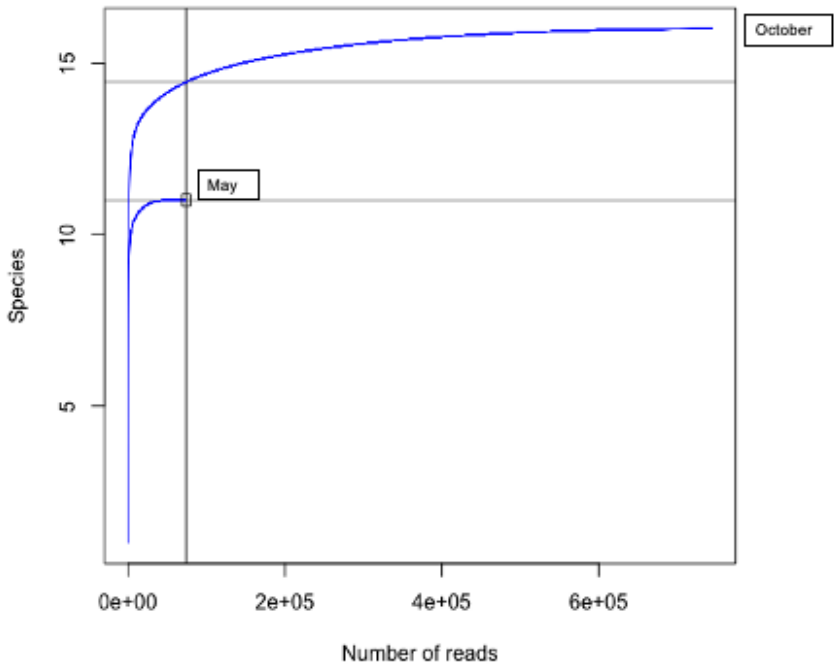
S4.5 Supplementary Figure: Plot showing number of sequencing reads for nsDNA.

Figure shows number of reads per sample for anemone nsDNA only between for both locations and months. Ellipses show 95% confidence.



S4.6 Supplementary Figure: Rarefaction curve showing nsDNA sequencing reads.

A rarefaction curve showing the number of sequencing reads for anemone nsDNA only, between May and October.


October
May
Species
15
10
5
0e+00
2e+05
4e+05
6e+05
Number of reads

8.3 Supplementary Material – Chapter 5

8.3.1 COI Molecular Marker

8.3.1.1 DNA Extraction

DNA extraction is described in the main manuscript in the section Material and Methods.

8.3.1.2 Library preparation

PCR amplification was performed in triplicate for each of the 18 Sterivex and 18 mussel samples, using the COI Leray primer, which target a ~313 bp fragment of the COI rRNA mitochondrial region (Leray et al., 2013). These were the same DNA extracts used for the 12S analysis, as described in the Chapter 5. Positive controls were put in place for each PCR batch. We used extracts of iridescent shark (*Pangasianodon hypophthalmus*) DNA at 0.05 ng/µl, choosing this organism as it is a tropical freshwater fish, unrelated to fish species in UK waters. Primer pairs were uniquely indexed to enable demultiplexing for downstream bioinformatic analysis. For each DNA sample, PCR amplification was performed in triplicate. The reaction mix included: 10µL Mifi DNA polymerase mastermix (2x), molecular grade water 5.84µL, BSA 0.16µL, 1µL of each primer (10 µM) and 2 µL of DNA with a thermocycling profile denaturation step at 95 °C for 15 min followed by a total of 30 cycles of 95 °c for 45 s (denaturation), 55 °C for 45 s (annealing), and 72 °C for 45 s (final extension) (Leray et al., 2013).

PCR products were run on 2% agarose gels stained with SYBR safe. PCR replicates were pooled and samples were purified with a 1:1 30µL Mag-Bind® TotalPure NGS magnetic beads and 30µL of pooled PCR product. The concentration of each purified PCR was quantified using Qubit 4 Fluorometer dsDNA HS assay kits and pooled in equimolar amounts. The pooled PCR product was quantified using a Tapestation 4200 using the high sensitivity D1000 assay. Then, a further 1x bead clean-up was performed on the pooled PCR product. Illumina libraries were prepared using the Perkin-Elmer NEXTFLEX® Rapid DNA-Seq Kit 2.0, using 1 µg as starting concentration of the pooled PCR product following the manufacturers guidelines with library amplification. The library and PhiX control were quantified using qPCR using the NEBNext® Library Quant Kit for Illumina®. The final library and PhiX control were diluted to 85pM (with PhiX 20% of the run) and loaded onto an Illumina® MiSeq™ 100 Reagent v2 (2 x 300 cycles).

8.3.1.3 Bioinformatics and downstream analysis

The bioinformatic process was based on the OBITOOLS pipeline (Boyer et al., 2016). FASTQC was used to assess the quality scores of the fastq files. ILLUMINAPAIREDEND was then used to merge the paired-end reads and remove alignments with low (<40) quality scores. NGSFILTER was used to de-multiplex samples. To remove sequences that were not in the target base pair range, we filtered the sequence lengths to 313bp by using OBIGREP. We dereplicated the samples using OBIUNIQ. Chimeras were then removed using the uchime-denovo chimera search function in VSEARCH (Rognes et al., 2016). Molecular operational taxonomic unit (MOTU) clustering was implemented using SWARM with "-d 13" (Mahé et al., 2014). Taxonomic assignment per sample was carried out using ECOTAG which was constructed using 'ecoPCR' *in silico* against the EMBL database (Release version r143 October 2023). The taxonomic assignments included seven levels of biological classification along with a percentage indicating the likelihood of accurate classification. After taxonomic assignment, non-native and unexpected taxa were cross-checked by manual BLAST against the NCBI nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide). MOTUs that could not be assigned to genus or species level were also cross-checked manually against the NCBI nucleotide database.

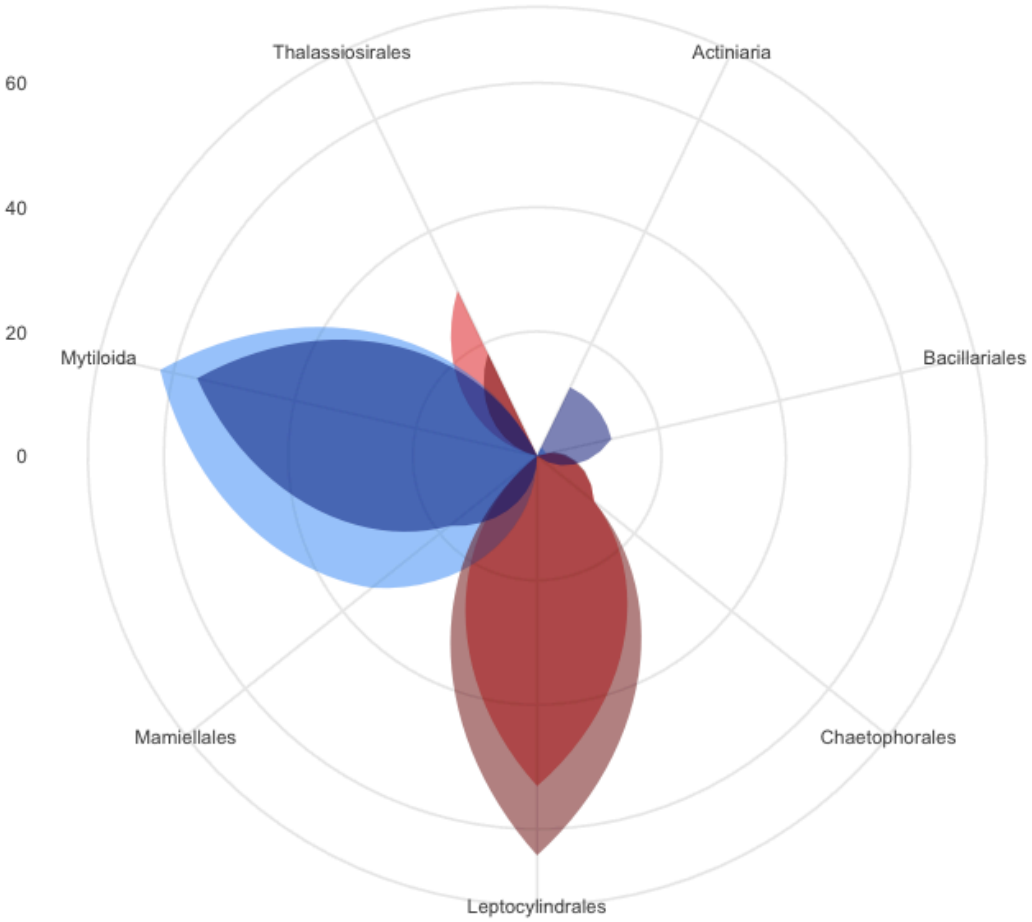
8.3.2 Results

A total of 1,669,571 sequencing reads were generated from 18 Sterivex filters and 18 blue mussel samples. Following the initial filtering process, 1,608,078 sequencing reads were retained for subsequent analysis. A total of 211,621 sequencing reads were for the Sterivex samples and 1,213,838 sequencing reads for the mussels.

Altogether, 39 distinct Orders were detected. In July, 27 orders were identified in the Sterivex samples, while 16 orders were found among the mussel samples. In November, 25 orders were detected in the Sterivex samples, and 16 orders were observed in the mussel samples.

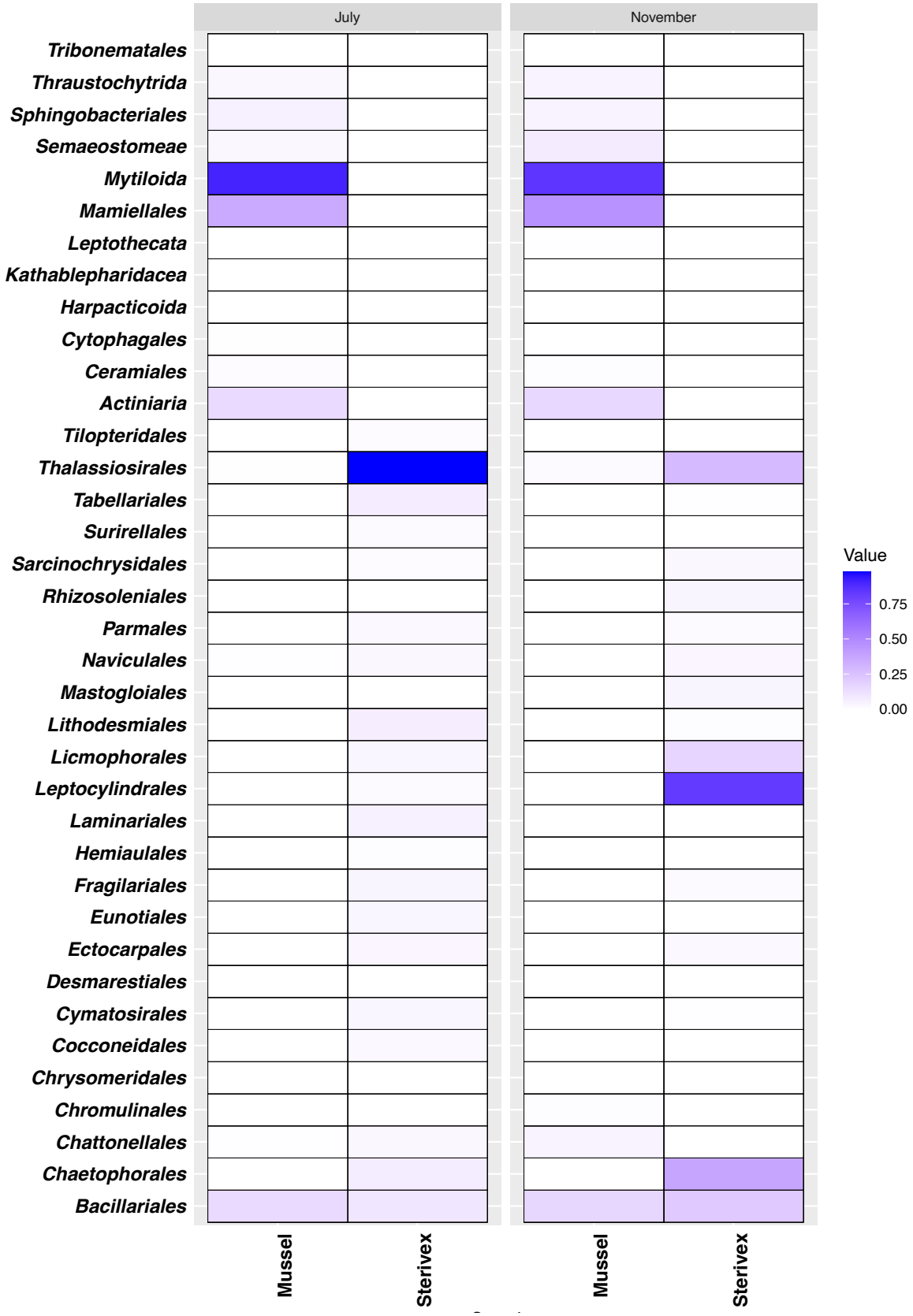
S5.1 Supplementary Figure: Spider of relative proportion of classes between nsDNA and eDNA COI data.

Spider plot illustrating the transformed of classes exceeding 5% relative proportionate reads, with taxa sourced from mussels depicted in blue and water samples in red. Darker shades represent data from November, while lighter shades represent data from July



S5.2 Supplementary Figure: Heatmap of proportionate taxa across nsDNA and eDNA COI data.

Heatmap illustrating the proportion of taxa based on Hellinger transformed data detected in the classification of order across both the Sterivex and mussel samples between July and November.



PERMANOVA showed significant differences in community composition with molecular marker (pseudo-F= 12.162, P=0.001).

S5.3 Supplementary Figure: Non-metric Multi-dimensional Scaling of nsDNA and eDNA COI data.

NMDS plot of COI data based on using Bray-Curtis dissimilarity on Hellinger transformed data.